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B. TYROSINE HYDROXYLASE

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It is of interest that, of the three steps involved in the biosynthesis of norepinephrine (NE), two are hydroxylations involving the direct addition of a hydroxyl moiety, derived from atmospheric oyxgen, to the substrate. In animals, tyrosine can be derived from dietary phenylalanine by another hydroxylase found in liver. However, NE synthesis may be considered to start with tyrosine, which represents a branch point for many important biologic processes in animal tissues (fig. 1). For a long time formation of the first intermediate, dopa, in the sympathetic nervous system was considered to be catalyzed by tyrosinase, the enzyme which forms dopa and melanin in melanin-forming tissues. It was even suggested that the conversion of tyrosine to dopa might proceed without an enzyme. With the isolation of tyrosine hydroxylase (12) it became apparent that sympathetic nervous tissues contain a highly specific enzyme for converting tyrosine to dopa and that this enzyme is not related to tyrosinase. The enzyme has now been demonstrated directly in adrenal medulla, brain, heart and other

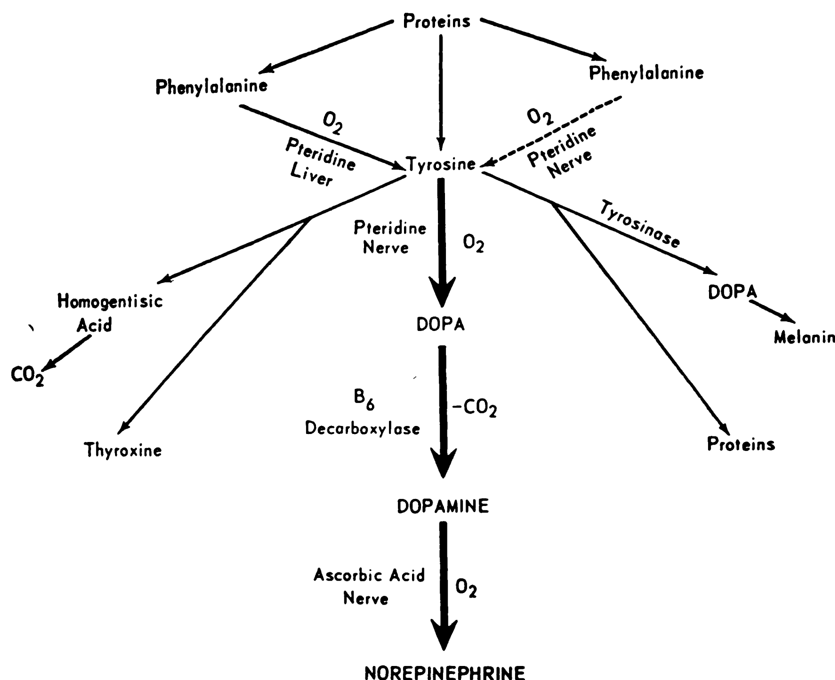


FIG. 1. Various routes of tyrosine metabolism in animal tissues

TABLE 1

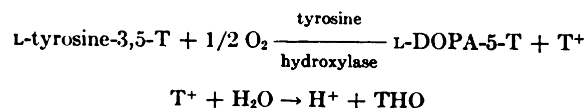
Requirements of purified tyrosine hydroxylase

Additions	Dopa Formed
Complete system	13.0
$-DMPH_4$	0.3
$-Fe^{++}$	2.8
−Enzyme	0.0
−Oxygen	0.1

tissues from a variety of animal species and has been purified several hundredfold from beef adrenal medulla. Some properties of the purified enzyme, its distribution in tissues and evidence for its role in the formation of the neurotransmitter are presented here.

PROPERTIES OF TYROSINE HYDROXYLASE

The enzyme in brain and adrenal medulla is associated with subcellular particles (see below) from which it is readily dissociated by prolonged homogenization. Following such homogenization the soluble enzyme can be purified by standard procedures. Tyrosine hydroxylase from beef adrenal medulla has been purified 400- to 500-fold and has been shown to require a tetrahydropteridine cofactor, Fe^{++} and oxygen (table 1). These requirements are the same as those previously reported for phenylalanine hydroxylase (6) and, in fact, it has been shown that the naturally occurring pteridine cofactor, dihydrobiopterin, which functions with liver phenylalanine hydroxylase, can activate adrenergic tyrosine hydroxylase (1). The enzyme is assayed most simply by measuring the release of tritium from 3,5-ditritiotyrosine (13). The tritium equilibrates with water, which is readily separable from excess substrate and other labeled products. The radioactivity in the water is equivalent to the dopa formed (fig. 2).



$$\text{L-tyrosine-3,5-T} + 1/2\,O_2 \xrightarrow[\text{hydroxylase}]{\text{tyrosine}} \text{L-DOPA-5-T} + T^+$$

$$T^+ + H_2O \rightarrow H^+ + THO$$

FIG. 2. Conversion of 3,5-tritiotyrosine to 5-tritio-dopa and tritium water by tyrosine hydroxylase.

The purified enzyme does not hydroxylate D-tyrosine, tyramine or tryptophan. It is of interest that *m*-tyrosine is not converted to dopa whereas phenylalanine is converted to *p*-tyrosine (5). In both instances the para position is available for hydroxylation (fig. 3). It would appear therefore that the *m*-hydroxyl group prevents the entry of an adjacent hydroxyl group. The ability of tyrosine hydroxylase to convert phenylalanine to tyrosine is of interest for many reasons. First, it represents a consecutive sequence of reactions catalyzed by one and the same enzyme. This is rather unusual but is comparable to the action of xanthine oxidase on xanthine and hypoxanthine. The ability to oxidize both phenylalanine and tyrosine should make it possible to distinguish among certain suggested

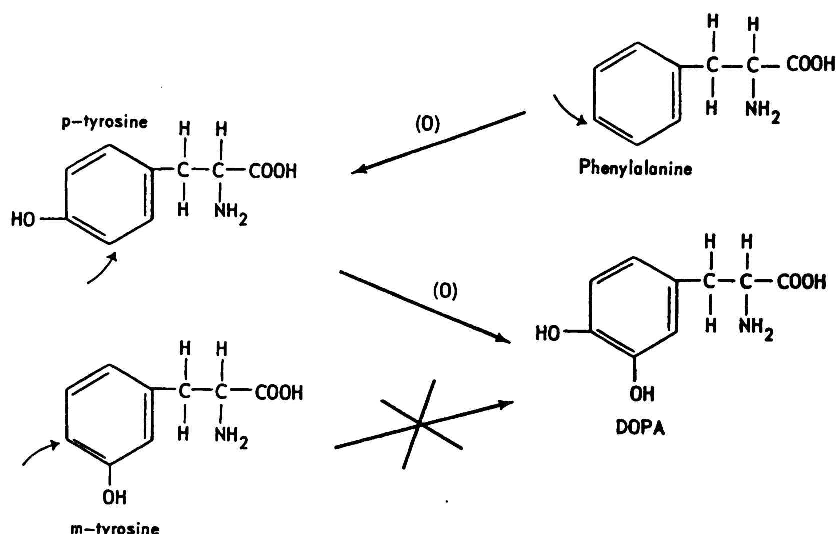


FIG. 3. Substrates of tyrosine hydroxylase

mechanisms of aromatic hydroxylation (fig. 4). When phenylalanine is incubated with tyrosine hydroxylase, both tyrosine and dopa are formed. It was found, however, that the appearance of free tyrosine in the incubation mixture preceded that of dopa (fig. 5) and that there were always appreciable amounts of the former in the incubation flask. This is true in spite of the fact that the K_m for tyrosine (5×10^{-5} M) is much lower than the K_m for phenylalanine (3×10^{-4} M). These data suggest that the enzyme first converts phenylalanine to tyrosine and that the enzyme-tyrosine complex is not directly oxidized to an enzyme-dopa complex. In other words, as suggested by Kaufman (6), the hy-

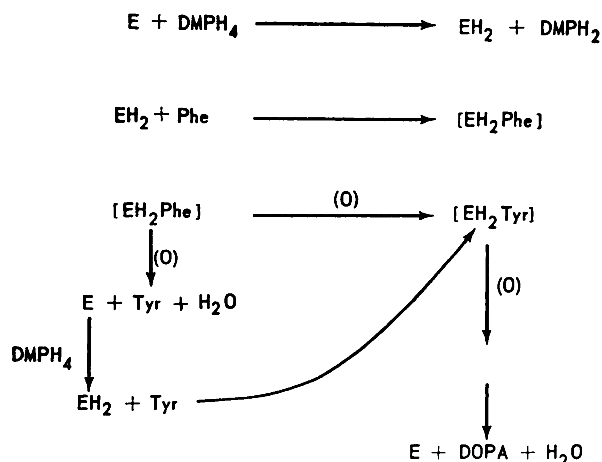


FIG. 4. Alternative mechanisms to explain consecutive hydroxylation of phenylalanine and tyrosine by tyrosine hydroxylase. E = tyrosine hydroxylase and DMP = pteridine.

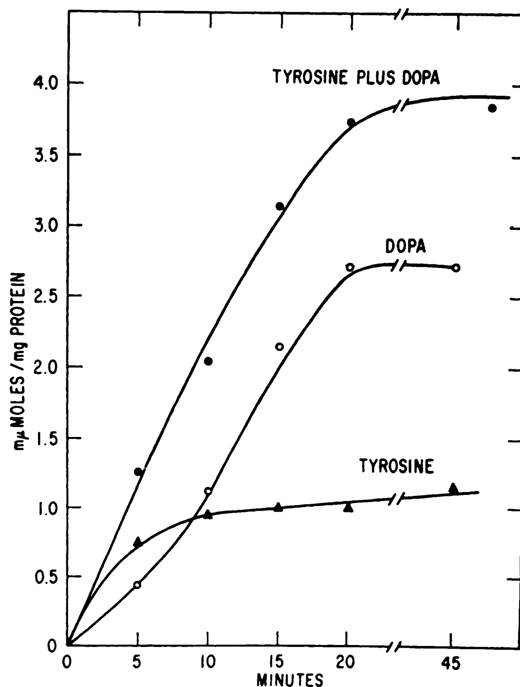


FIG. 5. Appearance of tyrosine and dopa during the oxidation of phenylalanine by tyrosine hydroxylase. (From Biochem. Biophys. Res. Comm. **18:** 482–488, 1965.)

droxylase must first be reduced to be available for hydroxylation. The oxygen oxidizes both the substrate (phenylalanine) and the enzyme. Of the two possible mechanisms in figure 4, the data favor the one showing that the oxidized enzyme and product (tyrosine) dissociate (fig. 4, lower left). It appears that the enzyme must again be reduced by the cofactor in order to be in a form capable of reacting with the tyrosine so as to convert it to dopa. Kinetic studies with this two-step reaction may provide important information concerning the mechanism of tetrahydropteridine hydroxylases.

The conversion of phenylalanine to tyrosine by adrenergic tyrosine hydroxylase indicates that the liver is not the sole site for tyrosine production. Earlier studies in patients with phenylpyruvic oligophrenia, where the liver phenylalanine hydroxylase is known to be missing, had shown a small amount of extrahepatic hydroxylation of phenylalanine (18). The phenylalanine hydroxylase activity of adrenergic tyrosine hydroxylase would explain the previous *in vivo* findings. It is quite likely that the demonstrable conversion of phenylalanine to tyrosine in phenylketonuria (about 5% of normal) is catalyzed by the enzyme in adrenergic tissues.

TYROSINE HYDROXYLASE INHIBITORS

Two classes of compounds have been studied as inhibitors of tyrosine hydroxylase. These are amino acids and catechols (4a, 20). Some of the inhibitors

TABLE 2

Some inhibitors of tyrosine hydroxylase

Compound	Concentration for 50% Inhibition
α-Methyl-tyrosine	2.5×10^{-5}
3-Iodo-tyrosine	5×10^{-7}
3,5-Diiodotyrosine	2×10^{-5}
3-Iodo-α-methyl-tyrosine	3×10^{-7}
Norepinephrine	1×10^{-3}
α-Methyl-dopa	1.5×10^{-3}
Hassle 22/54	2×10^{-5}

which were tested are listed in table 2, where it can be seen that of the amino acids α-methyl-*p*-tyrosine and some of the 3- or 3,5-halogenated tyrosines are the most potent. These inhibit by competing with substrate. The most potent inhibitor of the purified enzyme yet found is 3-iodo-α-methyl-*p*-tyrosine. Its K_I is 1.8×10^{-7} compared to a K_m for tyrosine of about 5×10^{-5}.

All the catechol compounds investigated were inhibitory. It should be noted (table 2) that NE and many of the normally occurring catechol metabolites are of about comparable activity. However, certain catechols such as 3,4-dihydroxyphenylpropylacetamide (Hassle 22/54) were far more potent than the others. The reason for the unusual inhibitory properties of compounds like Hassle 22/54 is not apparent but may emerge as the mechanism of catechol inhibition is explained. We have shown that catechol inhibition is not competitive with the substrate but with the pteridine cofactor (20). This would suggest some interaction between the catechols and the pteridine. Although the mechanism of this interaction is not yet understood, it is apparent that it is not limited to tyrosine hydroxylase. Other pteridine-requiring hydroxylases such as liver phenylalanine hydroxylase (2) and mast cell tryptophan hydroxylase (8) are inhibited by catechols. In these instances Hassle 22/54 is again a most potent catechol inhibitor.

Inhibitors of tyrosine hydroxylase lower the endogenous production of catecholamines in animals (17) and in man (4) and thereby bring about a reduction in the tissue levels of NE and dopamine. In Sections VI F and VII E Dr. A. Sjoerdsma and Dr. S. Spector summarize studies with tyrosine hydroxylase inhibitors in patients and experimental animals.

ROLE OF TYROSINE HYDROXYLASE IN THE OVERALL PRODUCTION OF NE

For a variety of reasons many investigators of catecholamine metabolism suspected that the hydroxylation of tyrosine would be the rate-limiting step in the production of NE. However, this could not be verified until all three enzymes involved in NE biosynthesis had been characterized. The recent isolation of tyrosine hydroxylase from sympathetically innervated tissues made possible such studies.

It has been shown previously that the perfused adrenal gland can form NE and epinephrine (E) from tyrosine (15) and that it therefore contains all the

necessary synthetic enzymes. Studies in our laboratory with the perfused guinea pig heart gave similar results (16). Chidsey *et al.* (3) and Musacchio and Goldstein (11) also showed that the perfused heart can form NE from labeled dopa and dopamine. In a subsequent study we were able to show that the isolated perfused guinea pig heart not only can form NE from tyrosine-C^{14} but also can do so at a rate comparable to that shown to obtain *in vivo* (7). Furthermore, under appropriate conditions, essentially all the newly formed, labeled NE remained in the heart and was not released into the perfusate. The perfused guinea pig heart provided, therefore, an ideal *in situ* system for evaluating NE synthesis from each of the precursors. The data summarized in figure 6 show the rates of NE production obtained on perfusing each of the three precursors. Several things are apparent: 1) all three precursors yielded NE; 2) saturation was achieved only with tyrosine; 3) the maximal rate achieved with tyrosine, about 0.15 to 0.20 μg/g per hr, agrees favorably with values estimated from *in vivo* studies (10 and 19); and 4) the concentration of tyrosine required to form NE at half its maximal rate was about 2×10^{-5} M. The measured K_m of tyrosine hydroxylase is approximately 5×10^{-5} M.

The data in figure 6 are consistent with the conclusion that tyrosine hydroxyl

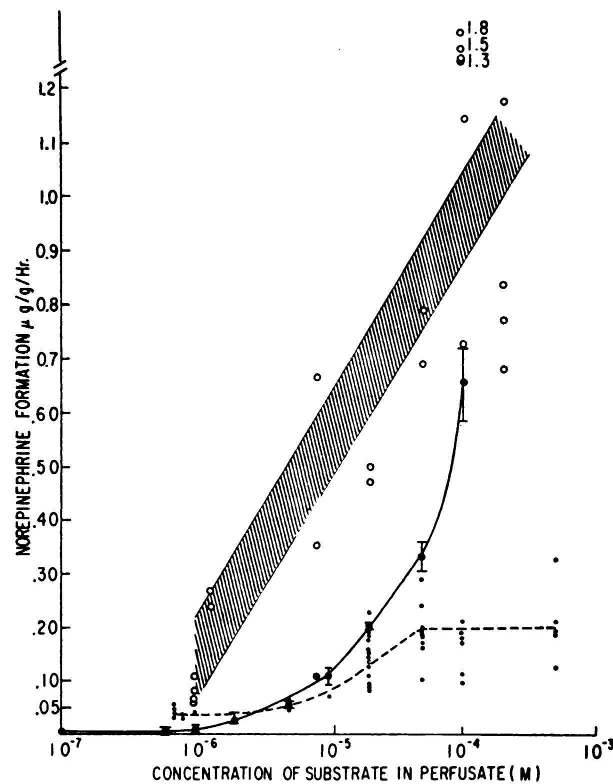


FIG. 6. NE formation from the three precursors in the isolated perfused **guinea pig heart.** Tyrosine O- - -O, dopa ●——●, and dopamine O/////O.

ase is the rate-limiting step in the formation of NE in the sympathetic nervous system. That the initial enzyme in a sequence should be the site of regulation is in agreement with current concepts of metabolic control. Although the latter information has been obtained largely from bacterial studies it is pertinent to animal systems too. However, several questions remain. The data in column 3 of table 3 show that the K_m for tyrosine hydroxylase is much lower than the K_m values for the other two enzymes. How can the enzyme with the lowest affinity for its substrate be the one that is rate-limiting? The data in column 4 of table 3 explain this. It can be seen that the apparent V_{max} for purified tyrosine hydroxylase is two or three orders of magnitude lower than the values for purified dopa decarboxylase and dopamine-β-hydroxylase. Where it has been possible to make measurements in intact tissues (slices and homogenates), tyrosine hydroxylase activity is usually measured in millimicromoles of tyrosine oxidized per gram of tissue per hour whereas the decarboxylase and dopamine-β-hydroxylase activities are measured in micromoles of substrate converted per gram of tissue per hour. Thus, tyrosine hydroxylation becomes limiting because the amount of enzyme is limiting. Furthermore, fasting tissue concentrations of tyrosine are about 10^{-4} M; at these concentrations tyrosine hydroxylase is fully saturated and normal variations in tyrosine concentration cannot influence NE formation.

That tyrosine hydroxylase is indeed rate-limiting is further suggested by studies *in vivo* with inhibitors. α-Methyl-*p*-tyrosine, α-methyl-phenylalanine, 3-iodotyrosine, 3-iodo-α-methyl-tyrosine and Hassle 22/54 all lower tissue levels of NE when administered to animals. This is significant since it had been reported previously that endogenous NE levels could not be lowered appreciably even when either of the other two enzymes was markedly inhibited (14).

The most direct eivdence to emerge from *in vivo* studies as to the rate-limiting nature of tyrosine hydroxylase was obtained recently in our laboratory (22). NE formation was estimated by administering tyrosine-C^{14} to guinea pigs, killing them 2 hr later, isolating the NE from individual tissues, and determining the total radioactivity. Under standard conditions the amount of tyrosine-C^{14} incorporated into NE in a given tissue was quite constant. When α-methyl-tyrosine was administered to the animals 30 min prior to the tyrosine-C^{14} much less radioactive NE appeared in the tissues. When the tissue levels of α-methyl-tyrosine and tyrosine were determined, it was possible to estimate the degree of inhibition of tyrosine hydroxylase by substituting the K_m and K_I values for tyrosine and the inhibitor in the equation shown in table 4. As shown in this table the inhibition of NE synthesis (measured by tyrosine-C^{14} incorporation)

TABLE 3

Comparison of properties of the three enzymes involved in NE biosynthesis

Enzyme	Purified from	$K_m \times 10^{-5}$	V_{max}
Tyrosine hydroxylase	Beef adrenal medulla	5	150
Dopa decarboxylase	Guinea pig kidney	40	33,000
Dopamine-β-oxidase	Beef adrenal medulla	580	50,000

TABLE 4

Comparison of measured inhibition of NE synthesis and calculated inhibition of tyrosine hydroxylase in guinea pig heart resulting from α-methyl-p-tyrosine

Time	α-Methyl-tyrosine	Calculated Inhibition of Tyrosine Hydroxylase Activity	Measured Inhibition of NE Synthesis	Radioactivity in Isolated NE
hr	*M*	%	%	*cpm/organ*
0	0	0	0	2522[a]
2	3.8×10^{-4}	92	95	124
4	4×10^{-4}	93	92	193
8	3.1×10^{-4}	91	96	97
16	8.7×10^{-4}	74	87	326
24	3.9×10^{-5}	56	61	988
36	—	—	6	2377

The average concentration of tyrosine in guinea pig heart was found to be 4.9×10^{-5} M.

[a] Average of replicate samples. Each calculated point $= 100\left(1 - \frac{V_I}{V_0}\right)$ where V_0 = normal rate of NE-C^{14} formation, V_I = rate in presence of inhibitor, $\frac{V_I}{V_0} = \frac{K_m + S}{K_p + S}$ and $K_p = \frac{i + K_I}{K_I} \times K_m$. $K_m = 6.25 \times 10^{-5}$ (for tyrosine); $S = 5 \times 10^{-5}$ M (measured concentration of tyrosine in heart); i = measured concentration of α-methyl-tyrosine in heart at each time interval; and $K_I = 1 \times 10^{-6}$ (for α-methyl-tyrosine).

and the calculated degree of tyrosine hydroxylase inhibition (estimated from tissue levels of tyrosine and α-methyl-tyrosine and their K_m and K_I values) coincide almost exactly. This could occur only if tyrosine hydroxylase were rate-limiting. Under such circumstances one would expect the residual tyrosine hydroxylase activity and NE synthesis to be the same.

CONCLUSIONS

What can be concluded from these properties of tyrosine hydroxylase? First, the amount of NE synthesis occurring at any one time is most probably related to the tyrosine hydroxylase activity. When the amount of enzyme is constant, then changes in its activity will be reflected by changes in NE. It has already been shown that the tyrosine concentrations normally occurring in tissues are sufficient to saturate the hydroxylase. Furthermore, even in starvation tyrosine levels do not fall below saturating values (9). One must conclude, therefore, that NE synthesis is not ordinarily influenced by variations in tyrosine concentration. On the other hand, inhibitors of tyrosine hydroxylase decrease NE synthesis in direct proportion to the degree of tyrosine hydroxylase inhibition. The latter observation makes it attractive to consider the possibility of other sympathetic control mechanisms which operate by influencing tyrosine hydroxylase activity. One such mechanism for which some evidence *in vitro* exists is end-product inhibition. Although the inhibition produced by catechols is not specific for tyrosine hydroxylase, one would expect that the concentration of catechols would be greatest in sympathetic nerve and that mainly in this tissue could the interaction with the reduced pteridine cofactor have any physiologic significance.

End-product inhibition would reduce NE synthesis in the presence of large amounts of the hormone. Such a situation could conceivably exist in patients with pheochromocytoma, where large amounts of catecholamines are secreted by the tumors. Much of this material is probably taken up by sympathetic nervous tissue, where it could block local synthesis of NE within the nerve.

The possibliity should be considered that tyrosine hydroxylase activity is increased during prolonged sympathetic stimulation. Conceivably this could represent increased synthesis of the enzyme or activation of the enzyme by one of the agents secreted during stimulation of sympathetic nervous system. With our more complete knowledge of the enzymes involved in NE synthesis it should be possible to apply biochemical methods to elucidate mechanisms which regulate sympathetic nervous activity.

A review of the overall biosynthesis of NE was presented by the author in a recent Harvey Society Lecture (21).

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