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CATECHOLAMINE FORMATION IN BRAIN FROM PHENYLALANINE AND TYROSINE—EFFECTS OF PSYCHOTROPIC DRUGS AND OTHER AGENTS*,†

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Abstract—The present report is a comparative study of phenylalanine and tyrosine as the precursors of cerebral catecholamines. Subcellular fractions of brain regions were incubated with a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine for the studies *in vitro*; the labeled amino acids in solution were injected into the lateral ventricle of rat brain for the experiments *in vivo*. After the experiments, the ratio of ^{14}C to ^{3}H (^{14}C dis./min/^{3}H dis./min × 100) in the isolated catecholamines [R(CA)] and tyrosine [R(TY)] was determined. The ratio R(CA)/R(TY) was taken as a measure of the divergence between the isotope ratio in tyrosine and that in the catecholamines. The experiments *in vitro* indicate that the rate of phenylalanine hydroxylation, the R(CA) value and the R(CA)/R(TY) value may be characteristic properties of specific brain regions. Several physical or chemical agents were found to have profound effects not only on the hydroxylation of phenylalanine but also on the R(CA) and R(CA)/R(TY) values. Lowering the incubation temperature from 37° to 0° decreased the hydroxylation of both the amino acids but favored the relative formation of (^{14}C)catecholamines. Lower incubation temperature also favored the formation of the consecutive hydroxylation product (^{14}C)catecholamines and very little (^{14}C)tyrosine was present in the incubation mixture. Pretreatment of the synaptosomal–mitochondrial enzyme source with any one of several detergents or with hypotonic sucrose almost completely inhibited the hydroxylation of both the substrates. The residual activity of such preparations favors the formation of (^{3}H)catecholamines. Time studies at 37° and at 0° indicate that the ratio R(CA)/R(TY) is always greater than unity within the time range of 5–60 min. After incubation of the particulate fraction at 0°, the R(CA) value continues to increase up to 60 min, at which time the formation of (^{14}C)catecholamines is about 1·4 times that of (^{3}H)catecholamines. The results of incubations at various phenylalanine substrate concentrations show that the value of R(CA)/R(TY) is relatively insensitive to changes in phenylalanine concentration in the presence of a constant level of tyrosine and the values were larger than unity. *In vivo*, several psychotropic drugs—chlorpromazine, amphetamine, apomorphine and morphine—were observed to either stimulate or inhibit the formation of catecholamines from (^{14}C)phenylalanine. In conclusion, our results suggest that: (1) the tyrosine that is formed *in vitro* from phenylalanine in brain tissue is not freely miscible with the endogenous free tyrosine; (2) the hydroxylation characteristics of the phenylalanine-derived tyrosine may be distinctly different from that of the free endogenous tyrosine; and (3) phenylalanine hydroxylation may be sensitive to some psychotropic drugs *in vivo*.

A number of studies have indicated that brain tissue may hydroxylate phenylalanine both *in vivo* [1, 2] and *in vitro* [3, 4], leading to the formation of tyrosine and catecholamines (CA). Although tyrosine is expected to be an obligatory intermediate during the formation of CA, the mechanism of the formation and subsequent hydroxylation of this amino acid intermediate is not clear. Ikeda *et al.* [5] studied the consecutive hydroxylation of phenylalanine to Dopa by a partially purified preparation of brain tyrosine hydroxylase (EC 1.14.3.a); their results were later confirmed by Shiman *et al.* [6] employing a highly purified preparation of adrenal tyrosine hydroxylase. According to the suggestion of Ikeda *et al.* [5], the tyrosine intermediate is free and it is subsequently converted to Dopa by a distinctly separate hydroxylation step. If it is assumed that this is the mechanism of phenylalanine hydroxylation in brain and that the tyrosine intermediate is miscible with the endogenous free tyrosine, the cerebral hydroxylation of phenylalanine should only contribute to the already present free tyrosine in brain. On the other hand, if the intermediate tyrosine does not mix with the endogenous tyrosine, such a restriction may possibly be imposed by some sort of binding, phenylalanine may prove to be a direct and independent precursor of brain catecholamines. Some of our recent results [7] have raised the possibilities that the intermediate tyrosine may not be miscible with the free tyrosine under conditions *in vitro* and that the mechanisms of the formation of catecholamines from these two types of tyrosine molecules may not be identical. For further examination of these possibilities, we have compared, employing homogenates and synaptosomal preparations, the

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formation of ^{3}H-labeled catecholamines from (^{3}H)tyrosine with the formation of ^{14}C-labeled catecholamines from (^{14}C)tyrosine resulting from the hydroxylation *in situ* of (^{14}C)phenylalanine. For these experiments we have used (^{14}C)phenylalanine and (^{3}H)tyrosine as co-substrates. We have also investigated the effects of several chemical and physical agents on the relative formation of ^{3}H- and ^{14}C-labeled catecholamines *in vitro* from the mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine. Because of the obvious interest in results obtained *in vivo*, we also report here the results of hydroxylation studies following the intraventricular injection of (^{14}C)phenylalanine in rats pretreated with some psychotropic drugs.

MATERIALS AND METHODS

Animals. Female Wistar rats (150–175 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, drugs and other materials. Uniformly ^{14}C-labeled L-phenylalanine and L-tyrosine (specific radioactivity, 350–400 mCi/m-mole) were purchased from New England Nuclear, Boston, Mass. Tritium-labeled L-tyrosine [L-*p*-hydroxyphenylalanine-2,3-^{3}H); specific radioactivity, 5 Ci/m-mole] was obtained from Amersham/Searle Corp., Don Mills, Ontario. Following are the drugs used and their sources: chlorpromazine (Largactil), Poulenc Ltd., Montreal, purchased locally; D-amphetamine sulfate, Sigma Chemical Co., St. Louis, Mo.; apomorphine hydrobromide, Eli Lilly & Co., Indianapolis, Ind. The administration of chlorpromazine and the isotonic saline solution of amphetamine and apomorphine was by the intraperitoneal route. The detergents used were Triton X-100 (J. T. Baker Chemical Co.), cetyltrimethylammonium bromide (CTA; Aldrich Chemical Co.) and sodium dodecylsulfate (SDS; Schwarz/Mann). The synthetic hydroxylation cofactor, 2-amino-4-hydroxy-6,7-dimethyl tetrahydropteridine ($DMPH_4$) was obtained from Calbiochem (Los Angeles).

Experiments in vitro. Dissected brain areas were homogenized (10%) in 0·32 M sucrose containing 10 μM calcium chloride. The sucrose homogenate was spun once at 27,000 *g* for 15 min and the sedimented particles were gently resuspended in the original volume of 0·1 M (pH 6·0) phosphate buffer. Aliquots were then incubated as previously described [3, 7]. Briefly, the brain preparations were incubated in the presence of (^{14}C)phenylalanine (0·1 μCi) and (^{3}H)tyrosine (0·1 μCi) after the addition of 2-mercaptoethanol and pargyline hydrochloride. Complete decarboxylation of the formed Dopa was achieved by the addition of crude kidney Dopa decarboxylase (EC 4.1.1.26). For a number of experiments, the crude mitrochondrial–synaptosomal fraction sedimenting at 27,000 *g* was hypotonically shocked by gently resuspending in 0·03 M sucrose and chilling in ice for 5 min, and then was mixed with 0·2 M sodium phosphate buffer (pH 6·0) for a final concentration of 100 mg wet tissue equivalent per ml of 0·1 M phosphate buffer (equivalent to 10% homogenate).

Experiments in vivo. For these experiments, the isotonic saline solution of either (^{14}C)phenylalanine or (^{3}H)tyrosine, or their mixture, was injected into the lateral ventricle of rat brain by slow infusion as described before [7].

Analytical methods. Analysis of the perchloric acid extract of the brain tissue was carried out as published before [3] for the separation and radioactivity assay of phenylalanine, tyrosine and catecholamines. Phenylalanine and tyrosine were purified by chromatography on Dowex-50(Na^+) and paper, while the same ion-exchange resin and acid Alumina were employed for isolation of dopamine. Recoveries of these compounds were checked by carrying samples of known labeled compounds through the complete analytical procedure. The average recoveries from a large number of such samples were 65 per cent for phenylalanine, 58 per cent for tyrosine and 55 per cent for CA. In view of the negligible differences between the recoveries, no corrections were applied to the radioactivities of tyrosine and CA reported in the Results. For the separation of 3,4-dihydroxyphenylacetic acid (Dopac), the acidic effluent from the Dowex-50 ion-exchange column was adjusted to pH 8·4 after addition of 100 mg ascorbic acid and 1·0 ml of 0·2 M EDTA solution. The mixture was stirred with approximately 300 mg of acid-washed Alumina which was then filtered, washed twice with water and once with 0·2 M sodium acetate (pH 8·3). The Dopac was eluted from the Alumina by slow percolation of 2·5 ml of 1 N H_2SO_4 through the Alumina contained in a glass column. The sulfuric acid eluate was saturated with sodium chloride and then shaken with 5·0 ml ethyl acetate for the extraction of Dopac. The organic layer was separated and mixed with 15 ml Aquasol scintillation fluid (New England Nuclear, Boston, Mass.) for radioactivity assay.

Determination of radioactivity. A liquid scintillation counter (Beckman Instruments Ltd., Pasadena, Calif.; model LS-100) was used for counting the radioactivities of either single-labeled or double-labeled (^{14}C/^{3}H) samples. The method for the assay of radioactivity and for the determination of the ratio of ^{14}C to ^{3}H has been described before [7].

Assay of endogenous tyrosine and phenylalanine. Fluorometric methods for the assay of phenylalanine [8] and that of tyrosine [9] were used and the reported results are the averages of three to four samples. For tyrosine assay, the perchloric acid extract of the brain tissue was initially fractionated employing ion-exchange resins for achieving a partial purification and concentration of the amino acid before reacting with the nitroso-napthol reagent. Such a partial purification of tyrosine to separate it from the acidic and amine derivatives was achieved by chromatography of the tissue extract at pH 2·0 on a Dowex-50(Na^+) column. The amphoteric fraction containing tyrosine was eluted from the resin by 0·1 M, pH 6·4, sodium phosphate buffer and rechromatographed on a fresh Dowex-50(Na^+) column. Tyrosine was then eluted by 2 N ammonia in 65% ethanol and reacted with the nitroso-napthol reagent after removal of the eluting agent by drying.

Assay of phenylalanine was performed by the fluorometric method as modified in our laboratory. The initial purification and fluorescence development methods were as described below. A cation-exchange resin, Dowex-50(Na^+), was used for the purification and concentration of the endogenous tissue phenylalanine starting with the perchloric acid extract. The

resin, besides being activated by alternate acid and alkali treatment, was washed twice with 1 N NH_4OH in 65% ethanol, which helped to reduce the fluorescence blank of the samples. The perchloric acid extract of brain tissue was adjusted to pH 2 and then run through the ion-exchange resin column. The resin was washed with water and then with 10 ml of 0·1 M sodium acetate buffer (pH 3·5). The buffer wash removed most of the glutamic acid normally present in brain tissue without eluting the phenylalanine absorbed in the resin. Phenylalanine was subsequently eluted with 10 ml of 0·1 M sodium phosphate buffer (pH 6·5). This fraction was readjusted to pH 2 and run again through a fresh column of Dowex-50(Na^+) resin. After a water wash, phenylalanine was eluted with 5 ml of 15% pyridine in water. This fraction was dried completely under N_2 at 50°, dissolved in a suitable small volume of water and reacted with the ninhydrin–peptide mixture for 2 hr at 60°. These incubated samples were diluted with copper sulfate solution made up in 0·5 M sodium phosphate buffer (pH 8·0) and the fluorescence was read at 485 nm after activation at 385 nm in an Aminco–Bowman spectrophotofluorometer. Dilute perchloric acid, containing no tissue, was carried through the entire procedure and was read for determination of the blank fluorescence of the tissue samples. Tissue samples (100 mg) were extracted with perchloric acid and simultaneously analyzed, in triplicate, with and without 1·0 μg of known phenylalanine added as an internal standard.

The fluorescence increment, due to this added 1·0 μg phenylalanine internal standard, was used to calculate the phenylalanine value of the brain tissue samples from their fluorescence readings. Our present method differs from the original method mainly by the steps of initial purification and concentration of phenylalanine present in the tissue acid extract. This modification served two purposes. First, the glutamic acid that is present in brain tissue in large excess (about 70 times that of phenylalanine) [10] was observed to suppress the fluorescence of added phenylalanine and was therefore removed by the initial pH 3·5 buffer wash of the ion-exchange resin. Second, the concentration of phenylalanine achieved by the present method allowed us to determine the endogenous level of the amino acid in small brain areas.

RESULTS

Formation of double-labeled CA in vitro. Homogenates of dissected brain areas, either as such or after centrifugal fractionation, were incubated with a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine as described. The R(CA) and R(TY) values and the ^{14}C contents of the CA and tyrosine were determined after analysis of the incubation mixture. The results (Table 1) show that the whole homogenate of caudate nucleus and the particulate fraction actively hydroxylated the substrate mixture. The soluble supernatant (27,000 *g*) fraction, however, does not seem to have any hydroxylating capacity. CA formed after incubation of the whole homogenate and the particulates had R(CA) values of 15·7 and 48·3, respectively, and these figures are considerably higher than the R(TY) values, 5·2 and 10·3. This apparent anomaly, the discrepancy between R(CA) and R(TY) values, may be expressed by the ratio R(CA)/R(TY). An R(CA)/R(TY) value of 1 would then mean that CA and tyrosine had equal ^{14}C to ^{3}H ratio. A value higher than 1 would suggest that the isotope ratio in CA was different from that in tyrosine and that there was a preferential incorporation of ^{14}C into the CA molecule. Clearly the R(CA)/R(TY) values from whole homogenate and particulates are considerably higher than 1. The particulates from several other brain areas (brainstem, hypothalamus and midbrain) were also incubated for comparison. The results (Table 1) show that these areas are also able to hydroxylate the substrate mixture, although with lesser activity. Brainstem, hypothalamus and midbrain all had R(CA)/R(TY) values higher than that obtained from the incubation of caudate particulates. The whole homogenate of spleen tissue formed no catecholamines

Table 1. Hydroxylation of (^{14}C)phenylalanine and (^{3}H)tyrosine co-substrates *in vitro* by brain areas and the isotope ratio in CA and tyrosine*

		CA		Tyrosine		
Brain area (N)	Cell fraction	R(CA)†	^{14}C (nCi/g/hr)	R(TY)†	^{14}C (nCi/g/hr)	R(CA)/R(TY)†
	Whole homogenate	15·7	36·4	5·2	51·0	3·0
Caudate	Supernatant	NS‡	1·0	NS	5·6	NS
nucleus	Particulate	48·3	386·4	10·3	212·8	4·7
(4)						
Brainstem	Particulate	19·1	7·2	1·5	45·2	12·7
(2)						
Hypothalamus	Particulate	21·4	13·8	1·5	29·6	14·3
(2)						
Midbrain	Particulate	18·6	6·6	1·8	55·8	10·3
(2)						
Spleen	Whole homogenate	NS	0·0	NS	1·7	NS
(2)						

* Tissue homogenates or their cell fractions were incubated with a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine as described in Materials and Methods. The isotope ratios were determined after separation of CA and tyrosine as described in Materials and Methods.

† R(CA), isotope ratio (^{14}C dis/min/^{3}H dis/min × 100) in catecholamines; R(TY), isotope ratio in tyrosine.

‡ NS, not significant.

Table 2. Effects of various additions or conditions on the relative hydroxylation of (^{14}C)phenylalanine and (^{3}H)tyrosine by caudate nucleus particulates*

Special condition or addition (N)		CA R(CA)	CA ^{14}C (nCi/g/hr)	CA (%)	Tyrosine R(TY)	Tyrosine ^{14}C (nCi/g/hr)	Tyrosine (%)	R(CA)/R(TY)
None† (6)		38·0	407·2	(100)	9·9	208·6	(100)	3·8
Incubation at (2)	10°	89·0	124·8	(31)	1·3	29·6	(14)	68·0
	0°	134·2	36·6	(9)	0·22	5·0	(2·4)	610·0
Detergent treatment‡ (4)	Triton X-100	NS	0·22	(0·05)	NS	1·6	(0·8)	NS
	CTA	21·5	6·4	(1·6)	0·7	17·2	(8·3)	30·7
	SDS	8·7	0·8	(0·2)	0·4	10·0	(4·8)	21·7
Hypotonic shock§ (4)		16·9	34·0	(8·4)	2·0	33·6	(16)	8·4
Hypotonic shock and 0° incubation (2)		99·6	9·0	(2·2)	0·08	1·6	(0·8)	1245·0
Dopamine, 4×10^{-5} M (2)		22·0	156·6	(38)	5·9	126·2	(61)	3·7
Hypotonic shock plus dopamine at 4×10^{-5} M (2)		10·6	7·8	(1·9)	0·8	14·2	(6·8)	13·2
Dopamine, 8×10^{-5} M at 10 (2)		53·7	49·4	(12)	0·9	22·8	(11·0)	59·7
Amphetamine, 10^{-4} M (2)		35·4	332·6	(82)	9·1	178·4	(86)	3·9

* Particulates prepared from caudate nucleus homogenate were incubated under standard conditions as described in Materials and Methods. Any special conditions or additions were as listed.

† The final concentrations and specific radioactivities were: phenylalanine, 3·5 μM and 58 nCi/nmole; tyrosine, 3·0 μM and 50 nCi/nmole.

‡ For detergent treatment, the particulate preparation in 0·1 M phosphate buffer (pH 6·0) was mixed with the detergent (0·1% final strength) and kept at 0° for 5 min before addition of the labeled substrates. SDS, sodium dodecylsulfate; CTA, cetyltrimethyl ammonium bromide.

§ Hypotonic shock was given by suspending the particulate fraction in 0·03 M sucrose for 5 min at 0°.

upon incubation and very little (^{14}C)tyrosine was formed. In this experiment the brain preparation was replaced by spleen homogenate in an otherwise identical incubation mixture. Therefore, low or no activity in tyrosine and CA indicates the absence of appreciable nonenzymatic product formation, in agreement with our previous report [3].

Influence of various additions and conditions on double-labeling of CA. The objective for carrying out the present experiments was to explore the possible factors, physical or chemical in nature, which may have discriminating effects on the hydroxylation of phenylalanine and tyrosine. For these experiments, particulates from caudate nucleus homogenate were incubated with a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine under standard conditions as described in Materials and Methods. Various compounds listed in Table 2 were added to the incubation medium to test their effects on hydroxylation of the substrates. Incubations were also done at temperatures lower than the standard 37° and, in some instances, the particulates were subjected to a hyptonic shock in 0·03 M sucrose as described in Materials and Methods. Various combinations of chemical and physical factors were also tried to investigate the interaction between these factors. The results (Table 2) show that, on incubation at a lower temperature of 10° or 0°, lesser quantities of the products were formed from both the substrates. The relative rate of hydroxylation as indicated by the R(CA) value was, however, profoundly affected by the change of incubation temperature. The R(CA) value went up sharply at 10° and at 0° from that at the standard incubation temperature of 37°. The lowering of incubation temperature had a different effect on the R(TY) value and it was reduced at 10° and at 0°. The net result was that the R(CA)/R(TY) value rose steeply with the lowering of the incubation temperature.

Detergents are known to disrupt synaptosomes [11] by virtue of their effects on the membrane. The effects of several detergents on hydroxylation by our synaptosomal–mitochondrial preparation were investigated. For this purpose, a suspension of caudate nucleus particulates in 0·1 M phosphate buffer (pH 6·0) was adjusted to a final concentration of 0·1% of the detergent and held at 0° for 5 min before addition of the substrate mixture and incubation. The results (Table 2) show the effect of Triton X-100 treatment and indicate an almost complete loss of hydroxylating activity. The other two detergents, CTA and SDS, although not as potent as Triton X-100, inhibited the hydroxylating activity over 97 per cent. Pretreatment with either of these two detergents lowered the R(CA) and R(TY) values but elevated the R(CA)/R(TY) value. In some experiments, the synaptosomal membranes were disrupted by suspending the particulates in 0·03 M sucrose for 5 min at 0°. The hypotonic shock considerably reduced the product formation from both the substrates and lowered the R(CA) and R(TY) values. After observing that both hypotonic shock and 0° incubation drastically affected hydroxylation, the combined effect was investigated in some experiments. The hydroxylation inhibition in this case was stronger than that observed after either hypo-

Table 3. Effects of incubation time and temperature on hydroxylation of (^{14}C)phenylalanine and (^{3}H)tyrosine co-substrates*

Enzyme source (incubation temp.)	Time (min)	CA R(CA)	CA ^{14}C (nCi/g/hr)	Tyrosine R(TY)	Tyrosine ^{14}C (nCi/g/hr)	R(CA)/R(TY)
Particulates (37°)	5	51·4	157·2	2·6	66·4	19·8
	10	45·2	218·2	4·2	95·2	10·8
	20	37·7	268·0	6·2	135·8	6·1
	30	37·7	407·2	9·9	188·8	3·8
	45	36·0	370·4	10·6	192·2	3·4
Particulates (0°)	5	37·6	3·2	0·25	6·7	150·4
	10	54·0	5·6	0·29	7·4	186·2
	20	85·0	13·9	0·32	8·2	265·6
	30	134·2	36·6	0·35	8·1	383·4
	60	139·2	54·4	0·39		356·9
Shocked suspension (37°)	5	18·2	18·6	1·0	21·0	18·2
	10	16·1	26·5	1·5	31·5	10·7
	20	14·1	30·8	2·1	43·1	6·7
	30	16·9	34·0	2·0	33·6	8·4
Shocked suspension (0°)	30	99·6	9·0	0·08	1·6	1245·0

* Particulates obtained from caudate nucleus homogenate were either used as such or after a hypotonic shock (suspended in 0·03 M sucrose for 5 min at 0°). The enzyme source was incubated with a mixture of labeled phenylalanine and tyrosine as described in Materials and Methods. The results are the averages of duplicate and quadruplicate experiments.

tonic shock or 0° incubation. The low radioactivity of the (^{14}C)tyrosine formed in this experiment led to a very low R(TY) value. The combined effect of hypotonic shock and addition of 4×10^{-5} M dopamine, an inhibitor of both the hydroxylations [7], was also investigated. Dopamine inhibited the hydroxylations by the shocked preparation and further lowered the R(CA) and R(TY) values. The effect of dopamine on the hydroxylations at 10° was also found to be inhibitory.

Isotope ratios at various incubation periods. The results summarized in Table 2 show the isotope ratios after 30 min of incubation. Experiments were also carried out to determine the time course of the isotope ratios when caudate nucleus particulates and their hypotonically shocked suspensions were incubated at 37° and 0°. When particulates were incubated at 37°, the R(CA) value steadily decreased (Table 3) while the R(TY) value rose. The R(CA)/R(TY) value, initially quite high, dropped gradually with the increase of the incubation period. In contrast, at 0° the R(CA) value increased steadily, whereas the R(TY) value, quite low at the shortest incubation period, increased only slightly with time. In some experiments the crude synaptosomal preparations were given hypotonic shock in 0·03 M sucrose as described before (see Table 2) to disrupt the synaptosomal membrane. The shocked preparations were then incubated for various time periods and the results (Table 3) show that at 37° the R(CA)/R(TY) value was 18·2 at 5 min and then decreased with the increase of the incubation period.

Effect of the synthetic cofactor $DMPH_4$ on hydroxylation. The widespread use of the cofactor $DMPH_4$ for studying enzymatic hydroxylation of phenylalanine and tyrosine suggested the present experiment. Particulate preparations from caudate nucleus and brainstem were incubated under the standard conditions and after addition of $DMPH_4$ to the incubation medium to various final concentrations. The cofactor solution was prepared fresh in ice-cold 1 mM 2-mercaptoethanol and added to the medium just before the start of incubation. Some samples of the particulate preparations were pretreated with Triton X-100 as described before. $DMPH_4$ inhibited (Table 4) the formation of both the ^{14}C- and ^{3}H-labeled CA in a concentration-dependent manner. Inhibition of ^{3}H-CA formation, although quite strong, was relatively less and the R(CA) value dropped from 48·3 to 20·5 at 4·0 mM $DMPH_4$ concentration. The effect of $DMPH_4$ on the formation of (^{14}C)tyrosine was of a mixed nature. Initially, at the lower concentrations of 0·4 and 1·0 mM, some inhibition was apparent; at the 4·0 mM cofactor concentration, however, there was no inhibition. It is interesting to observe that addition of 4·0 mM $DMPH_4$ to the Triton X-100-treated synaptosomal preparation completely restored the loss of (^{14}C)tyrosine formation that occurs after the detergent treatment. The addition of $DMPH_4$, however, barely restored the formation of either ^{14}C- or ^{3}H-labeled CA. The formation of ^{14}C-CA, almost completely blocked by Triton X-100 treatment, did not increase significantly after addition of the synthetic cofactor to the detergent-treated preparation. The formation of ^{3}H-CA, although increased 27-fold, was only 6·4 per cent of the control. In a parallel study employing brainstem particulates, the results were very similar (Table 4). There was no increase of the formation of ^{14}C-CA when $DMPH_4$ was added to the detergent-treated particulates. There was some

Table 4. Effects of the synthetic hydroxylation cofactor ($DMPH_4$) on hydroxylation of (^{14}C)phenylalanine and (^{3}H)tyrosine*

$DMPH_4$ concn† (mM)	Treatment	CA R(CA)	CA ^{14}C (nCi/g/hr)	CA ^{3}H‡ (nCi/g/hr)	Tyrosine R(TY)	Tyrosine ^{14}C‡ (nCi/g/hr)
Caudate nucleus§						
None	None	48·3	386·4	849·0	10·3	212·8
(4)			(100)	(100)		(100)
0·4	None	31·9	110·8	347·4	5·5	137·4
(2)			(29)	(41)		(64)‖
1·0	None	26·5	86·8	327·8	6·8	177·6
(4)			(22)	(39)		(83)
4·0	None	20·5	44·0	225·4	7·4	206·4
(2)			(11)	(26)		(97)
None	Triton	NS	0·2	2·0	NS	1·6
(4)	X-100¶		(0·05)	(0·2)		(0·7)
4·0	Triton	1·4	0·6	54·8	6·7	203·2
(2)	X-100		(0·2)	(6·4)		(95)
Brainstem						
None	None	19·1	7·2	37·8	1·5	45·2
(2)			(100)	(100)		(100)
None	Triton	NS	0·2	1·6	NS	2·6
(2)	X-100		(2·7)	(4·2)		(5·7)
1·0	Triton	NS	0	5·8	5·5	180·4
(2)	X-100		(0)	(15·3)		(400)
4·0	Triton	NS	0	9·2	7·5	207·0
(2)	X-100		(0)	(24·3)		(458)

* Particulate fractions obtained from tissue homogenates were incubated with (^{14}C)phenylalanine and (^{3}H)tyrosine and various concentrations of the synthetic cofactor. The cofactor was freshly dissolved in ice-cold 1 mM 2-mercaptoethanol.

† N is given in parentheses.

‡ The per cent is given in parentheses.

§ For the final concentration and specific radioactivities of the substrates, see Table 5.

‖ When tissue was omitted from the incubation mixture, 8·7% of (^{14}C)tyrosine was formed. The corresponding figures for the experiments with 1·0 and 4·0 mM $DMPH_4$ were 10·9 and 14·7% respectively.

¶ The particulate preparation was kept at 0° for 5 min in the presence of 0·1% Triton X-100 before mixing with the other components of the incubation mixture.

increase of the formation of ^{3}H-CA, about 3·6-fold and 5·7-fold, when $DMPH_4$ was added to 1·0 and 4·0 mM concentrations respectively. Addition of the cofactor resulted, as with the caudate preparation, in the appearance of a large amount of radioactivity in the (^{14}C)tyrosine fraction.

Isotope ratios at various phenylalanine substrate concentrations. The isotope ratios obtained after incubations at a low concentration (3·5 μM) of phenylalanine are listed in Table 2. It was thought that a higher phenylalanine concentration might significantly affect the ratios. To investigate this possibility, a series of incubations were carried out at various phenylalanine substrate concentrations. The results (Table 5) show that formation of total ^{14}C-labeled products increased with increased substrate concentration up to $1{\cdot}88 \times 10^{-4}$ M, the maximum concentration tested. The results also show that the increased total ^{14}C product formation was all due to increased (^{14}C)tyrosine formation. There was a 150 per cent rise of the (^{14}C)tyrosine product when the substrate concentration was increased from $0{\cdot}28 \times 10^{-4}$ M to $1{\cdot}88 \times 10^{-4}$ M. At the same time, however, formation of ^{14}C-CA dropped somewhat. Both the R(CA) and R(TY) values changed inversely with the change in substrate concentration. The R(CA)/R(TY) value rose, however. The R(CA) values at various phenylalanine concentrations were always higher than the corresponding R(TY) values, and the R(CA)/R(TY) values were much greater than unity.

CA formation in brain in vivo *from (^{14}C)phenylalanine–(^{3}H)tyrosine mixture—Effect of CPZ.* Our present results (Table 1) indicate that caudate tissue may hydroxylate (^{14}C)phenylalanine and (^{3}H)tyrosine cosubstrates *in vitro*, forming double-labeled CA. It was therefore of interest to us to determine the double labeling of CA that may occur *in vivo* from a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine. Chlorpromazine (CPZ) treatment has been reported to increase the formation of CA from (^{14}C)phenylalanine [7] and from labeled tyrosine [12] in brain *in vivo*. We have considered that any effect CPZ treatment may have on the formation of double-labeled CA and on its isotope ratio may be indicative of the relative drug effects on CA formation from these two amino acids. For these experiments, groups of rats were fasted overnight and were given i.p. either saline or CPZ (15 mg/kg) 2 hr before intraventricular administration of a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine, 15 μCi each, as mentioned in Materials and Methods. Fifteen min after radioisotope administration, the animals were decapitated and the caudate regions were analyzed for labeled tyrosine, CA and Dopac as described in Materials and Methods. The radioactivity of ^{14}C and ^{3}H appearing in the amino acid fraction during the ion-exchange analysis was taken as a mea-

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Table 5. Hydroxylation of (^{14}C)phenylalanine and (^{3}H)tyrosine at various phenylalanine substrate concentrations*

Phenylalanine concn (μM)† (N = 2)	^{14}C-CA		^{14}C-tyrosine		Total ^{14}C product‡ (nmoles/g/hr)	^{3}H-CA (nCi/g/hr)	R(CA)	R(TY)	R(CA)/R(TY)
	(nCi/g/hr)	(nmoles/g/hr)	(nCi/g/hr)	(nmoles/g/hr)					
28·1	28·8	5·4	85·2	16·0	26·8	209·4	13·7	4·3	3·2
54·7	13·6	5·0	49·0	18·0	28·0	150·6	8·9	2·3	3·9
99·2	7·4	5·0	32·6	21·8	31·8	104·0	7·2	1·6	4·5
188·1	3·2	4·0	19·0	24·0	32·0	61·4	5·2	0·9	5·8

* Particulate fraction from caudate nucleus homogenate was incubated *in vitro* with a mixture of (^{14}C)phenylalanine-(^{3}H)tyrosine and various concentrations of phenylalanine.
† Tyrosine concentration was approximately 3 μM during these incubations.
‡ Total product is the sum of nmoles/g/hr of (^{14}C)tyrosine and 2 × nmoles/g/hr of ^{14}C-CA.

Table 6. Isotope ratios in Dopac and CA in the caudate nucleus and brainstem and the effects of CPZ pretreatment*

Drug treatment (N)	Residual precursor		R values and ^{14}C in various fractions					
			Dopac		CA		Tyrosine	
	$^{14}C/^{3}H \times 100$	^{14}C† (nCi/g)	R(Dopac)	^{14}C† (nCi/g)	R(CA)	^{14}C† (nCi/g)	R(TY)	^{14}C† (nCi/g)
Caudate nucleus								
None (5)	104·0 ± 4·4	1265·0 ± 145·0 (100)	7·1 ± 0·7	1·0 ± 0·17 (100)	6·0 ± 1·2	4·0 ± 1·0 (100)	1·60 ± 0·16	15·0 ± 1·6 (100)
CPZ (4)	107·1 ± 8·6	1170·0 ± 190·0 (92)	14·8 ± 1·3‡	2·5 ± 0·28§ (250)	11·9 ± 1·2‖	14·3 ± 3·8‖ (357)	2·6 ± 0·3¶	18·3 ± 4·0** (122)
Brainstem								
None (5)	74·8 ± 2·6	1015·0 ± 90·0 (100)	20·9 ± 1·3	1·6 ± 0·3 (100)	19·2 ± 1·1	2·6 ± 0·4 (100)	2·4 ± 0·2	25·8 ± 3·2 (100)
CPZ (3)	81·7 ± 8·3	1600·0 ± 330·0 (158)	16·6 ± 0·9**	1·1 ± 0·06** (69)	13·0 ± 1·6‖	1·8 ± 0·6** (69)	2·1 ± 0·2**	30·4 ± 7·5** (118)

* For these experiments, a mixture containing 15 μCi each of (^{14}C)phenylalanine and (^{3}H)tyrosine was injected. CPZ was given intraperitoneally (15 mg/kg) 2 hr before the radioisotope administration. The results are averages ± S.E.M.

† The per cent is given in parentheses.

‡ Drug vs. saline, $P < 0.001$.

§ Drug vs. saline, $P < 0.005$.

‖ Drug vs. saline, $P < 0.025$.

¶ Drug vs. saline, $P < 0.05$.

** Drug vs. saline, $P > 0.05$.

sure of the unutilized (^{14}C)phenylalanine and (^{3}H)tyrosine precursors. The results (Table 6) show that, in the untreated group, the isotope ratio in CA is significantly higher than that in tyrosine. The R(CA)/R(TY) in the caudate was 5·0, which was not widely divergent from the values of 3·0 and 4·7 obtained from the experiments *in vitro* (Table 1). The R(CA)/R(TY) value in the brainstem was even higher, a fact which has also been observed *in vitro*. Dopac, which is a major metabolite of caudate CA [13], had an isotope ratio close to that of CA in both brain regions. CPZ treatment did not significantly alter the concentration of (^{14}C)tyrosine in caudate or the concentrations of the residual precursors. We have observed before [7] that CPZ did not affect the endogenous phenylalanine or tyrosine level. It is likely therefore, that the drug did not have much effect on the specific radioactivities of the precursor amino acids. The drug treatment, however, resulted in a marked rise of the ^{14}C-CA concentration and of the R(CA) value in the caudate and a parallel rise of radioactivity and of isotope ratio in Dopac. The rise of radioactivity in both ^{14}C-CA and ^{14}C-Dopac is probably indicative of increased ^{14}C-CA synthesis. Such an effect of CPZ, however, may be specific for caudate, since neither ^{14}C-CA nor ^{14}C-Dopac increased in brainstem after the drug treatment.

Effect of other psychotropic drugs on formation of CA from (^{14}C)phenylalanine in vivo. In view of the effect of CPZ on the formation of CA from (^{14}C)phenylalanine *in vivo*, we wished to test several other drugs for their possible effects on ^{14}C-CA formation. For these experiments various groups of rats were fasted overnight and were given intraperitoneally either saline, amphetamine (10 mg/kg, 1·5 hr), apomorphine (20 mg/kg, 1·0 hr) or morphine (15 mg/kg, 1·0 hr) at the time indicated within the parentheses, before injection of a solution of (^{14}C)phenylalanine (8 μCi). The brain areas were dissected and analyzed 15 min after injection of the radioisotope. The results (Table 7) show the effects of the drug pretreatments on the formation of labeled CA and tyrosine. The concentration of residual (^{14}C)phenylalanine precursor in the caudate nucleus of each drug-treated group was only slightly different from that in the control group caudate. Morphine, however, lowered the ^{14}C concentration in the residual precursor in brainstem to 44 per cent of the control. The concentration of labeled CA was significantly altered by the drug treatments. Amphetamine reduced the ^{14}C-CA concentration both in the caudate nucleus (18 per cent of control) and in the brainstem (24 per cent of control). Amphetamine treatment, however, did not have a significant effect on the (^{14}C)tyrosine concentration in this tissue. Apomorphine, as can be seen from the results, did have an effect on the caudate labeled CA which was very similar to that induced by amphetamine on the same brain area; there was a considerable reduction of the ^{14}C-CA concentration (18 per cent of control). Again, no significant effect of apomorphine on (^{14}C)tyrosine concentration was apparent. In contrast to the effect of apomorphine on caudate CA, no reduction of ^{14}C-CA concentration was apparent in the brainstem of apomorphine-treated animals. The results (Table 7) show that morphine treatment had only a moderate effect on caudate ^{14}C-CA concentration (73 per cent of control). A rather prominent effect of morphine on brainstem, however, was indicated; considerable reduction of ^{14}C-CA concentration (27 per cent of control) resulted. We have considered that the drugs amphetamine, apomorphine and morphine may influence the specific radioactivity of labeled phenylalanine in brain areas. We have therefore assayed the endogenous level and determined the specific radioactivity of the amino acid after injection of saline and the above-mentioned drugs. The results in Table 7 do not suggest any significant effect of amphetamine or apomorphine on the specific activity of phenylalanine in caudate nucleus and brainstem. Morphine, however, significantly reduced the specific radioactivity in the brainstem.

DISCUSSION

The results in Table 1 show that various brain

Table 7. Effect of drug treatment on (^{14}C)tyrosine and ^{14}C-CA formation from (^{14}C)phenylalanine*

Drug treatment (N)	^{14}C in various fractions†			Residual (^{14}C)phenylalanine sp. act. (nCi/nmole)
	Residual precursor (nCi/g)	CA (nCi/g)	Tyrosine (nCi/g)	
Caudate nucleus				
None (11)	655·0 ± 55·0 (100)	2·2 ± 0·4 (100)	7·8 ± 0·6 (100)	5·6
Amphetamine (8)	800·0 ± 110·0 (122)	0·39 ± 0·16‡ (18)	7·9 ± 0·7§ (101)	5·7
Apomorphine (7)	805·0 ± 75·0 (123)	0·30 ± 0·09‡ (18)	7·4 ± 0·7§ (95)	6·7
Morphine (7)	735·0 ± 140·0 (112)	1·6 ± 0·3§ (73)	9·9 ± 1·5§ (127)	6·0
Brainstem				
None (13)	650·0 ± 75·0 (100)	1·1 ± 0·1 (100)	10·6 ± 1·1 (100)	6·2
Amphetamine (9)	680·0 ± 100·0 (105)	0·26 ± 0·06‖ (24)	15·7 ± 2·6§ (148)	5·0
Apomorphine (9)	670·0 ± 90·0 (103)	0·96 ± 0·2§ (87)	12·6 ± 1·3§ (119)	7·0
Morphine (6)	285·0 ± 60·0 (44)	0·30 ± 0·08‡ (27)	8·3 ± 1·8§ (78)	2·5

* Female rats were given intraventricularly (^{14}C)phenylalanine (8 μCi) following either saline, amphetamine (10 mg/kg, 1·5 hr), apomorphine (20 mg/kg, 1 hr) or morphine (15 mg/kg, 1 hr) at the time indicated. After 15 min, brain areas were analyzed as described in Materials and Method. The results are average ± S.E.M.

† The per cent is given in parentheses.

‡ Drug vs. saline, $P < 0.005$.

§ Drug vs. saline, $P > 0.05$.

‖ Drug vs. saline, $P < 0.001$.

regions may hydroxylate a mixture of (^{14}C)phenylalanine and (^{3}H)tyrosine substrates. The CA formed is labeled with both ^{14}C and ^{3}H. Furthermore, the R(CA)/R(TY) value, an indicator of the divergence between R(CA) and R(TY) values, may be characteristic of a region and is always larger than unity. The results of our further studies *in vitro* (Tables 2–5) suggest that a number of factors, either chemical or physical in nature, may affect the relative conversions of (^{14}C)tyrosine and (^{3}H)tyrosine to CA. The effects of these factors are indicated by the resultant shift of the R(CA) and R(CA)/R(TY) values. The temperature of incubation is one of the physical factors which has a remarkable effect; the R(CA)/R(TY) value may rise precipitously with the lowering of the incubation temperature. It is rather obvious from our results (Table 2) that incubation temperature change has widely divergent effects on the hydroxylation of phenylalanine and tyrosine, and further study of this discriminating effect of temperature may prove useful. It is also apparent that phenylalanine is favored, by a large factor, over tyrosine as the hydroxylation substrate at lower incubation temperatures. Keeping in mind that every molecule of a catecholamine formed requires two hydroxylations of phenylalanine but only one of tyrosine, an R(CA) value of 134·2 at 0° (Table 2) means that the hydroxylation of phenylalanine proceeds at a 2·68 times (134·2 × 2/100) faster rate. Lower incubation temperature has another distinct effect that seems to favor formation of the final product ^{14}C-CA; not much (^{14}C)tyrosine is left in the incubation medium at 10° and even less at 0°. It may be that an inverse effect, in relative terms, of temperature lowering on two consecutive hydroxylation steps may lead to the non-accumulation of (^{14}C)tyrosine. We also found that the effects of lower temperature may be further accentuated if a hypotonically shocked enzyme preparation is employed. Hardly any activity is observed in (^{14}C)tyrosine after incubation of such a preparation at 0°. It appears that the effects of lower temperature and of disruption of membranes by hypotonic shock are additive and that some membrane-related phenomenon is involved in both cases. Our observed results (Table 2) of detergent treatment further suggest that membranes may play a vital role in the hydroxylation process. Triton X-100 treatment eliminated more than 99 per cent of the hydroxylating activity and no significant calculation of the isotope ratio was possible. Two other detergents, CTA and SDS, although extremely effective inhibitors (>98 per cent), permitted hydroxylations sufficient for calculation of the ratios. It may be pointed out here that the detergents appear to be far more potent inhibitors of phenylalanine hydroxylation than alpha-methyl *p*-tyrosine or dopamine [7]. The results of our time study (Table 3) point out that at any time from 5–60 min, the R(CA)/R(TY) value is much larger than unity. A comparison of the isotope ratios at 37° with those at 0° for various time periods suggests that the process of phenylalanine hydroxylation may be relatively more sensitive to the higher incubation temperature. The downward shift of the R(CA)/R(TY) value at 37° is reversed when the incubation temperature is lowered to 0°. The lower incubation temperature may possibly have a variety of effects, including prevention of deterioration of the membrane and/or the hydroxylating enzyme.

The results of our present experiments (Table 4) with added $DMPH_4$ confirm our previous findings [3] that this synthetic cofactor inhibits phenyl-

alanine hydroxylation in a concentration-dependent manner. The results also indicate that this cofactor has a relatively less pronounced inhibiting effect on the formation of catecholamines when tyrosine is the initial substrate. The R(CA) value progressively dropped with the increase of cofactor concentration. It also appears that formation of ^{14}C-CA in relation to that of (^{14}C)tyrosine is highly distorted by added $DMPH_4$. The ratio of ^{14}C-CA to (^{14}C)tyrosine is 1·8 in the absence of $DMPH_4$, but after addition of 1·0 and 4·0 mM cofactor, the ratio becomes 0·49 and 0·21 respectively. These results suggest therefore that $DMPH_4$ may not be suitable as a cofactor for studying the formation of catecholamines from phenylalanine in brain. While studying the hydroxylation of phenylalanine by adrenal tyrosine hydroxylase, Shiman *et al.* [6] have arrived at the similar conclusion that $DMPH_4$ may have questionable value as a cofactor.

The results presented in Table 5 suggest no substrate inhibition of phenylalanine hydroxylation up to $1{\cdot}88 \times 10^{-4}$ M and indicate that the ratio R(CA)/R(TY) was larger than unity in all of these experiments employing a wide range of phenylalanine substrate concentrations and phenylalanine to tyrosine ratios. These results suggest that the formation of CA from phenylalanine may be able to continue at a relatively normal rate in phenylketonuria, which is characterized by a high phenylalanine to tyrosine ratio.

Our present results (Table 6) confirm our previous observation [7] that CPZ significantly stimulates CA formation from (^{14}C)phenylalanine. Furthermore, the marked increments of R(CA) and R(Dopac) values after CPZ treatment suggest that the formation of ^{14}C-CA is stimulated to a greater degree than that of ^{3}H-CA. After presentation (see footnote to Title) of some of these data, we became aware of the results obtained by Nybäck *et al.* [14]. Their data indicated that a preferential stimulation, in agreement with our results, of CA formation from phenylalanine may occur after CPZ treatment. Amphetamine and apomorphine (Table 7) significantly lowered the concentrations of ^{14}C-CA in brain areas. The mechanisms underlying such effects, however, are not clear, since altered synthesis, release and further metabolism of CA may be caused by these drugs. Some results of our preliminary studies (not reported here) have indicated that amphetamine and apomorphine not only reduce ^{14}C-CA formation but also lower the R(CA) value from a (^{14}C)phenylalanine–(^{3}H)tyrosine mixture, suggesting stronger effects on ^{14}C-CA, as observed with CPZ. It has been discussed [15] that amphetamine may act on dopaminergic and noradrenergic systems, and apomorphine has been suggested [16, 17] to be a specific stimulator of dopaminergic receptors. Such properties of these two drugs could well be responsible for their observed effects on ^{14}C-CA. To summarize, the presently observed effects of CPZ, amphetamine, apomorphine and morphine on ^{14}C-CA suggest that phenylalanine may be an important physiological precursor of brain CA. We wish to point out, however, that the present studies of the effects of these psychotropic drugs are mainly intended as preliminary tests and further work is planned on these drug effects.

Our main findings may therefore be summarized in the following way: (1) The tyrosine intermediate formed *in vitro* during consecutive hydroxylations of phenylalanine may not be freely miscible with endogenous free tyrosine. This is suggested by the fact that the isotope ratio in tyrosine differs widely from R(CA) under a variety of conditions. (2) A number of factors, physical or chemical in nature, have powerful discriminating effects on the hydroxylations of these two amino acids. (3) The synthetic hydroxylation cofactor, $DMPH_4$, may not be suitable for studying the hydroxylation of phenylalanine. (4) The formation of CA from phenylalanine in brain may be sensitive to several psychotropic drugs such as CPZ, amphetamine, apomorphine and morphine.

Since 1964, when tyrosine hydroxylase activity was demonstrated [18, 19] in brain tissue *in vitro*, a large number of publications have appeared based directly or indirectly on the supposed fact that the action of this enzyme constitutes the rate-controlling step for CA biosynthesis, starting from freely available endogenous tyrosine. We are suggesting the possibility that phenylalanine, another aromatic amino acid freely available from diet, is an alternate direct source of cerebral CA without any obligatory participation of free endogenous tyrosine. Furthermore, the process of CA formation from this alternate source may be responsive to a variety of psychotropic drugs.

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