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Dopa and dopamine formation from phenylalanine in human brain*

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Little work has been done on the formation of the putative neurotransmitter dopamine (DA) in the CNS of human despite the invaluable nature of the data obtained directly from human tissue. A limited number of publications [1–3] on tyrosine hydroxylation, considered to be the critical step in DA synthesis [4], have appeared reporting data on postmortem human brain tissue. No study however, to the best of our knowledge, has been reported on DA formation from phenylalanine in the same tissue although it is known that both DA and norepinephrine may be formed from this amino acid in laboratory animal brain [5–8]. We have now examined human brain tissue for the presence of any activity forming DA and its precursor Dopa from phenylalanine.

Uniformly ^{14}C-labelled L-phenylalanine and L-tyrosine (specific radioactivity, 350–400 mCi/m-mole) were purchased from New England Nuclear, Boston, MA. Triton X-100 was obtained from J. T. Baker Chemical Co. and the decarboxylase inhibitor NSD-1034 was a kind gift from Smith and Nephew Co., England. Crude Dopa decarboxylase was prepared by the method of Clark, *et al.* [9]. The determination of phenylalanine concentration was done by fluorometry as described before [10]. Human brain tissue samples were obtained following autopsies at various local hospitals. Wistar rats (female, 150–170 g) were supplied by the Carworth division of Charles River Co., Wilmington, MA. The brain region of caudate nucleus was dissected out in each case and employed for the experiments. The chilled tissue samples were homogenized in ice-cold 0.32 M sucrose containing 10 micromolar calcium chloride [11]. The whole homogenate (10%) was spun once at 27,000 *g* and the particulate fraction was suspended in 0.125 M sodium phosphate buffer (pH 6.0). The particulates were then incubated with [^{14}C]phenylalanine as described before [12]. Briefly, the method consisted of incubating 0.5 ml of the phosphate buffer suspension of the particulates (50–160 mg wet tissue equivalent from human samples; 20 mg of rat brain) diluted to approximately 675 microliters of final vol. containing the final concentrations of 0.05 M mercaptoethanol, 0.4 mM pargyline hydrochloride (MAO inhibitor) and 0.092 M sodium phosphate at pH 6.0. The incubation was for 30 min at 37°. At the end of the incubation, crude Dopa decarboxylase and 0.5 M sodium phosphate buffer (pH 7.0) was added and the mixture was shaken for 15 more min to complete the decarboxylation of any remaining labelled Dopa to DA. The reaction was stopped by adding ice-cold 0.8 N perchloric acid and then the whole mixture was extracted twice by homogenizing, centrifuging and rehomogenizing the pellet followed by recentrifugation. The analysis of the combined extracts was carried out as described in detail before [6, 12] for the separation of the products, tyrosine and DA formed from phenylalanine substrate. The tyrosine formed was purified by chromatography on Dowex-50 (Na^+) and paper. The same ion-exchange resin and acid alumina was employed for the isolation of DA which contained only a trace (< 10%) of norepinephrine as reported before [6].

The analytical recoveries were checked by analyzing known labelled compounds and the average values from a number of such analyses were 58 per cent for tyrosine and 55 per cent for DA; the reported results were not corrected for the losses. The presence of any contaminating compound in the labelled phenylalanine substrate was checked by incubating either perchloric acid or Triton X-100 treated (tissue preparation in either 0.4 N perchloric acid or 0.1% final Triton concentration at 0° for 15 min) brain tissue samples with the labelled substrate and analyzing for the radioactivities in the purified tyrosine and DA fractions. Such blank radioactivities were quite low; tyrosin and DA radioactivities formed from the uninhibited human brain (Table 1) samples were between 5 and 50 times of those from the blanks. All the purified tyrosine and DA fractions were counted in model 100C Beckman liquid scintillation counter and the counting efficiencies were checked by internal standards. The results reported are the averages of 2–4 experimental values usually differing by less than 10 per cent.

The results in Table 1 show the radioactivities in DA and tyrosine formed from the incubation of [^{14}C]phenylalanine with the particulates from human brain samples. The results also indicate the effects of various agents and alterations of incubation conditions upon the product formations. DA products were 8.6 and 4.2 nCi/gm/hr from case 3 and 7 respectively and the corresponding tyrosine formations were 48.5 and 53.7 nCi/gm/hr. Alpha methyl *p*-tyrosine strongly (98 per cent and 87 per cent) inhibited the formations of DA and tyrosine (case 3). Potent (92 per cent and 45 per cent) inhibitory effects of tyrosine as well were indicated by the results (case 7). In one of our experiments (case 3), 1.7×10^{-5} M phenylalanine inhibited 74 per cent of DA formation from [^{14}C]tyrosine substrate suggesting mutual inhibition. Detergents are known to disrupt synaptosomes [13] and Triton X-100 pretreatment resulted in complete loss of DA forming activities. DA formation (case 3) was also potently blocked by the decarboxylase inhibitor compound NSD-1034 and the results indicated that the product which accumulated as Dopa could be, subsequent to its purification, decarboxylated to DA by the kidney decarboxylase preparations. The human samples in our studies (Table 1) had 3.5 hr and 29 hr postmortem delay of experiment and it is quite likely that enzyme activity loss may occur in that period. Although human and rat brain may not be strictly compared regarding such activity losses, we have carried out some preliminary studies with rat brain. Rat heads, following decapitation, were stored at 4° for varying periods and then assayed for product formation from phenylalanine by the standard method. The results (Table 1) indicate that some postmortem losses may occur; tyrosine formation was, compared to 0 hr postmortem delay, 98 per cent and 81 per cent after 5 hr and 17 hr respectively. DA formation was reduced to 87 per cent and 51 per cent at the corresponding periods.

It is apparent from the results (Table 1) that human brain tissue may form DA and its precursors, Dopa and tyrosine, from phenylalanine. In our previous studies [6, 12] demonstrating phenylalanine hydroxylation by rat brain, we have used various approaches to rule out non-enzymatic product formations. Several observations

* Some of the results were presented at the annual meeting of the American Society for Neurochemistry, March, 1975, at Mexico City, Mexico.

Table 1. DA formation from [^{14}C]phenylalanine incubation with human and rat brain particulates

Sample	Addition/ Condition	Product radioactivity (nCi/gm/hr) Tyrosine (%)	DA (%)
Human brain:			
Case No. 3	None	48.5 (100)	8.6 (100)
Age—49; Sex—M	MPT (3.2×10^{-4} M)*	6.3 (13)	0.2 (2)
Cause of death:	Triton X-100**	7.4 (15)	0.2 (2)
respiratory failure	DA (7×10^{-5} M)	17.8 (36)	1.0 (12)
Expt. 3.5 hr	NSD-1034 (4×10^{-4} M)†	61.6 (127)	0.2 (2)
postmortem.	23 hr at 0°‡	27.6 (57)	3.7 (43)
	No decarboxylase†	43.7 (90)	1.2 (14)
Human brain:			
Case No. 7	None	53.7 (100)	4.2 (100)
Age—80; Sex—F	Triton X-100**	19.7 (37)	0.2 (5)
Cause of death:	Tyrosine (3.1×10^{-4} M)	29.6 (55)	0.3 (8)
coronary arterio-sclerosis	18 hrs. at 0°‡	30.9 (58)	1.9 (45)
Expt. 29 hr postmortem.			
Rat brain ($n = 4$)	Expt. 0 hr postmortem	284.0 (100)	171.0 (100)
	Expt. 5 hr (at 4°) postmortem.	278.1 (98)	149.1 (87)
	Expt. 17 hr (at 4°) postmortem.	230.8 (81)	87.5 (51)

* Alpha methyl *p*-tyrosine

** Particulates in buffer kept at 0° for five min in 0.15% Triton X-100 before the incubation.

† Dopa fractions (Dowex-50 columns) isolated from these experiments were decarboxylated by the crude decarboxylase and purified as DA before radioactivity assay; case 3 had 13.7 nCi/gm/hr of Dopa in the presence of NSD-1034 and 5.1 nCi/gm/hr when decarboxylase was omitted.

‡ Whole homogenate kept at 0° for 18–23 hr before the separation of the particulates.

The particulates from human and rat brain were incubated with [^{14}C]phenylalanine under the standard conditions or any listed variation. The data indicate the formation of the products, DA, Dopa and tyrosine as radioactivity per gram wet tissue per hour. The final concentrations (micromolar) and specific radioactivities (nCi/nM) of phenylalanine in the incubation mixtures were: case 3, 11.4 and 27.2; case 7, 4.0 and 74.6; rat brain 11.7 and 18.2 respectively.

suggest that the appearance of products from phenylalanine in human brain is also enzymatic, (a). We have observed before [12] that tyrosine and DA formation from phenylalanine is strongly inhibited when rat brain particulates are pretreated with detergents as Triton X-100, sodium dodecyl sulphate, etc. In our present experiments, Triton X-100 pretreatment of the human brain particulates potently blocked tyrosine (63–85%) and DA (95–98%) formations (Table 1). We have also ensured that the presence of Triton X-100 did not affect our analyses of tyrosine and DA by actual recovery analyses of authentic labelled tyrosine and DA in the presence of the detergent. (b). In our present experiments, alpha methyl *p*-tyrosine, tyrosine and DA added to the incubation mixture markedly inhibited labelled tyrosine (45–87%) and DA (88–98%) formations as also we have observed [6, 12] with rat brain preparation; these compounds are not likely to be inhibitory towards a non-enzymatic product formation. (c). The results of our experiments (Table 1) with the Dopa decarboxylase inhibitor, NSD-1034, clearly indicate the enzymatic nature of the reaction. The radioactivity in DA was reduced to 2 per cent in the presence of this inhibitor. The accumulated Dopa, following its purification, could be decarboxylated to DA by the action of the decarboxylase. (d). There were activity losses of both human and rat brain upon storage (17–23 hr) at 0–4° (Table 1). Such activity decrement also support the enzymatic character of the product formation.

The actual nature of the enzyme responsible for tyrosine, Dopa and DA formations from phenylalanine has not been established in our present study. Of the three likely enzymes, tyrosine, tryptophan and phenylalanine hydroxylases, as discussed before [6], only tyrosine hydroxylase may however form Dopa from phenylalanine [14]. Further studies on the nature of the human brain enzyme are planned.

The present results suggest some broad similarities between the enzyme activities in human and rat brain. Human brain tissue, like that from rat [6, 12], may form Dopa and DA in addition to tyrosine, from phenylalanine substrate. Inhibition of the product formation in the presence of DA, alpha methyl *p*-tyrosine and tyrosine may be observed with both the tissues. Triton X-100 treatment of the particulates is as effective in blocking the rat brain activity [12] as it is towards that in human tissue. It is also possible that such comparisons of human to rat brain may be extended to cow, guinea-pig (our observations, not reported), mouse [8], monkey and cat [7] brains all of which have been observed to form Dopa and DA from phenylalanine. Our recent data [10] indicate that some psychoactive drugs, commonly employed for human use, may affect catecholamine formation from phenylalanine in rat brain. If indeed the human and rat brain activities are of the same nature, further studies of Dopa/DA formation in human brain may prove useful.

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Effect of parenteral dimethyl sulfoxide (DMSO) on drug metabolizing enzyme activities in the phenobarbital pretreated rat

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Since many substrates are nonpolar compounds, small volumes of a solvent or detergent are frequently utilized to enhance their aqueous solubilization. It is recognized that the addition of such organic solvents may stimulate or inhibit biotransformation studies involving the microsomal mixed function oxygenase enzyme system [1, 2] and, therefore, misinterpretation of biologic events may occur unless the solvent effect is determined. Because we were interested in investigating drug metabolizing enzyme activities associated with experimental hepatic injury, and DMSO was to be used as the solubilizing agent for a known hepatotoxin, we assessed the effect of the *in vivo* administration of DMSO upon hepatic microsomal enzyme activities in the phenobarbital pretreated rat.

Normal Sprague-Dawley rats (240–260 g), as well as those pretreated with phenobarbital (75 mg/kg/day) intraperitoneally for 4 days, were studied. They had free access to Purina Rat Chow and water, and were caged in a constant temperature room (22°) with alternating 12 hr of light and darkness. All experiments were done in the early morning. Animals were divided into the following groups: Group 1—normal rats received a single injection of 0.55 gm (0.5 ml) undiluted DMSO (Fisher Scientific, spectral grade) intraperitoneally 72 hr prior to sacrifice; Group 2—on the day 4 of the phenobarbital pretreatment, rats received a single injection i.p. of 0.55 gm undiluted DMSO and were sacrificed 72 hr later. Control rats (no DMSO injection) were also studied in each group.

Following decapitation, blood was collected for SGOT (Sigma Kit) and serum bilirubin [3] determinations. The liver was rapidly biopsied and specimen were fixed in formalin and stained with hemotoxylin and eosin for evaluation by light microscopy. The liver was then perfused *in situ* with 75 ml of ice cold 0.15 M sodium chloride and excised. Microsomes were prepared by differential centrifugation at 105,000 *g* for 60 min in a Beckman L2-65 B centrifuge. The suspended microsomal pellet obtained from the centrifugation was used for the following assays: Cytochrome P-450 content [4], aminopyrine demethylase [5], aniline hydroxylase [6], and bilirubin glucuronyl transferase [7]. Protein concentrations were determined by the method of Lowry *et al.* [8] using bovine albumin as standard.

Statistical indices were calculated by standard methods and are expressed as the mean ∓ SEM. A t test of the difference between two sample means was utilized to assess the significance between parameters [9]. P values equal to or less than 0.01 were considered significant.

DMSO had no effect on the drug metabolizing enzyme activity in the normal rats (Group 1). A previous report [10], utilizing larger doses of DMSO i.p. in normal Sprague-Dawley rats, demonstrated that aniline hydroxylase activity was increased within the first 24 hr, however, this had returned to control level by 48 hr. Further, there was no change in the cytochrome P-450 levels. Therefore, the results reported here are in agreement with this report as our assays were determined 72 hr after the lower dose of DMSO administration.

The phenobarbital pretreated rats, however, responded differently to the DMSO. The cytochrome P-450 content, and the activities of aminopyrine demethylase and aniline hydroxylase were reduced by 27, 32 and 19 per cent, respectively (Table 1). All reductions were significantly different from the phenobarbital controls. To determine whether the reduction of either the aminopyrine demethylase or aniline hydroxylase activities were disproportionate to the reduction in cytochrome P-450 content, we calculated the ratio of the enzyme activity per nmole of cytochrome P-450 [11]. For aminopyrine demethylase, the ratio was essentially the same in the phenobarbital control and DMSO treated animals; 3.4 and 3.2 respectively. Similarly, the ratios for aniline hydroxylase were essentially the same (0.55) and 0.62) between control and DMSO treated animals. This would imply that the reduced enzyme activities were largely related to a diminished cytochrome P-450 content, and not due to specific alteration of the two enzyme activities. We did assess, however, the substrate spectral binding curve for DMSO in rat microsomes, and this revealed a modified Type II with a lambda maximum at 417 nm and lambda minimum at 395 nm [12]. Thus, the DMSO could competitively inhibit at both Type I (aminopyrine) and Type II (aniline) substrate binding sites