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FORMATION OF CATECHOLAMINES FROM PHENYLALANINE IN BRAIN—EFFECTS OF CHLORPROMAZINE AND CATRON*†

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Abstract—Phenylalanine hydroxylation in brain was studied *in vitro* and *in vivo*. The products were tyrosine and catecholamines. Dopamine was the major catecholamine formed in the region of caudate nucleus *in vivo*. Brainstem and hypothalamus formed a significant amount of norepinephrine. Excluded was the possibility that the hepatic hydroxylation of phenylalanine may contribute to the products present in brain in our experiments *in vivo*. Among various inhibitors of the hydroxylation *in vitro*, catechol compounds were found to be quite active. Tyrosine and phenylalanine mutually inhibited the hydroxylation *in vitro*. Pretreatment of the rats with the monoamine oxidase (MAO) inhibitor Catron strongly depressed the hydroxylation *in vivo*, and inhibition of the hydroxylation *in vitro* resulted after the addition of dopamine. Pre-treatment of the rats with chlorpromazine was found to stimulate the formation of catecholamines from phenylalanine in the caudate nucleus *in vivo* but not in the brainstem. After the hydroxylation of a mixture of ^{14}C-phenylalanine and ^{3}H-tyrosine by the homogenate of caudate nucleus, the ratio of ^{14}C to ^{3}H present in the tyrosine differed significantly from the ratio of the two isotopes present in the catecholamines. The results indicate that: (a) phenylalanine may be actively hydroxylated by brain tissue both *in vivo* and *in vitro* to form tyrosine and catecholamines, (b) the hydroxylation of phenylalanine *in vivo* may be controlled by a negative feedback-like mechanism, (c) chlorpromazine may stimulate catecholamine formation from phenylalanine in the caudate nucleus and (d) phenylalanine may serve as a primary precursor of catecholamines in brain.

WE HAVE reported earlier that labeled norepinephrine was formed in brainstem *in vivo* after intraventricular injection of ^{14}C-phenylalanine.[1] While investigating the hydroxylation of phenylalanine to tyrosine *in vitro* by brain areas, we have observed[2] a significant formation of dopamine as well from ^{14}C-phenylalanine when incubated with the homogenate of caudate nucleus. Because of the implication of brain catecholamines with various neurological diseases and emotional states, we decided to investigate further the formation of catecholamines from phenylalanine in brain both *in vivo* and *in vitro*. The high hydroxylating activity of caudate nucleus region to form dopamine *in vitro* suggested the selection of this brain area for the present studies. We have investigated the effect of chlorpromazine (CPZ) pretreatment on the catecholamine formation from phenylalanine in caudate nucleus, since various studies[3–5] indicate that this widely used drug may affect the metabolism of dopamine,

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the major catecholamine present in caudate nucleus. It has been demonstrated that the inhibition of monoamine oxidase (MAO) also affects the synthesis of brain catecholamines,[6] possibly via a feedback inhibition mechanism. We have, therefore, conducted experiments to study any effect that Catron (pheniprazine), a known potent inhibitor of MAO, may have on the cerebral hydroxylation of phenylalanine leading to the synthesis of catecholamines.

Our preliminary results[2] have suggested that the brain enzyme system(s) hydroxylating phenylalanine may have some properties similar to those of the enzyme tyrosine hydroxylase, also present in brain. Therefore, for the purpose of identifying any common properties or any dissimilarities of these two hydroxylation reactions, we have duplicated a number of our experiments substituting labeled tyrosine for the labeled phenylalanine.

MATERIALS AND METHODS

Animals. Female Wistar rats (160–180 g) were used for all the experiments. The animals were fasted overnight to minimize the fluctuation of free phenylalanine and tyrosine in brain.

Labeled compounds and drugs. Uniformly labeled L-phenylalanine and L-tyrosine (specific radioactivity 350–400 mCi/m-mole) was purchased from New England Nuclear, Boston, Mass. Labeled dopa (DL-3(3,4-dihydroxyphenyl)alanine-2-^{14}C, specific radioactivity 52 mCi/m-mole) and tritium labeled tyrosine (L-*p*-hydroxyphenyl(alanine-2,3-^{3}H), specific radioactivity 5000 mCi/m-mole) was obtained from Amersham/Searle Corp., Don Mills, Ontario. Chlorpromazine (Largactil, Poulenc Ltd., Montreal) was purchased locally. Lakeside Laboratories, Milwaukee, Wis., kindly supplied Catron (pheniprazine).

Experiments in vitro. The method for experiments *in vitro* is essentially the same as described before.[2] Briefly, dissected areas of brain were homogenized in ice-cold 0·1 M sodium phosphate buffer at pH 6·0 and aliquots of the homogenates were incubated at 37° with either ^{14}C-phenylalanine or ^{14}C-tyrosine and the standard additions of 2-mercaptoethanol and pargyline hydrochloride. Additionally, the following modification was employed. At the end of the incubation period, to each tube was added 0·1 ml of a solution containing crude dopa decarboxylase enzyme and 0·7 ml of a 0·5 M sodium phosphate buffer (pH 7·0) containing sufficient L-phenylalanine and L-dopa to achieve concentrations of 1 mM and 0·01 mM, respectively, of the amino acids. Shaking of the samples was continued for another 15 min and then 1·5 ml of ice-cold 0·8 N perchloric acid solution was added to each tube to stop the enzyme reactions. This modification ensured the complete decarboxylation of any labeled dopa formed during the hydroxylation of phenylalanine or tyrosine, that might not have been converted to dopamine due to the relative weak concentration of dopa decarboxylase in brain tissue. The crude preparation of dopa decarboxylase enzyme was obtained for this purpose by precipitating the enzyme from 10,000 *g* supernatant of rat kidney homogenate between 37 and 55 per cent ammonium sulphate concentration as described by Clark *et al.*[7] The crude enzyme preparation was kept frozen in small aliquots in the presence of 10^{-3} M pyridoxal phosphate. Frequently, the activity of this enzyme preparation was checked by substituting 2-^{14}C-dopa for ^{14}C-phenylalanine in our standard incubation mixture and measuring the formation of ^{14}C-dopamine. All the results of the studies *in vitro*

are the averages of 2–3 experimental values differing usually by no more than 10 per cent.

Experiments in vivo. For the experiments *in vivo*, solution of labeled phenylalanine or tyrosine in isotonic saline was directly injected into the cerebral ventricle of the rat under ether anesthesia as described before.[1] The only modification employed was that the solution was administered by slow infusion. The plunger of the microsyringe holding 40 μl of the labeled amino acid solution was slowly pushed by a home made hydraulic device driven at a constant speed by a compact infusion pump (model 975, Harvard Apparatus Company, Willis, Mass.). The radioactive solution was delivered into the cerebral ventricle at the rate of 10 μl/25 sec. At the end of the infusion period, the animal was allowed to recover from the anesthesia within 10–20 sec. Fifteen min after the start of the infusion, the brain was removed following quick decapitation and immediately chilled in ice-cold 0·25 M sucrose. The required brain areas were then dissected in the cold and after wiping off excess fluid, weighed and extracted twice with cold 0·4 N perchloric acid by homogenization and centrifugation.

Analytical methods. The analysis of tissue extract was carried out as described before.[2] The procedure involved initial fractionation on Dowex-50 ion-exchange column to separate the acids, neutrals (amino acids) and the amines. The column effluent containing the acidic compounds was concentrated to 2–3 ml under vacuum at 60–70° after the addition of 100 mg of ascorbic acid and 100 μg of 3,4-dihydroxyphenylacetic acid (dopac). The concentrate was then saturated with sodium chloride, adjusted to pH 2 and shaken with 10 ml of *n*-butyl acetate to extract the dopac. The organic layer was then evaporated to dryness at 50° under nitrogen. The dopac present in the residue was separated by chromatography on 3 MM paper employing benzene–acetic acid–water (125:72:3) solvent mixture for 6 hr. The area containing dopac was located by spraying with diazotized sulphanilic acid spray reagent. The formed chromophore was eluted with 2·0 ml of water and assayed for radioactivity in a scintillation counter after adding the scintillation fluid Aquasol (New England Nuclear, Boston, Mass.). The methods employed for the separation of phenylalanine, tyrosine, dopamine and norepinephrine and the assay of radioactivity present in these fractions are as described before.[2] Briefly, a combination of ion-exchange and paper chromatography was used for phenylalanine and tyrosine separation. Ion-exchange analysis followed by acid alumina chromatography was utilized for the catecholamines. Where necessary, dopamine and norepinephrine were separated by paper chromatography of the acetic acid eluate of the alumina column.

Assay of endogenous phenylalanine and tyrosine. Endogenous phenylalanine was assayed by a micro adaptation of the fluorometric method of McCaman and Robins.[8] Our modified method separates phenylalanine from the glutamic acid which interferes with the assay, and achieves a concentration of phenylalanine suitable for the application of the fluorometric assay starting with small brain regions. Details of this method will be published elsewhere. Endogenous tyrosine was assayed by the fluorometric method[9] after initial fractionation and concentration of this amino acid employing ion-exchange column.

Assay of ^{14}C and ^{3}H in the double labeled samples. The determination of the radioactivity of either a single labeled or double labeled ($^{14}C/^{3}H$) sample was done using a liquid scintillation counter (model LS-100; Beckman Instruments Ltd., Pasadena).

Wide open window-setting was used for single isotope counting. For the sample containing both ^{3}H and ^{14}C, narrow windows were employed which afforded very little spillover (0·3 per cent) of ^{3}H into the ^{14}C channel. The spillover of ^{14}C into the ^{3}H channel was, however, significant (19 per cent) and internal standards were employed for accurate calculation of the spillover. However, each of our double labeled samples contained much more ^{3}H than ^{14}C thereby reducing the effect of ^{14}C spillover into the ^{3}H channel. After the radioactivity assay of a double labeled sample, the ratio (R) of ^{14}C to ^{3}H ($R = {}^{14}C/{}^{3}H$) was calculated.

RESULTS

Formation of catecholamines from ^{14}C-phenylalanine in brain in vivo. Solution of either ^{14}C-phenylalanine or ^{14}C-tyrosine was infused into the lateral ventricle of rat brain and after 15 min brain areas were analyzed as described in Materials and Methods. The results are summarized in Table 1. The data indicate that significant

TABLE 1. CEREBRAL FORMATION OF LABELED CATECHOLAMINES AND TYROSINE FROM ^{14}C-PHENYLALANINE

Brain region	Expt. No.	Unchanged precursor (nCi/g)	^{14}C in the products (nCi/g)		Relative radioactivity (dopamine/ norepinephrine)
			Tyrosine	Catechol-amines	
		Intraventricular ^{14}C-phenylalanine*			
Caudate nucleus	1	427·0	12·0	3·7	90/10
	2	471·0	12·5	3·2	
Brainstem	1	329·0	12·9	2·2	40/60
	2	291·0	17·5	1·8	
Hypothalamus	1	1014·0	21·0	3·0	40/60
	2	1471·0	25·8	3·0	
		Intraventricular ^{14}C-tyrosine*			
Caudate nucleus	1	942·0		35·6	
	2	1000·0		24·3	
Brainstem	1	569·0		7·8	
	2	671·0		8·9	
		Intraperitoneal ^{14}C-phenylalanine†			
Caudate nucleus	1	7·1	0·7	0	
	2	5·2	1·0	0	
Brainstem	1	6·8	0·8	0	
	2	5·0	1·1	0	

* Eight μCi of the amino acid in 40 μl of saline was injected into the cerebral ventricle.
† Eight μCi of ^{14}C-phenylalanine in 0·5 ml of saline was given intraperitoneally.

formation of labeled tyrosine and catecholamines, both dopamine and norepinephrine, occurs in brain from ^{14}C-phenylalanine and that the hydroxylation of phenylalanine is not limited to any specific area of brain. Brain tissue also catalyzes the formation of catecholamines from tyrosine[10] and, for comparative purposes, we have determined the concentration of labeled catecholamines in brain after the intraventricular administration of ^{14}C-tyrosine under experimental conditions identical with that employed for the administration of ^{14}C-phenylalanine. The results indicate that, after ^{14}C-

tyrosine administration, a large concentration of labeled catecholamines was present in the brain areas. It is also indicated that the concentration of the residual ^{14}C-tyrosine precursor was approximately twice that of the residual ^{14}C-phenylalanine after the injection of the latter.

Since liver tissue contains phenylalanine hydroxylase which can hydroxylate phenylalanine, we have considered the remote possibility of the following sequence of events. The ^{14}C-phenylalanine administered into the cerebral ventricle may reach liver via bloodstream, then be converted to ^{14}C-tyrosine by the action of hepatic phenylalanine hydroxylase and subsequently the ^{14}C-tyrosine may be transported to cerebral tissue. This ^{14}C-tyrosine and any catecholamines formed from it by the action of brain tyrosine hydroxylase[10] may then be present in the cerebral tissue without any hydroxylation of ^{14}C-phenylalanine actually occurring in brain. To test this possibility, we have injected intraperitoneally 8 μCi of ^{14}C-phenylalanine, the same radioactivity as was used for the intraventricular injections. The ^{14}C-phenylalanine thus injected is directly taken up by the hepatic tissue according to the findings of Lukas *et al.*[11] and is expected to be rapidly converted to ^{14}C-tyrosine by the hepatic phenylalanine hydroxylase. Our results (Table 1) show that, after the i.p. administration of the labeled phenylalanine, very little ^{14}C was present in the isolated brain tyrosine and the catecholamines did not contain detectable radioactivity. It is, therefore, apparent that even with a direct uptake of the ^{14}C-phenylalanine by the hepatic tissue, very little of the ^{14}C-tyrosine formed there may reach brain during the short term of our experiments.

Hydroxylation of phenylalanine by brain homogenate. Pooled caudate tissue obtained from several rats was homogenized in phosphate buffer and then incubated in the presence of various additions as described in Materials and Methods. The results in Table 2 indicate the effects of various additions on the formation of tyrosine and catecholamines from phenylalanine *in vitro*. The well-known tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine, almost completely inhibited the formation of tyrosine and catecholamines. The inhibitor of L-dopa decarboxylase, NSD-1034, completely inhibited the formation of labeled catecholamines (Table 2) without significantly affecting the formation of labeled tyrosine. Radioactivity, representing the catecholamines, was however found to be present in dopa isolated from these incubation samples. Among the various other additions tested, catechol compounds, dopa and dopamine, at 5×10^{-5} M concentration strongly inhibited (72 and 82 per cent, respectively) the catecholamine formation from ^{14}C-phenylalanine which suggested the participation of a pteridine-like cofactor.[12] Of the two aromatic amino acids, tryptophan and tyrosine, which are also hydroxylated by brain tissue,[10,13] tryptophan did not have any noticeable effect but tyrosine was found to be a potent inhibitor (Table 2). We have also tested the effect of the addition of L-phenylalanine on the hydroxylation of ^{14}C-tyrosine by the same system and the results indicate significant inhibition as reported before.[10]

We have determined the hydroxylation of phenylalanine and tyrosine by the same preparation at various substrate concentrations and the results (Table 2) show that the hydroxylation of phenylalanine by caudate homogenate continued to increase up to $2{\cdot}02 \times 10^{-4}$ M substrate concentration, the highest we have tested. In contrast, tyrosine hydroxylation reached maximum only at $2{\cdot}6 \times 10^{-5}$ M and a slight inhibition was observed at $2{\cdot}0 \times 10^{-4}$ M. A similar observation has been made by

TABLE 2. HYDROXYLATION OF PHENYLALANINE TO TYROSINE AND CATECHOLAMINES BY CAUDATE NUCLEUS HOMOGENATE—EFFECTS OF VARIOUS ADDITIONS*

Substrate concn (10^{-5} M)	Addition		Products (nmoles/g tissue/hr)		
	Compound	Concn (10^{-5} M)	Tyrosine (%)	Catechol-amines (%)	Total (%)
		Labeled substrate: ^{14}C-phenylalanine			
0·8	None		9·1 (100%)	5·7 (100%)	14·8 (100%)
0·8	None†		0·6 (7%)	0 (0%)	0·6 (4%)
0·8	MPT‡	5	1·6 (18%)	0·06 (1%)	1·7 (11%)
0·8	Dopa	5	4·0 (44%)	1·6 (28%)	5·6 (37%)
0·8	Dopamine	5	3·6 (40%)	1·0 (18%)	4·6 (31%)
0·8	Tyrosine	4	2·3 (25%)	0·2 (4%)	2·5 (16%)
0·8	Tryptophan	5	7·0 (77%)	4·9 (86%)	11·9 (80%)
0·8	NSD-1034	40	11·5 (126%)	0 (0%)	11·5 (77%)
5·8	None		23·3 (100%)	5·7 (100%)	29·0 (100%)
5·8	None§		2·6 (11%)	0 (0%)	2·6 (8%)
5·8	None‖		11·9 (51%)	4·2 (74%)	16·1 (55%)
5·8	None¶		13·3 (57%)	4·0 (71%)	17·3 (59%)
5·8	NSD-1034	40	21·0 (90%)	0·3 (5%)	21·3 (73%)
5·8	Tyrosine	2·6	8·4 (36%)	0·6 (11%)	9·0 (31%)
20·2			37·7 (161%)	5·1 (89%)	42·8 (147%)
		Labeled substrate: ^{14}C-tyrosine			
2·6	None			25·7 (100%)	
2·6	Phenylalanine	2·9		16·7 (65%)	
2·6	Phenylalanine	5·8		11·6 (45%)	
10·3	None			26·8 (104%)	
20·0	None			19·6 (76%)	

* The expressed final concentrations of phenylalanine and tyrosine take into account the endogenous levels. The reported values of the products are not corrected for the blank values.
† Tissue omitted (blank experiment).
‡ DL,α-Methyl-*p*-tyrosine.
§ Caudate homogenate substituted by identically prepared spleen homogenate.
‖ Halved tissue, i.e. 25 mg employed.
¶ Halved incubation period, i.e. 15 min used.

Shiman *et al.*[14] while studying the hydroxylation of phenylalanine and that of tyrosine by highly purified adrenal tyrosine hydroxylase in the presence of tetrahydrobiopterin. Their data indicate a low saturation concentration of tyrosine substrate in contrast to a high value when phenylalanine is the substrate of the adrenal enzyme. A double reciprocal plot of phenylalanine concentration against the total phenylalanine hydroxylation (nanomoles of tyrosine product plus 2 × nanomoles of catecholamines product) by our caudate homogenate system was linear (Fig. 1) up to $2{\cdot}02 \times 10^{-4}$ M and the approximate K_m value was 5×10^{-5} M.

Phenylalanine hydroxylation in rat brain after Catron pretreatment. Two different drug treatment schedules have been used for these experiments. For the purpose of achieving a significant and sustained build-up of the brain catecholamines, Catron (10 mg/kg, i.p.) was given to a group of rats 18 hr and 2 hr prior to the intraventricular injection of labeled phenylalanine. Another group of rats, which was used for a relatively shorter term MAO inhibition, received only one injection of Catron

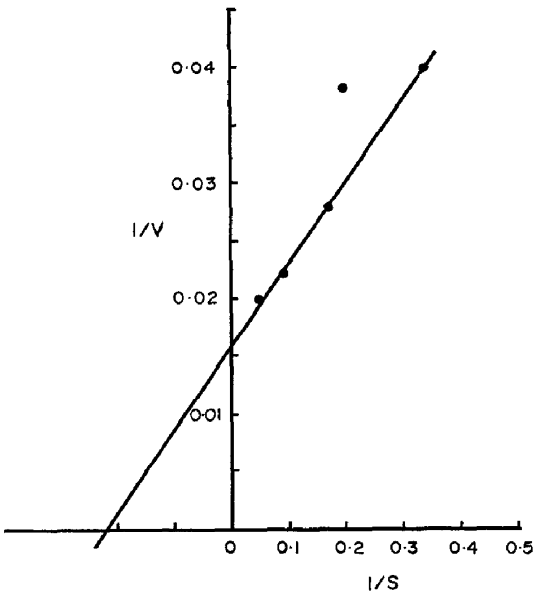

FIG. 1. Homogenate of pooled caudate nuclei was incubated at pH 6·0 for 30 min with 110,000 dis/min of ^{14}C-phenylalanine, mercaptoethanol, pargyline and various concentrations of phenylalanine. The methods for the incubation and for the determination of the products, ^{14}C-tyrosine and ^{14}C-catecholamines, were as described in Materials and Methods. The results are plotted as 1/v (v = nmoles of tyrosine per g tissue per hr plus 2 × nmoles of catecholamines per g tissue per hr) against 1/s (s = phenylalanine concentration, 10^{-5} M). Each point represents the average of 3–5 experiments.

(10 mg/kg, i.p.) 2 hr prior to the ^{14}C-phenylalanine administration and the control group received saline at the same time. Eight μCi of ^{14}C-phenylalanine was given intraventricularly, and after 15 min brain areas were dissected and analyzed to determine the products, labeled tyrosine and catecholamines, as described in Materials and Methods. For comparative purposes, ^{14}C-tyrosine solution was injected into the cerebral ventricle of a number of rats pretreated with Catron, and the radioactivity in the resulting labeled catecholamines was measured. The data (Table 3) show that the concentration of labeled catecholamines in the Catron-pretreated groups receiving ^{14}C-tyrosine was quite low and amounted to only 19 and 26 per cent of that present in the corresponding control group brain. These results suggest that significant reduction of the formation of catecholamines from tyrosine precursor may occur after MAO inhibition. Our present results (Table 3), furthermore, indicate that the concentration of labeled catecholamines when ^{14}C-phenylalanine was employed as the precursor was also sharply reduced (9–20 per cent of the corresponding control group value) after MAO inhibition. A parallel reduction of the formation of labeled tyrosine was also observed (31–35 per cent of the control value). Catron pretreatment does not appear to have any noticeable effect on either the endogenous concentration of the amino acids or the level of the residual ^{14}C-phenylalanine.

Inhibition of phenylalanine hydroxylation by dopamine in vitro. Pooled caudate nuclei from a number of rats were homogenized in phosphate buffer at pH 6·0 and incubated in the presence of ^{14}C-phenylalanine, various concentrations of dopamine

TABLE 3. LABELED TYROSINE AND CATECHOLAMINES IN THE BRAIN OF CATRON-PRETREATED RATS AFTER THE INTRAVENTRICULAR INJECTION OF ^{14}C-PHENYLALANINE

Brain region	Pre-treatment* (N)	^{14}C (nCi/g)			Endogenous concn (nmoles/g)	
			Products			
		Residual precursor	Tyrosine	Catechol-amines	Phenyl-alanine	Tyrosine
		Intraventricular ^{14}C-phenylalanine				
Caudate nucleus	Saline (N = 5)	459·8 ± 32·7† (100%)	12·0 ± 1·4 (100%)	3·1 ± 0·9 (100%)	105·5	101·5
	Catron, 2 × (N = 5)	570·2 ± 108·0 (124%)	3·7 ± 0·6‡ (31%)	0·28 ± 0·0‡ (9%)	101·8	89·0
	Catron, 1 × (N = 4)	493·8 ± 120·0 (107%)	4·1 ± 1·0§ (34%)	0·54 ± 0·26‡ (17%)		
Brainstem	Saline (N = 5)	320·0 ± 38·6 (100%)	15·8 ± 1·8 (100%)	1·5 ± 0·3 (100%)	81·8	71·1
	Catron, 2 × (N = 7)	353·5 ± 69·8 (100%)	4·9 ± 0·4‡ (31%)	0·22 ± 0·02‡ (15%)	60·6	67·7
	Catron, 1 × (N = 4)	263·3 ± 53·0 (82%)	5·5 ± 1·3§ (35%)	0·30 ± 0·08‡ (20%)		
		Intraventricular ^{14}C-tyrosine				
Caudate nucleus	Saline (N = 5)	913·0 ± 58·0 (100%)		34·9 ± 2·8 (100%)		
	Catron, 2 × (N = 4)	681·0 ± 58·0 (75%)		6·6 ± 0·2‖ (19%)		
	Catron, 1 × (N = 4)	617·0 ± 125·0 (68%)		9·1 ± 1·7‖ (26%)		

* Pretreatments: saline at 2 hr; Catron 2 ×, 10 mg/kg at 18 hr and 2 hr; and Catron 1 ×, 10 mg/kg at 2 hr before the sacrifice.
† The values are expressed as the average ± S.E.M.
‡ $P < 0·001$ (saline vs drug-treated).
§ $P < 0·005$ (saline vs drug-treated).
‖ $P < 0·05$ (saline vs drug-treated).

and the standard additions as described in Materials and Methods. The formation of labeled tyrosine and catecholamines was determined after the incubation. The results (Table 4) show that dopamine added *in vitro* may inhibit the formation of both the products of phenylalanine hydroxylation, tyrosine and catecholamines. The inhibition, based on the total products formed, was 23 per cent at 10^{-5} M dopamine, 59 per cent at 5×10^{-5} M and 69 per cent at 10^{-4} M, the highest concentration of dopamine tested. At a dopamine concentration of 10^{-4} M, the formation of labeled catecholamines was almost completely blocked, only 15 per cent of the control.

Effect of CPZ on the formation of catecholamines from phenylalanine in vivo. Two groups of rats received intraventricular injections of ^{14}C-phenylalanine solution as described in Materials and Methods. One group received CPZ (22 mg/kg; i.p.) and the other group saline 2 hr before the labeled amino acid administration. Following decapitation 15 min after the intraventricular injection, the brain areas were dissected and analyzed. Similar experiments were performed substituting ^{14}C-tyrosine for the labeled phenylalanine with groups of CPZ and saline-treated rats. The results are summarized in Table 5. The data indicate that there was a large increase (516 per cent

TABLE 4. INHIBITION OF THE HYDROXYLATION OF PHENYLALANINE *in vitro* BY DOPAMINE*

Dopamine added (10^{-5} M)	Products (nmoles/g/hr)		
	Tyrosine	Catecholamines	Total
0	23·3 (100%)	5·7 (100%)	29·0 (100%)
1	18·6 (80%)	3·8 (67%)	22·4 (77%)
5	10·7 (46%)	1·1 (20%)	11·8 (41%)
10	8·1 (35%)	0·8 (15%)	8·9 (31%)

* Homogenate of pooled caudate nuclei was incubated at pH 6·0 as described in Materials and Methods. The substrate concentration was $5·8 \times 10^{-5}$ M and dopamine was added at the beginning of the incubation.

of the control value) of the concentration of labeled catecholamines formed from ^{14}C-phenylalanine in the caudate after CPZ treatment. No significant increase in the concentration of labeled catecholamines in the brainstem of the same animals could be observed and, if anything, a slight reduction (66 per cent of the control value) took place. CPZ pretreatment did not noticeably affect either the uptake of ^{14}C-phenylalanine or the formation of labeled tyrosine in any of the brain regions. Similarly unchanged was the endogenous concentration of phenylalanine and that of tyrosine after CPZ treatment. In the parallel experiments employing ^{14}C-tyrosine as the precursor of catecholamine, CPZ did not have a great effect on the formation of labeled catecholamines (Table 5) in the caudate nucleus. Therefore, the only CPZ-induced change that was observed in our experiments was the increase in the concentration of labeled caudate catecholamines after ^{14}C-phenylalanine injection.

Effect of CPZ pretreatment on the phenylalanine hydroxylation in vitro. A group of rats was given CPZ (22 mg/kg; i.p.) and 2 hr later the rats were decapitated. The dissected caudate nuclei were pooled and homogenized in phosphate buffer. The homogenate was then incubated with ^{14}C-phenylalanine and the standard additions, after which the products formed, catecholamines and tyrosine, were isolated and assayed for radioactivity as described in Materials and Methods. The results which are summarized in Table 6 show that the formation of tyrosine (21·4 nmoles/g/hr) and that of the catecholamines (5·7 nmoles/g/hr) from phenylalanine *in vitro* by the CPZ-pretreated rat caudate was not significantly different from the formation of tyrosine and that of the catecholamines, respectively, by the untreated rat caudate. This observed absence of any effect of CPZ pretreatment may mean that this drug or any of its metabolites present in caudate at the time of decapitation may not have a direct effect *in vitro* on the enzyme system hydroxylating phenylalanine.

Hydroxylation of a mixture of ^{14}C-phenylalanine and ^{3}H-tyrosine in vitro. Caudate nuclei, obtained from rats which had received no pretreatment, were pooled and homogenized in phosphate buffer. Aliquots of the homogenate were incubated with a mixture of 132,000 dis/min of ^{14}C-phenylalanine (uniformly labeled) and 231,000

Table 5. Labeled tyrosine and catecholamines in the brain of CPZ-pretreated rats after the intraventricular injection of ^{14}C-phenylalanine

Brain region (N = 5)	Pretreatment	Residual precursor (nCi/g)	^{14}C in the products (nCi/g)			Endogenous concn (nmoles/g)	
			Tyrosine	Catecholamines	Dopac	Phenylalanine	Tyrosine
			Intraventricular ^{14}C-phenylalanine				
Caudate nucleus	Saline*	459·8 ± 32·7 (100%)	12·0 ± 1·4 (100%)	3·1 ± 0·9 (100%)	0·5 ± 0·1 (100%)	105·5	101·5
	CPZ†	576·4 ± 36·6 (125%)	16·3 ± 1·9 (135%)	16·0 ± 4·9‡ (516%)	1·2 ± 0·0§ (225%)	98·1	90·6
Brainstem	Saline*	320·0 ± 38·6 (100%)	15·8 ± 1·8 (100%)	1·5 ± 0·3 (100%)		81·8	71·1
	CPZ	351·3 ± 66·5 (110%)	14·6 ± 2·5 (92%)	1·0 ± 0·2 (66%)		84·2	81·4
			Intraventricular ^{14}C-tyrosine				
Caudate nucleus	Saline*	913·0 ± 58·0 (100%)		34·9 ± 2·8 (100%)	13·4 ± 2·4 (100%)		
	CPZ	625·0 ± 37·7 (68%)		35·6 ± 5·0 (102%)	14·2 ± 2·7 (106%)		
Brainstem	Saline	588·0 ± 62·0 (100%)		8·4 ± 1·0 (100%)			
	CPZ	535·0 ± 134·3 (91%)		5·0 ± 1·1 (60%)			

* Saline given, i.p., 2 hr before the sacrifice. Data from Table 3.
† CPZ given, i.p., 22 mg/kg, 2 hr before the sacrifice.
‡ $P < 0.08$ (saline vs drug-treated).
§ $P < 0.001$ (saline vs drug-treated).

TABLE 6. HYDROXYLATION OF PHENYLALANINE *in vitro* BY CAUDATE NUCLEUS PRETREATED WITH CPZ *in vivo**

Caudate from rats	Products (nmoles/g/hr)		
	Tyrosine	Catecholamine	Total
Untreated†	23·3 (100%)	5·7 (100%)	29·0 (100%)
CPZ treated	21·4 (92%)	5·3 (93%)	26·7 (92%)

* Rats were given CPZ (22 mg/kg; i.p.) and 2 hr later decapitated. The dissected caudate nuclei were pooled, homogenized and incubated at pH 6·0 in presence of $5{\cdot}8 \times 10^{-5}$ M labeled phenylalanine as described in Materials and Methods.

† Data from Table 4.

dis/min of ^{3}H-tyrosine (side chain labeled at carbons 2 and 3) after adding the standard additions (see Materials and Methods). To avoid dilution of the labeled substrates, no carrier phenylalanine or tyrosine was added to the incubation mixture; thus maximum radioactivity was obtained in the hydroxylation products which facilitated double isotope counting. The final concentrations of the substrates in the incubation mixture were determined by the free phenylalanine and tyrosine present endogenously in the tissue and were $8{\cdot}4 \times 10^{-6}$ M for phenylalanine and $8{\cdot}1 \times 10^{-6}$ M for tyrosine. The method for the separations of tyrosine and catecholamines and the determination of the R value after double isotope radioassay were as described (see Materials and Methods). Any possibilities of either non-enzymatic formation of labeled products or the presence of catechol-like impurities in the labeled substrates were checked. The results (Table 2) of the studies employing ^{14}C-phenylalanine establish the purity of this substrate. When the ^{3}H-tyrosine was incubated with a heated tissue homogenate sample (75 sec at 100°) and the standard additions, the radioactivity in the isolated catecholamines was extremely low (2 times the scintillation counter background) and was only 0·2 per cent of the radioactivity in the catecholamines obtained from a test incubation employing caudate homogenate as the enzyme source.

The results of the determination of the R values of tyrosine and catecholamines after the incubation of caudate homogenate with mixed ^{14}C-phenylalanine and ^{3}H-tyrosine are summarized in Table 7. The results show that the R values of catecholamines in three experiments were 6·66/100, 6·24/100 and 6·90/100 and were significantly higher than the R values of tyrosine, 2·44/100, 2·21/100 and 2·47/100. It should be considered that unlike the radioactivity of ^{3}H-tyrosine, which remains virtually the same during the incubation period, the radioactivity of ^{14}C-tyrosine formed from ^{14}C-phenylalanine starts at zero and attains a maximum value near the end of the incubation period. It follows therefore that the R value of tyrosine is likely to be very small at the beginning of the incubation period and may reach the maximum value near the end, the value that was found by the analysis (Table 7). The average of the R values of tyrosine at various time points during the incubation is therefore less than the experimentally found R value. The R value of catecholamines, however, could have been higher, if not for the extra hydroxylation required

TABLE 7. HYDROXYLATION OF A MIXTURE OF ^{14}C-PHENYLALANINE AND ^{3}H-TYROSINE BY CAUDATE NUCLEUS HOMOGENATE; THE ISOTOPE RATIO (R = ^{14}C/^{3}H) IN TYROSINE AND IN CATECHOLAMINES*

Expt. No.	R, tyrosine	R, catecholamines	
		Calculated†	Actually found
1	2·44/100	< 2·44/100	6·66/100
2	2·21/100	< 2·21/100	6·24/100
3	2·47/100	< 2·47/100	6·90/100

* Homogenate of caudate nucleus was incubated at pH 6·0 with a mixture of 132,000 dis/min of ^{14}C-phenylalanine and 231,000 dis/min of ^{3}H-tyrosine and the standard additions of mercaptoethanol and pargyline. The methods employed for the incubation, analysis and the determination of R were as described in Materials and Methods. The final concentration of phenylalanine and tyrosine was $8{\cdot}4 \times 10^{-6}$ M and $8{\cdot}1 \times 10^{-6}$ M respectively.

† Calculated value is based on the assumption that the formation of dopa (catecholamines) occurred after the mixing of the ^{3}H-tyrosine present and the ^{14}C-tyrosine formed *in situ* from ^{14}C-phenylalanine.

for the synthesis of ^{14}C-catecholamines and for the accumulation of ^{14}C-tyrosine that must precede that synthesis.

DISCUSSION

It is apparent from the results in Table 1 that phenylalanine may be hydroxylated to form tyrosine and catecholamines *in vivo* in various brain areas. It was also observed, when the individual catecholamines were separated and assayed for radioactivity, that the preponderance of the specific catecholamines, dopamine in caudate and norepinephrine in the brainstem, is in general agreement with the known distribution[15] of the endogenous catecholamines. For comparative purposes, the hydroxylation of ^{14}C-tyrosine was studied (Table 1) under the same conditions. If the radioactivities of the residual ^{14}C-phenylalanine and ^{14}C-tyrosine may be taken as rough indicators of the precursor specific radioactivities, then it appears that the total hydroxylation of phenylalanine in cerebral tissues may be as large as that of tyrosine if not actually larger. It should also be borne in mind that the formation of a catecholamine molecule from tyrosine involves one ring hydroxylation which is half as required to form a catecholamine from phenylalanine.

The results in Table 2 showing the inhibitory effects of several compounds, dopamine, α-methyl-*p*-tyrosine, etc., clearly demonstrate the enzymatic nature of the hydroxylation reaction *in vitro* in support of our previous[2] findings. Furthermore, the addition of NSD-1034 completely inhibited the formation of labeled catecholamines. The radioactivity of the isolated catecholamines was evidently due to the decarboxylation of labeled L-dopa and thus the absolute stereospecificity of the decarboxylase[16] further supports the enzymatic nature of the hydroxylation. The results of studies *in vitro* also indicate that L-tyrosine and L-phenylalanine are mutually inhibitory toward their hydroxylation by the caudate nucleus homogenate. It is possible, therefore, that the hydroxylation of phenylalanine in brain *in vivo* is partially inhibited by the endogenous tyrosine. While the preparation of this manuscript was under progress, Karobath and Baldessarini[17] reported similar mutual inhibition of catecholamine formation from phenylalanine and tyrosine when incubated with

brain mitochondrial preparation. No data on the inhibition of the formation of tyrosine from phenylalanine in these experiments were presented, however, by these authors.

The present observation of the effect of a MAO inhibitor on the formation of catecholamines from ^{14}C-tyrosine precursor (Table 3) is not new. Reduction of catecholamine formation from ^{14}C-tyrosine in MAO-inhibited rat brainstem[6] and caudate nucleus[18] has been observed before and ascribed to the feedback inhibition of the tyrosine hydroxylation. Our present observation (Table 3) that the formation of catecholamines and that of tyrosine from phenylalanine precursor is strongly reduced as well in MAO-inhibited rat brain is entirely new, and this observation leads to the interesting possibility that the mechanism of feedback inhibition may also control cerebral hydroxylation of phenylalanine. It is further apparent from the effect of dopamine *in vitro* (Table 4) that the catecholamine products may exert feedback inhibition *in vivo* on the hydroxylation of phenylalanine. It should be pointed out that only a small fraction of the tyrosine present is lost by hydroxylation during the incubation *in vitro* and, therefore, the observed inhibition of phenylalanine hydroxylation by dopamine may not be due to the tyrosine build-up resulting from the inhibition of tyrosine hydroxylation.

The most prominent effect of CPZ observed in our experiments was the increased radioactivity in the caudate catecholamines (Table 5) after ^{14}C-phenylalanine injection. This increase, however, might have been due to either increased synthesis of catecholamines or their reduced removal via oxidation to acidic metabolites. Since dopac is a major metabolite of caudate dopamine,[18] the concentration of labeled dopac may be taken as a rough indicator of the oxidative loss of labeled catecholamines. It is apparent from the values of labeled dopac in caudate formed from ^{14}C-phenylalanine (Table 5) that the radioactivity in dopac also increased in CPZ-treated rats. It is, therefore, indicated that the CPZ-induced increase in the concentration of labeled catecholamines in caudate may not be simply due to reduced degradation.

A number of studies[3–5] on the other hand have indicated that CPZ pretreatment increases the formation of brain catecholamines from ^{14}C-tyrosine precursor. Our present results (Table 5) of the experiments employing ^{14}C-tyrosine as the precursor do not afford a clear conclusion regarding any effect of CPZ on caudate catecholamines, since the uptake of ^{14}C-tyrosine was somewhat affected (68 per cent of the control value). In the brainstem, however, CPZ did not have any noticeable effect on the precursor uptake, and the formation of catecholamines, if anything, was slightly reduced (60 per cent of the control value). The reason for this apparent lack of the stimulating effect of CPZ on brain catecholamines formation, in contrast with the previous results,[3–5] is not quite clear. The hypothermic effect of CPZ on rats is well known, and some studies have shown that the effect of CPZ on brain catecholamines is either less prominent[19,20] or not quite clearcut[3] when the drug-induced hypothermia was not prevented by higher ambient temperature. In our experiments the obvious hypothermia of the CPZ-treated rats was not prevented by any means, and the rats were kept at room temperature (21°) along with the saline-treated controls. It is conceivable that any stimulating effect of CPZ on the catecholamine formation from ^{14}C-tyrosine in our experiments was masked by some effect of hypothermia. In contrast, the results (Table 5) of our experiments employing ^{14}C-phenylalanine as the

catecholamines precursor clearly show that CPZ caused a large increase in the concentration of labeled catecholamines in the caudate nucleus of the rats maintained under the same hypothermic condition. The sequence of events that may lead to this apparent increased synthesis of catecholamines from ^{14}C-phenylalanine in the caudate nucleus is not clear at this time. It has been suggested[5,20] that CPZ may cause a blockade of dopaminergic pathway, and the resultant feedback activation may increase the synthesis of dopamine in the caudate nucleus. It has been shown[5] that this increased synthesis of caudate dopamine may occur from ^{14}C-tyrosine precursor. But further experiments are necessary to understand how this drug affects the formation of dopamine from ^{14}C-phenylalanine precursor and to discover the possible relevance of this new observation to the therapeutic and/or side effects of this widely used drug.

The present observation has implication for another interesting question, and it is concerning the mechanism of the formation of dopa from phenylalanine. It is expected that tyrosine is an intermediate in the formation of dopa, which is decarboxylated to dopamine by the ubiquitous dopa decarboxylase. Ikeda *et al.*[21] have studied the hydroxylation of phenylalanine to tyrosine and dopa by a purified preparation of tyrosine hydroxylase. These authors have discussed the alternate possibilities that the formed tyrosine may either (a) remain bound with the enzyme forming it, in some fashion, until further hydroxylated to dopa or (b) become free to be subsequently hydroxylated by a distinctly separate reaction. According to the first mechanism, the intermediate tyrosine molecule is not expected to be freely miscible with any free tyrosine that may be present. On the other hand, if the intermediate tyrosine is free, any overall increase in the formation of labeled dopa from ^{14}C-phenylalanine is likely to be accompanied by an increase in the level of radioactivity of the tyrosine due to the trapping of the ^{14}C-tyrosine intermediate by the free endogenous tyrosine present. Our present results (Table 5) indicate that the CPZ-induced increase of the radioactivity of catecholamines formed from ^{14}C-phenylalanine was disproportionately larger than the rise in the radioactivity present in tyrosine. It appears, therefore, that the intermediate ^{14}C-tyrosine formed during the hydroxylation of ^{14}C-phenylalanine by the enzyme system present in caudate may not be miscible with the endogenous free tyrosine present and, therefore, may be bound or restricted in some fashion.

The results of the double labeled experiment indicate a large difference between the R value of the catecholamines and that of the precursor tyrosine. If it is assumed that the intermediate ^{14}C-tyrosine, formed during the consecutive hydroxylation of ^{14}C-phenylalanine, is present in the incubation mixture as a free molecule easily miscible with the ^{3}H-tyrosine added to the medium, it is difficult to explain the observed difference between the R value of the precursor tyrosine and the R value of the product catecholamines. It is also difficult to envisage any physical compartmentation in our *in vitro* system employing caudate homogenate and, therefore, the observed difference between the R values may not be the result of simple inhomogeneity. Consequently, it may have to be considered that the intermediate tyrosine may be bound or restricted in some manner. Some sort of restriction, by enzyme binding or any other means, is also compatible with our observed effect of CPZ *in vivo* (Table 5). If it is so that the intermediate tyrosine does not mix with the endogenous free tyrosine present in tissue, an independent role of phenylalanine as a primary precursor of brain catecholamines may be indicated.

In view of our present and previous[1,2] findings and those of others,[14,17,21] it seems a discussion regarding the possible functions of phenylalanine hydroxylation in brain is in order. It is becoming increasingly clear that the hydroxylation of phenylalanine in brain may yield both tyrosine and dopa, the immediate precursor of catecholamines. It is difficult to assume that the tyrosine formation by itself may be of great importance since the plasma-borne transport of this amino acid, formed in liver and kidney by phenylalanine hydroxylase and obtained from dietary sources, may provide the requirement of brain tissue. In comparison, catecholamines are generally thought to be formed and utilized locally in brain. Since we have observed that catecholamines are synthesized from phenylalanine in a number of brain areas, such synthesis could conceivably serve the requirement of the individual areas. A question must be asked, however, regarding the physiological role of catecholamines formed from phenylalanine vis-a-vis the role of the catecholamines formed from the free endogenous tyrosine. The relative importance of the catecholamines derived from phenylalanine is not entirely apparent at this time, since a much larger quantity of these amines may be formed directly from tyrosine under identical conditions of experiment (Table 1). It is conceivable, however, that phenylalanine may serve as the precursor of the catecholamines belonging to a small pool. The existence of a small but active pool of catecholamines has been suggested and discussed[22–25] but never adequately proved for general acceptance. If, indeed, phenylalanine does have such a function, it is at least compatible with our present results which suggest that the formation of phenylalanine-derived catecholamines may be (a) controlled by a negative feedback-like mechanism and (b) stimulated by such neural activation as may be caused by CPZ.[5] The possibility of a connection between phenylalanine hydroxylation and the small active pool of catecholamines has not been raised before and further work is necessary before the import of the cerebral hydroxylation of phenylalanine may be fully understood.

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