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Inhibition of Tyrosine Aminotransferase Activity by L-3,4-Dihydroxyphenylalanine*

J. H. Fellman† and E. S. Roth

ABSTRACT: Purified liver cytosol tyrosine aminotransferase was shown to be capable of transaminating 3,4-dihydroxyphenylalanine (DOPA). The ratios of activities of the enzyme to both tyrosine and DOPA remained constant throughout the purification procedure. A study of DOPA as an inhibitor of the enzyme was undertaken. DOPA, *m*-tyrosine, and other *m*-hydroxyphenylethylamines behave as noncompetitive inhibitors of tyrosine aminotransferase activity. Thus DOPA, *m*-tyrosine, and norepinephrine were noncompetitive inhibitors with respect to tyrosine concentration. The inhibitory activity could be ascribed to the formation of an inhibitor, the corresponding isoquinoline derivative of pyridoxal 5-phosphate. A number of aromatic amino acid derivatives were studied as inhibitors of tyrosine aminotransferase. The implications of these findings in patients treated with L-DOPA for the relief of Parkinsonism are discussed.

Analysis of brain tissue obtained at autopsy from patients afflicted with Parkinsonism revealed a marked decrease of dopamine content in the basal ganglia (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1962). The tentative hypothesis that defects in catecholamine biosynthesis is responsible for the symptoms associated with this disease is supported by a number of ancillary observations including the provocation of tremor by agents which decrease central nervous system catecholamine content (Hornykiewicz, 1966). Although no clear animal model exists which corresponds precisely to the human condition, lesions in certain areas of midbrain of monkeys lead to a decrease in dopamine content in the striatum and pallidum, and to the appearance of extrapyramidal disfunction (Poirier and Sourkes, 1965; Sourkes and Poirier, 1966). These studies have encouraged a rational therapeutic approach to the treatment of Parkinsonism based upon replacement of brain dopamine with the precursor amino acid DOPA.[1] While early reports of therapeutic attempts utilizing DOPA were conflicting (Birkmayer and Hornykiewicz, 1961; Barbeau, 1962; Gerstenbrand *et al.*, 1963; Fehling, 1966), more recently success has been claimed in controlling the disability utilizing carefully regulated dosages

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[1] The following abbreviations were used: DOPA, 3,4-dihydroxyphenylalanine; tyrosine aminotransferase, L-tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5); *m*-tyrosine, 3-hydroxyphenylalanine.

TABLE I: Ultraviolet Absorption Spectra for Inhibitors Investigated.

Inhibitor	Peaks (mμ)	Mol Extinction Coeff
II DOPA–pyridoxal	327	8320
	288	4050
III Dopa–P-5-P	326	7300
	289	3950
IV *m*-Tyrosine–pyridoxal	327	8210
	281	2240
	251	5640
V *m*-Tyrosine–P-5-P	326	7700
	283	2400
	251	5580
VI Pyridoxyltyrosine	327	4310
	252	2700
VII P-5-P–tyrosine	326	4280
	251	2700

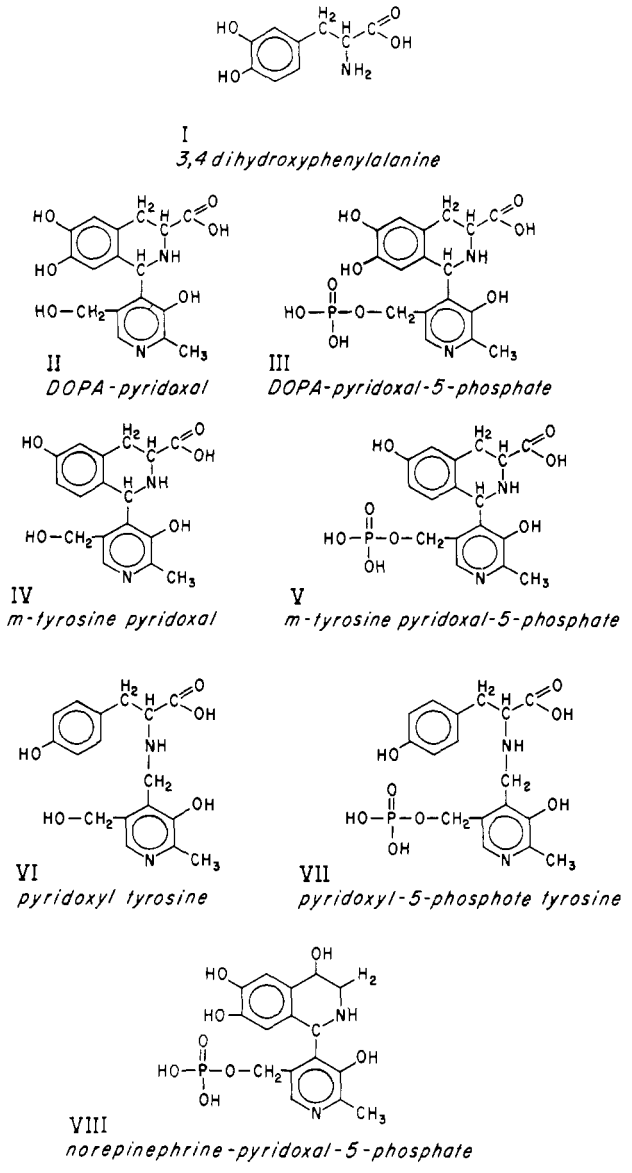

FIGURE 1: Structural formulae and trivial names for inhibitors investigated.

of L-DOPA (Cotzias *et al.*, 1969; Yahr *et al.*, 1969; Mawdsley, 1970).

Interestingly the salutary effects as well as the serious side effects experienced by patients treated with L-DOPA were reversed by vitamin B6 (pyridoxal) (Duvoisin *et al.*, 1969). It is well established that L-DOPA and *m*-hydroxyphenylethylamines react nonenzymatically with aldehydes including pyridoxal and pyridoxal 5-phosphate to corresponding tetrahydroisoquinoline derivatives (Schott and Clark, 1952). These products are stable and can account for the premature cessation of enzymic decarboxylase activity observed when L-DOPA is employed as substrate in *in vitro* studies. In this paper we wish to report our observations on the effects of DOPA and its pyridoxal 5-phosphate condensation product on the activity of tyrosine aminotransferase. We examined the behavior of L-DOPA as substrate and as an inhibitor of tyrosine aminotransferase, an enzyme that transaminates L-DOPA and contains pyridoxal 5-phosphate as coenzyme.

While these investigations were in progress a study of the effect of norepinephrine on tyrosine aminotransferase was published (Black and Axelrod, 1969). The conclusion by these investigators that norepinephrine inhibits transaminase activity by competing for the coenzyme was examined in the light of our current findings. The results of these studies are included.

Experimental Sections

Chemicals L-Tyrosine, DL-*m*-tyrosine, pyridoxal 5-phosphate, α-ketoglutarate, and L-DOPA were obtained from Sigma Chemical Co. L-tyrosine-*side-chain-2,3-*^3H was obtained from Amersham Searle. D-L-DOPA-*side-chain-2,3-*^3H was purchased from Tracerlab Inc. 6,7-Dihydroxy-1-(3′-hydroxy-5′-(methyl phosphate)-2′-methyl-4′-pyridyl)-1,2,4-tetrahydro-3-carboxylisoquinoline (L-DOPA, pyridoxal 5-phosphate condensation product III) was prepared as described by Schott and Clark (1952) after Heyl *et al.* (1948). To 197 mg (1 mM) of L-DOPA dissolved in a minimum volume of water at pH 7.0 was added 247 mg of (1 mM) pyridoxal 5-phosphate adjusted to the same pH. The clear solution is warmed on a steam bath for 1–2 hr. The pH is adjusted to 5.0 and the solution was placed in an ice chest for 5 hr during which needles of the tetrahydroisoquinoline separated out. The product is recrystallized from water. In a similar manner, DL-*m*-tyrosine–pyridoxal condensation product IV, its 5-phosphate derivative V, and norepinephrine–pyridoxal 5-phosphate condensation product VIII, were prepared. The infrared absorption spectra are consistent with the expected products and are given in Figure 2. The ultraviolet absorption spectra and extinction coefficients are given in Table I. The CH analysis was in satisfactory agreement with the calculated values for these compounds. Paper and thin-layer chromatography of the phosphate esters were attempted using a variety of solvent systems with little success. However thin-layer electrophoresis at 5 mA for 1 hr of 0.05 M borate (pH 9.3) buffer in a Desaga–Brinkmann electrophoresis apparatus afforded separation of these substances from their parent compounds. The pyridoxal–L-tyrosine VI and pyridoxal 5-phosphate–L-tyrosine VII were prepared by a modification of the method of Heyl *et al.* (1948) using borohydride to reduce the Schiff-base intermediate. L-Tyrosine (800 mg) was

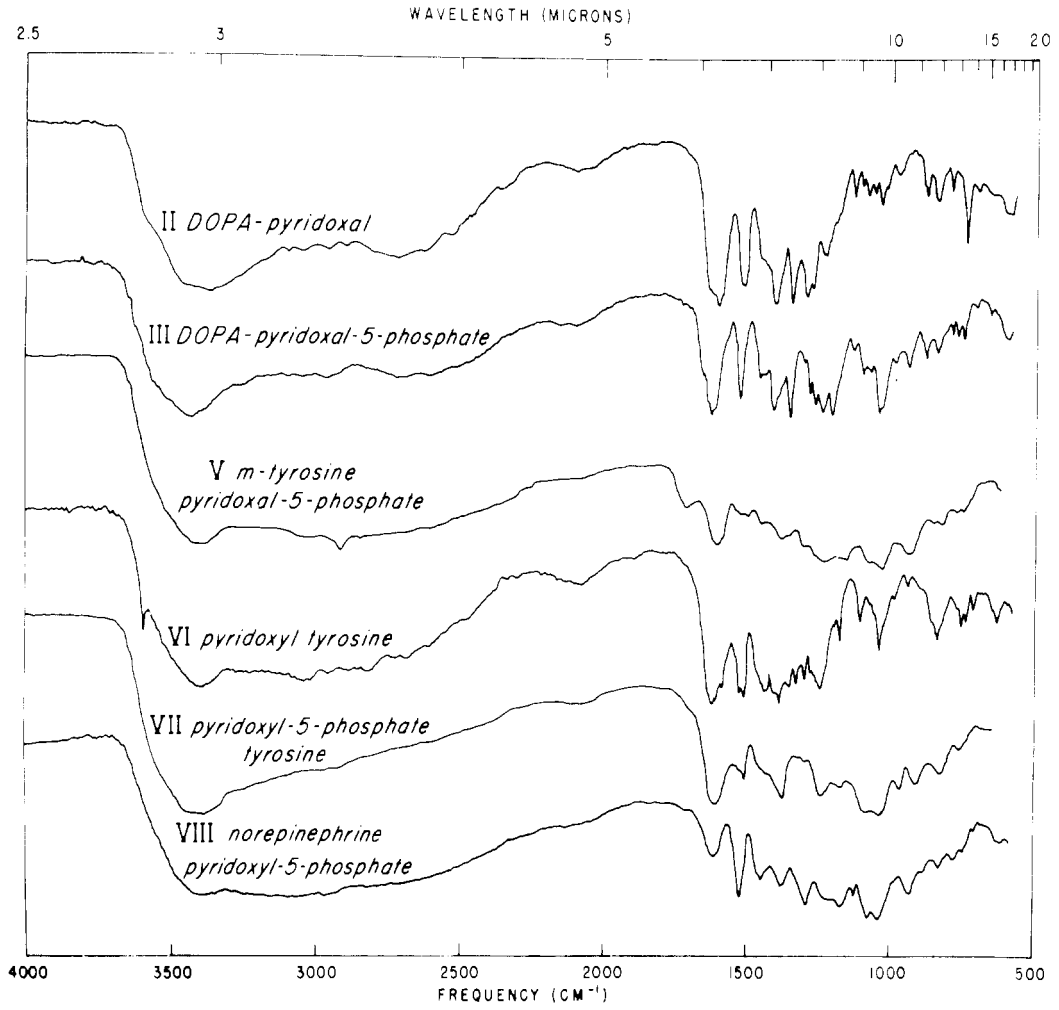

FIGURE 2: Infrared spectra of inhibitors studied were determined in a Perkin-Elmer 221-G infrared spectrophotometer using KBr pellets.

dissolved in a minimum amount of 1 N NaOH and added to 247 mg (1 mM) of pyridoxal 5-phosphate dissolved in a minimum amount of 1 N NaOH. The yellow solution rapidly turned orange indicating Schiff-base formation. After 1 min 53 mg (1 mM) of KBH_4 was added and the reduction was allowed to proceed. The loss of color indicated the completion of the reduction. The pH was adjusted with dilute HCl to pH 7.0, cooled and the excess tyrosine was filtered off. The residue was evaporated in a rotary evaporator, taken up in hot ethanol, and recrystallized from ethanol and ethanol–ether mixtures.

Enzyme Methods

Assay of Tyrosine Aminotransferase Activity. This activity was measured by the radiochemical method previously reported (Fellman *et al.*, 1969). A simplified means of sampling the released $[^3H]H_2O$ was employed in this assay. The assay was carried out in Thunberg tubes. The following modifications were made in sampling the tritiated water. The reaction was terminated by the addition of 0.1 ml of freshly prepared 1 M hydroxylamine at pH 8.0. The release of tritium from the substrate was measured in the following manner. After the addition of the hydroxylamine to stop the enzyme, a pledget of glass wool was introduced into the Thunberg tube and the top was fitted in place. The tube was then evacuated briefly with a water pump; the top was rotated to maintain the vacuum and the Thunberg tubes were then placed in a water bath maintained at 60° with the side arms resting in a trough of ice. The distillation proceeded rapidly and twelve or more tubes can be handled easily at one time. After distillation was complete (this precluded any isotope effect on the rate of distillation) the tubes were uncapped and 0.5 ml of the distillate in the side arm was placed in scintillation vials; 19 ml was added of a mixture containing 2 g of 2,5-diphenyloxazole and 50 mg of 1,4-[bis-2-(5-phenyloxazolyl)]benzene in 250 ml of absolute ethanol plus 250 ml of toluene (Buhler, 1965).

Assay of DOPA Aminotransferase Activity. Two procedures were followed; both are a modification of the methods of Fellman *et al.* (1969).

RADIOCHEMICAL. A standard 1.75-ml final volume contained 5.1 mM [2,3-^{3}H]-L-DOPA, 1 μCi/ml, 4.3 mM α-ketoglutarate, 0.002 mM pyridoxal 5-phosphate, and 200 mM phosphate buffer (pH 7.4) was placed in a standard Thunberg vessel. Standard assay incubation was carried out at 37° for 30 min with shaking. Boiled controls were routinely run with each experiment. The reaction was stopped by the addition of hydroxylamine (0.1 ml of a 1.0 M, pH 8.0). In some experiments, described in the Results section, all additions less

TABLE II: Ratios of Tyrosine–DOPA Aminotransferase Activities During Enzyme Purification.

Fraction	μmoles/mg per 30 min		
	Tyrosine	DOPA Amino-trans-ferase	Ratio Tyrosine/ DOPA
1. Supernatant[a]	0.028	0.004	7.1
2. After heat 60° 5 min	0.139	0.019	7.5
3. After ammonium sulfate	0.163	0.024	7.0
4. After DEAE-cellulose	1.60	0.140	11.0
5. After sucrose gradient	2.31	0.237	9.9
6. Disc electrophoresis[b]			10.2

[a] The purification procedure detailed by Valeriote *et al.* (1969) was followed. Fractions were submitted to multiple assay of rat liver extracts brought through purification. The livers of six rats were used as a source of enzyme. [b] The fraction obtained after DEAE-cellulose was submitted to electrophoresis in Tris-glycine buffer at pH 8.3, 5 mA/tube in a Buchler disc electrophoresis apparatus. The buffer contained 10^{-3} M EDTA, 10^{-3} dithiothreitol, and 2×10^{-4} M pyridoxal 5-phosphate to protect the enzyme during electrophoresis. The extruded disk was cut into fractions and the homogenized gels were assayed and their ratio expressed above.

the α-ketoglutarate were included as a control to establish that the tritium released from the DOPA was predicted on the presence of the α-ketoglutarate.

CHEMICAL. The chemical method was carried out using the identical additions and volumes given above save the absence of radioactive DOPA. The reaction was terminated by the addition of 0.5 ml of a 10% solution of sulfosalicylic acid. The tubes were centrifuged and 0.5 ml of supernatant was transferred into tubes containing 0.5 ml of 2 M hydrazine. After 15 min, 3 ml of 4-chloro-*o*-phenylenediamine, 100 mg in 100 ml of 85% phosphoric acid, was added. The tubes are mixed and placed in a boiling-water bath for 10 min, cooled, and the optical density at 380 mμ is determined in a Beckman DB spectrophotometer. Controls were run excluding the α-ketoglutarate acceptor.

The purification of rat liver tyrosine aminotransferase was effected following the procedure of Valeriote *et al.* (1969).

Results

L-*DOPA—a Substrate for Tyrosine Aminotransferase.* The fact that L-DOPA may serve as a substrate for tyrosine aminotransferase has been reported by other investigators (Cammarata and Cohen, 1950; Jacoby and La Du, 1964). Since in no study was purified enzyme employed, we undertook the stepwise isolation and assay of this enzyme using both L-DOPA and tyrosine as substrates and an exhaustive purification procedure of tyrosine aminotransferase (Valeriote *et al.*, 1969). Table II gives the results of this study. The purification of the rat liver cytosol enzyme included an additional procedure, *i.e.*, disc gel electrophoresis which helped establish that the enzymatic activity toward both substrates resides in a single enzyme. Thus the ratio of tyrosine to DOPA aminotransferase activities remained roughly constant.

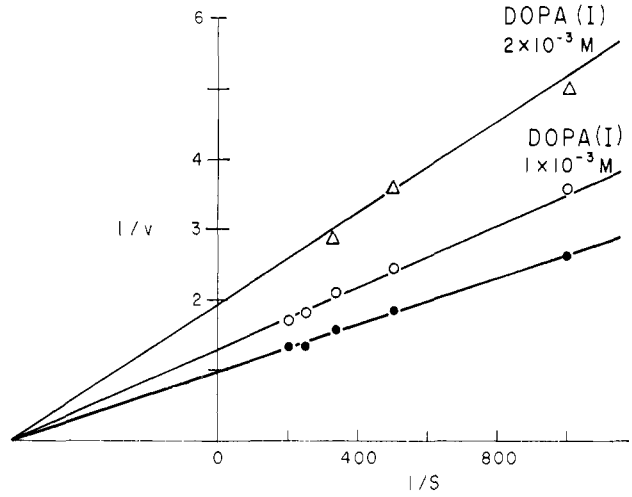

FIGURE 3: Reciprocal plot of velocity of tyrosine aminotransferase activity (*V*) *vs.* tyrosine concentration [S] (●—●). The effect of two concentrations of DOPA (I) (○—○) = 1×10^{-3} M, (△—△) = 2×10^{-3} M is shown. The enzyme used was partially purified rat liver cytosol fraction, specific activity of 1.60 μM transaminated/mg of protein per 30 min.

L-*DOPA and Derivatives as Inhibitors of Tyrosine Aminotransferase.* Observing that a single cytosol enzyme is responsible for transaminating both tyrosine and DOPA, we undertook to determine whether L-DOPA was an inhibitor of tyrosine and the type of inhibition observable. Since DOPA can nonenzymatically react with the coenzyme pyridoxal 5-phosphate we speculated that the inhibition would be noncompetitive with respect to the substrate tyrosine. Figure 3 shows that indeed DOPA behaved as a noncompetitive inhibitor; *m*-tyrosine behaved in a similar manner. Evidence for a reaction to form an inhibitor is given in Figure 4.

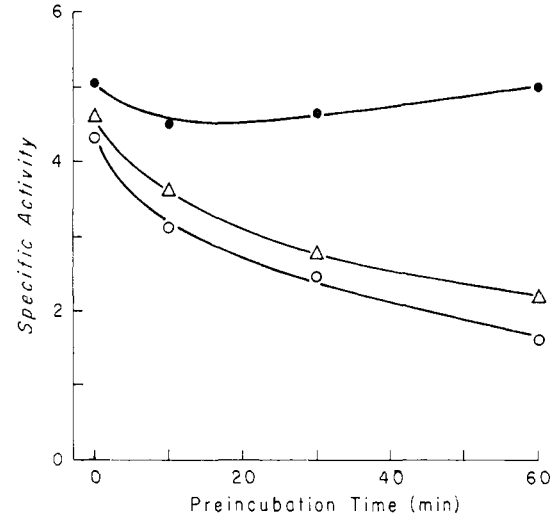

FIGURE 4: The effect of preincubation of tyrosine aminotransferase with L-DOPA (△—△) and DL-*m*-tyrosine (○—○). Controls (●—●) were treated in the same manner as experimental, using buffer in place of the corresponding inhibitors. Both inhibitory amino acids were added at 1×10^{-3} M final concentration. Assays were initiated by the addition of substrate tyrosine and carried out as described in the Enzyme Methods section.

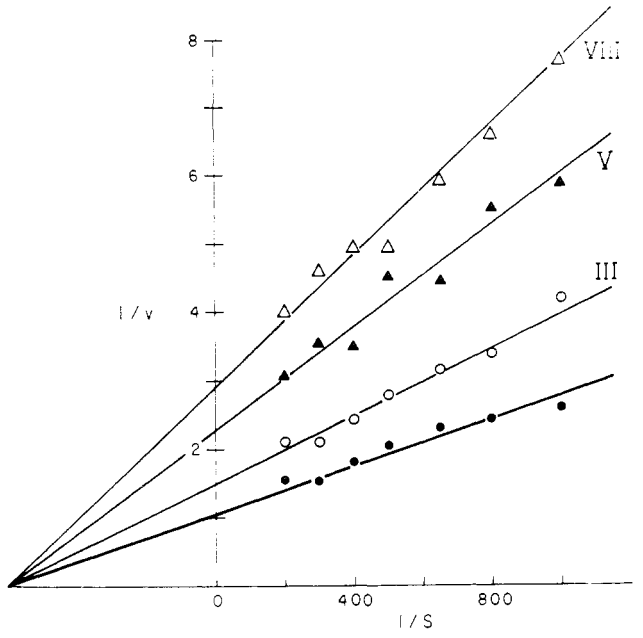

FIGURE 5: Reciprocal plot of the velocity of tyrosine aminotransferase activity (V) *vs.* tyrosine [S] concentration (●—●). The effects of various inhibitors are shown. (○—○) DL-DOPA-pyridoxal 5-phosphate (III) 3×10^{-3} M final concentration, (▲—▲) DL-*m*-tyrosine-pyridoxal 5-phosphate (V) 5×10^{-4} M final concentration and (△—△) norepinephrine-pyridoxal 5-phosphate (VIII) 5×10^{-3} M final concentration. The enzyme used was as described in Figure 3.

TABLE III: Inhibitory Effects of DOPA and Tyrosine Derivatives on Tyrosine Aminotransferase Activity.

Inhibitor[a]	Final Concn (M)	% Inhibn[a]
L-DOPA (I)	1×10^{-3}	32
	2×10^{-3}	55
L-DOPA–pyridoxal (II)	2×10^{-3}	0
DL-DOPA–P-5-P (III)	1×10^{-3}	10
	2×10^{-3}	20
DL-*m*-Tyrosine–pyridoxal (IV)	3×10^{-3}	0
	5×10^{-3}	31
	1×10^{-2}	50
DL-*m*-Tyrosine–P-5-P (V)	1×10^{-3}	51
	2×10^{-3}	65
L-Pyridoxyltyrosine (VI)	5×10^{-4}	35
	2×10^{-3}	50
L-Pyridoxyl 5-phosphate–tyrosine (VII)	5×10^{-4}	59
	2×10^{-3}	69

[a] The inhibitors were added to the reaction vessel without preincubation. Assays of activity were conducted as described in the Enzyme Methods sections and the decrease of enzyme activity is calculated as per cent inhibition.

Here increased inhibition of tyrosine aminotransferase is observed when either DOPA or *m*-tyrosine is preincubated with the enzyme assay mixture. The progression of inhibition was thought to be in part due to the nonenzymatic appearance of the condensation product formed from DOPA and pyridoxal 5-phosphate. Thus we examined the inhibitory effects on tyrosine aminotransferase activity of the DOPA and *m*-tyrosine condensation product formed from pyridoxal 5-phosphate. The Lineweaver–Burk plots of these data are given in Figure 5.

The inhibitory effects of several of these compounds on mitochondrial tyrosine aminotransferase were examined. Rat brain mitochondria were isolated as previously described (Fellman *et al.*, 1969). Table IV includes data which indicate that these compounds do not inhibit mitochondrial tyrosine aminotransferase at the concentrations tested. In other experiments the mitochondria were preincubated with the inhibitors at 2×10^{-3} M for 20 min before assaying for tyrosine aminotransferase activity. No inhibitory activity was evident.

The inhibition of tyrosine aminotransferase by norepinephrine has been described (Black and Axelrod, 1969). These authors demonstrated a product formed from pyridoxal 5-phosphate and the *m*-hydroxyphenylethylamine of their interest—norepinephrine. However, they conclude that a more complex product than the isoquinoline derivative was formed from 2 moles of pyridoxal 5-phosphate and 1 mole of norepinephrine reversibly. This product formation competed with the enzyme for available coenzyme. We examined an alternate hypothesis, namely that an inhibitory product was formed by condensation of pyridoxal 5-phosphate and norepinephrine to the corresponding isoquinoline. Since the recognition sites of the enzyme include the pyridoxal 5-phosphate moiety, we again predicted that the inhibition would appear noncompetitive with respect to the substrate tyrosine. Figure 5 includes data consistent with this prediction.

The conclusion that two pyridoxal 5-phosphate molecules react reversibly with 1 mole of norepinephrine was examined by attempting to reverse this product formation. Figure 6 shows the spectra obtained before and after incubation of the addition product of norepinephrine and pyridoxal 5-phosphate with hydroxylamine. Since pyridoxal 5-phosphate forms a stable oxime with hydroxylamine, we added a 200-fold excess of this amine in an attempt to reverse the putative complex. We observed no shift in the spectra.

A number of other derivatives of tyrosine–pyridoxal were studied and their inhibitory activities are recorded in Table III.

Discussion

One of the central questions examined in this investigation deals with the convergence of DOPA metabolism with tyrosine metabolism. The issue emerges under the special circumstances encountered when, for example, patients with Parkinsonism are fed large quantities of DOPA. The evidence summarized in Table II supports the conclusion that cytosol tyrosine aminotransferase can transaminate DOPA. Throughout rigorous purification the ratio of enzymatic activity toward tyrosine and DOPA remains approximately constant.

Another aromatic amino acid aminotransferase activity is found in the mitochondria which contributes to the transamination activity of tissue. In some tissue such as rat brain it represents the greatest amount if not the exclusive form of the enzyme (Fonnum and Larsen, 1965; Fellman *et al.*, 1969). In contrast to the liver cytosol enzyme, the brain mitochondrial tyrosine aminotransferase was not sensitive to these inhibitors at 2×10^{-3} M. The preincubation of the

TABLE IV: Effects of DOPA and Tyrosine Derivatives on Brain Mitochondrial Tyrosine Aminotransferase Activity.[a]

Inhibitor[b]	Final Concn (M)	% Inhibn[b]
L-DOPA (I)	2×10^{-3}	0
L-DOPA–P-5-P (III)	2×10^{-3}	0
L-Pyridoxal 5-phosphate–tyrosine (VII)	2×10^{-3}	0

[a] Washed brain mitochondria were preincubated for 20 min before being assayed for tyrosine aminotransferase activity. These results were identical with those obtained when no preincubation period was employed. [b] The inhibitors were added to the reaction vessel without preincubation. Assays of activity were conducted as described in the Enzyme Methods sections and the decrease of enzyme activity is calculated as per cent inhibition.

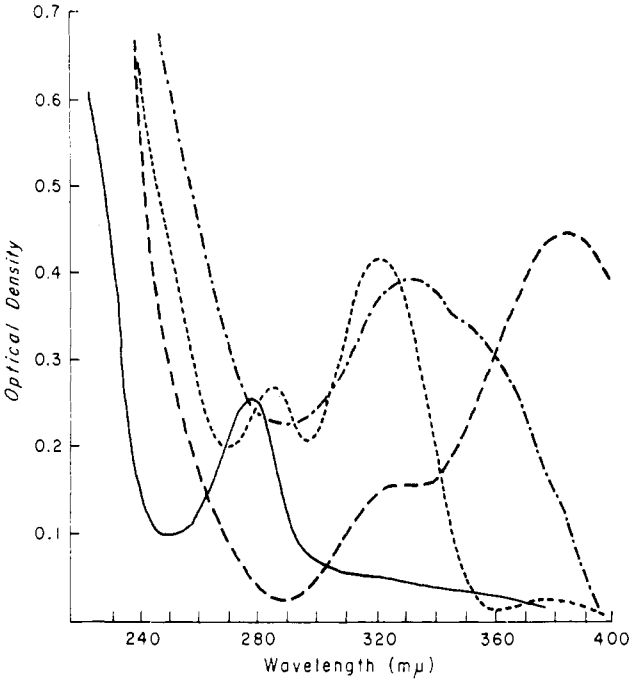

FIGURE 6: Ultraviolet absorption spectra of norepinephrine, 1×10^{-4} M (——), pyridoxal 5-phosphate, 1×10^{-4} M (— — —), the resultant condensation product after 1-hr incubation at 25° (- - -) and pyridoxal 5-phosphate oxime (·—·—). The spectrum of condensation product of norepinephrine and pyridoxal 5-phosphate did not change after the addition of hydroxylamine at 0.02 M final concentration. These spectra were carried out with a Cary Model 15 spectrophotometer.

mitochondria with L-DOPA did not effect the activity of the enzyme. These differences can in part be explained from the affinity of the pyridoxal 5-phosphate for the mitochondrial enzyme. Brain mitochondrial tyrosine aminotransferase does not require any additional pyridoxal 5-phosphate for maximum activity (Miller and Litwack, 1969). This suggests a firmer bond between the coenzyme and apo enzyme in the mitochondrial form which is not easily dislodged by the DOPA or DOPA–pyridoxal 5-phosphate III.

Since both substrates can be transaminated by the same liver cytosol enzyme, the possibility of competitive inhibition (*i.e.*, substrate competition) of tyrosine transamination by DOPA was examined. A noncompetitive form of inhibition was observed (Figure 3). This observation adds to the view that DOPA forms a condensation product which is a noncompetitive inhibitor with respect to the substrate tyrosine. An earlier investigation (Black and Axelrod, 1969) of the inhibitory effects of norepinephrine in tyrosine aminotransferase activity included some data from which was concluded that a derivative was formed from one molecule of norepinephrine and two of pyridoxal 5-phosphate. This condensation product was claimed to be in equilibrium with the precursors. We examined this point by attempting to reverse the product formed from the additional of norepinephrine and pyridoxal 5-phosphate. Since pyridoxal forms a stable oxime with hydroxylamine, we added 0.02 M hydroxylamine (200-fold excess) to 1×10^{-4} M condensation product. No shift to the oxime occurred. We conclude that the pyridoxal 5-phosphate forms a stable isoquinoline derivative with norepinephrine as it does with all *m*-hydroxyphenylethylamines examined.

The inhibition of tyrosine aminotransferase by norepinephrine was competitive with respect to the coenzyme pyridoxal 5-phosphate (Black and Axelrod, 1969) but noncompetitive with respect to the substrate tyrosine (*vide supra*). These observations point to an inhibitor whose molecular character provides recognition sites for the enzyme that includes the pyridoxal 5-phosphate moiety. Thus the inhibitory effect norepinephrine on tyrosine aminotransferase involves the generation of an inhibitory product (*i.e.*, the isoquinoline derivative from pyridoxal) and not exclusively or at all the deprivation of the coenzyme from the reaction.

The data in Table III present evidence for the "geometry of fit" for the inhibitors of tyrosine aminotransferase activity. DOPA (I) or DOPA–pyridoxal 5-phosphate are effective inhibitors of the enzyme—in the former case by generation of the isoquinoline derivative with pyridoxal 5-phosphate. DOPA–pyridoxal (II) lacking the phosphate moiety clearly is a poorer fit for the enzyme since it possessed no inhibitory activity at the concentration tested. Other quantitative examples of this relationship for inhibitor fit as a characteristic of the presence of crucial functional groups is shown in the cases of *m*-tyrosine–isoquinoline derivative (IV and V) and pyridoxal–tyrosine derivatives (VI and VII).

The effects of feeding DOPA to patients with Parkinsonism could be expected to alter the blood plasma tyrosine concentration by the inhibition of tyrosine aminotransferase. Since this enzyme is rate limiting and thus controls the major pathway for tyrosine metabolism, the inhibition of this enzyme should lead to elevated tyrosine blood levels, all other factors being unaltered. The aromatic amino acids in the plasma of 12 patients with Parkinsonism treated with oral doses ranging from 1 to 8 g of L-DOPA per day for up to 40 days were studied to investigate this question. Using the standard conditions employed in the Beckman 120C amino acid analyzer, a shoulder appears on the tyrosine peak in plasma samples from treated patients, which was shown to be 3-methoxytyrosine. This is a prominent metabolite of L-DOPA and could be resolved from the other amino acids employing a pyridine–formate buffer (Fellman *et al.*, 1970, unpublished data). With this correction no significant alteration in tyrosine blood levels was observed. Recently the acute effects of DOPA injection in human subjects have been reported (Grundig *et al.*, 1969). A rise in cerebrospinal

fluid tyrosine and phenylalanine concentration was reported; while no change in serum tyrosine was observed. However, a compensatory increase in tyrosine aminotransferase by enzyme induction could maintain hepatic tyrosine metabolism and thus sustain normal plasma tyrosine concentration. The increase in cerebrospinal fluid tyrosine cannot have resulted from inhibition of brain tyrosine aminotransferase since this enzyme does not appear to be sensitive to L-DOPA inhibition. More recently other workers report a small but significant drop in plasma tyrosine concentration during DOPA therapy and no change in cerebrospinal fluid tyrosine concentration (Van Woert and Bowers, 1969). This apparently conflicting data cannot be easily rationalized. While one might suspect that feeding large therapeutic levels of DOPA will exercise some impact of aromatic amino acid metabolism, the variables have not been sorted out to allow one to predict which, if any, amino acids are affected, and in which direction.

Acknowledgments

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