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DOPAMINE-β-HYDROXYLASE

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The first demonstration of the conversion of tyrosine to norepinephrine and epinephrine in cell-free preparations of adrenal medulla was made less than 10 years ago. It is a testimony to the rapid progress in our understanding of the enzymology of this process that one could, today, carry out the biosynthesis of these hormones in a test tube with a mixture of purified or partially purified enzymes and the appropriate cofactors.

In this review, the biochemical properties of one of these enzymes, Dopamine-β-hydroxylase,[2, 3] the enzyme that catalyzes the conversion of Dopamine to norepinephrine, will be discussed. Studies with this enzyme have led to insight into the mechanism of the hydroxylation reaction; they have led also to an understanding of some of the factors that might play a role in the regulation of the rate of biosynthesis of pressor amines *in vivo*.

HISTORICAL

Knowledge concerning the location of the catecholamines *in vivo* is based on their striking reducing property. Although not specific for catecholamines (4), it was this property that first brought them to the attention of the early histologists. Vulpian (165) was the first to discover the reducing characteristics of adrenal medullary tissue when he stained it with a number of oxidizing agents. It was not until 1895, however, that a hormonally active substance was isolated from the suprarenal medulla by Oliver and Schäfer (130, 131), and shown by Moore (119) to possess reducing properties. The substance, epinephrine, was purified by Takamine (159) in 1901. Stolz (157) and Dakin (31) synthesized both epinephrine and norepinephrine and described their physiological properties. Langley (104) demonstrated that the effects of epinephrine on smooth muscle simulate those caused by stimulation of their sympathetic nerve supply. As a result of both Langley's and his own work, Elliot (38) suggested that a positive reaction to epinephrine indicated the existence and nature of sympathetic nerves in an organ and, therefore, that this substance may be a neurotransmitter. It was not until many years later that the true neurotransmitter was isolated and identified as norepinephrine by von Euler (41).

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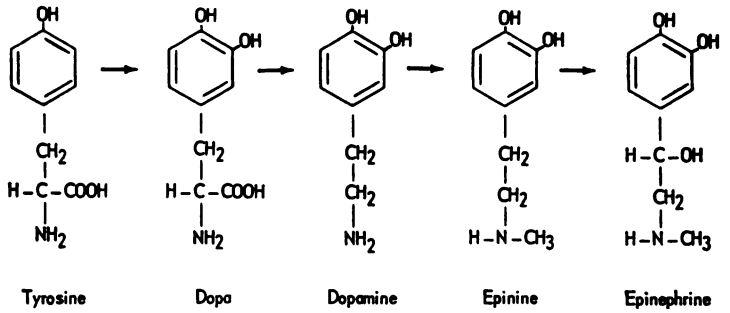
[2] Although this enzyme has not yet been named by the International Union of Biochemistry, "3,4-dihydroxyphenylethylamine-β-hydroxylase" (or its abbreviation, "Dopamine-β-hydroxylase") appears to be consistent with the trivial names assigned to other aerobic hydroxylases by the I.U.B. The enzyme also has been called "Dopamine-β-oxidase" by some workers, but this name can be misleading, since it is not descriptive of the type of reaction catalyzed by the enzyme.

[3] The abbreviations used in this review are: Dopamine, 3,4-dihydroxyphenylethylamine; DOPA, 3,4-dihydroxyphenylalanine.

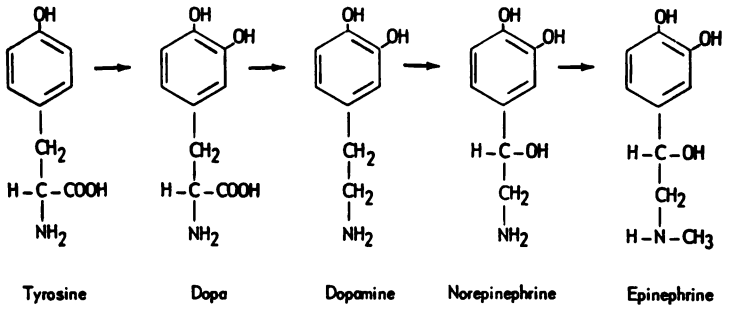
Because of the resemblances between epinephrine and phenylalanine or tyrosine, it is not surprising that, shortly after the elucidation of the structure of epinephrine, it was suggested that these aromatic amino acids were the biological precursors of this adrenal hormone.

For the direct conversion of tyrosine to epinephrine, 4 reactions are required: A) ring hydroxylation, B) side-chain hydroxylation, C) decarboxylation, and D) N-methylation. These reactions can obviously be arranged in different sequences to give 24 theoretically possible biosynthetic pathways; during the last 60 years, many, and perhaps all, of these possibilities have been proposed. Clearly, no useful purpose would be served by a consideration of all of these proposals. The first one, however, made by Halle in 1906 (61), was as follows:



It is noteworthy both for historical reasons and for its remarkable similarity to today's accepted scheme. It was derived on the basis of analogous reactions known at that time, and differs from today's version only by a reversal of the final 2 steps.

The pathway for the biosynthesis of both pressor amines first proposed by Blaschko in 1939 (10), is the following:



It is now widely accepted as the only quantitatively important one. If alternate routes exist (and some, including Halle's, are still consistent with the known specificity of the enzymes involved) they probably make only minor contributions to the metabolic traffic that leads to norepinephrine and epinephrine synthesis.

The experimental evidence that permitted a choice between the alternative biosynthetic pathways lagged far behind these perspicacious theoretical proposals. Indeed, it was not until 1947 that phenylalanine was proved to be the

biological precursor of epinephrine. Gurin and Delluva (59) administered ^{14}C- and ^{3}H-labeled phenylalanine to rats and isolated radioactive epinephrine from the adrenal glands of the animals. Udenfriend and Wyngaarden (163) demonstrated that ^{14}C-labeled tyrosine also could act as a precursor of epinephrine in the rat. Since it is known that phenylalanine can be converted to tyrosine by enzymes present in mammalian liver (86, 116) [more recently also found in mast cells (109)], it is likely that, as far as the adrenal is concerned, the only major precursor for norepinephrine synthesis is tyrosine.[4]

Ever since DOPA decarboxylase was discovered by Holtz *et al.* (74), it has been proposed as being involved in the biosynthesis of norepinephrine. Several reviews of the properties and specificity of this enzyme have been published (27, 75). Its role in catecholamine metabolism was supported by its discovery in the adrenal medulla (103). The first, indirect evidence that DOPA was a precursor of norepinephrine was obtained from dietary studies (1). Demis *et al.* (34) proved that it is converted to norepinephrine in homogenates of bovine adrenal glands. These workers were able to show that virtually all the ^{14}C-DOPA added was rapidly decarboxylated, and that 2 to 3% of the radioactivity appeared as norepinephrine. In 1956, Leeper and Udenfriend (105) demonstrated *in vivo* that when ^{14}C-Dopamine was administered to rats, ^{14}C-norepinephrine could be isolated from the adrenal gland. The norepinephrine isolated after Dopamine injection had 5 to 10 times the specific activity of that found after DOPA administration; this ratio indicated that Dopamine was a more immediate precursor of norepinephrine than DOPA.

Goodall and Kirshner (54) finally demonstrated, with isotope dilution techniques, that the pathway of norepinephrine proposed by Blaschko (10) was correct. They incubated ^{14}C-tyrosine and DOPA with bovine adrenal slices and showed that Dopamine became labeled rapidly and reached a maximum in 2 hours before declining. The total counts in norepinephrine increased over a 6-hour period before declining, whereas labeled epinephrine was formed very slowly. By adding unlabeled DOPA and Dopamine, Goodall and Kirshner were able to decrease the amount of radioactivity incorporated into norepinephrine.

Kirshner also showed that Dopamine-β-hydroxylase was contained in the particulate fraction of adrenal homogenates (95) and that it could be removed by centrifugation at 25,000 × *g*, whereas DOPA decarboxylase was in the soluble portion of the homogenate. Much evidence has now been accumulated that Dopamine-β-hydroxylase is intimately associated with the catecholamine-containing granules and that many of its properties *in vivo* are determined by the properties of these particles (23, 95, 97, 122).

The catecholamine-containing vesicles

The existence of granular elements in the medullary cells had been suggested in the last century (36, 78, 111). These granules were observed in the medulla

[4] The hydroxylation of phenylalanine to tyrosine reported to occur in adrenal tissue (47) is, as the authors concluded, probably a nonenzymatic reaction and, therefore, of doubtful physiological significance. A phenylalanine-hydroxylating system similar to the one found in liver tissue (86) has not as yet been detected in adrenal tissue.

after fixation with bichromate. They were thought to be different from mitochondria and to represent oxidized catecholamines (4). Not until they were isolated *in vitro* by Blaschko and Welch (14) from adrenal tissue was any evidence obtained for their occurrence *in vivo* as structural elements. Hillarp *et al.* (67, 68) demonstrated that the isolated granules were identical in staining properties to those present in fixed tissues and that they could be observed in vital tissue preparations by phase-contrast and dark-field microscopy. Lever (106) used electron microscopy to demonstrate that the cells in the rat adrenal medulla contained osmiophilic granules invested by a delicate membrane or sac. There was a depletion of this granular substance after stimulation of adrenal secretion (by exposure of the animals to 3 to 5°C) or by denervation of the medulla (4, 106). Two to three days after denervation the granules appear distorted and lose their saccular investment. Associated with this histologic change is a decrease in epinephrine output and manufacture (106). After stimulating the splanchnic nerves of the rabbit, De Robertis and Vaz Ferreira (35) were able to demonstrate that the granules were depleted, but that the membranes remained intact. The histologic evidence, therefore, indicates that these structures are vesicles. The granular appearance is a staining-fixation artifact representing the precipitation and condensation of oxidized catecholamines.

Many workers have been able to isolate these vesicles from bovine adrenal glands (13, 14, 60). They are readily separated from mitochondria by centrifugation in a sucrose gradient and contain only small amounts, if any, of succinic oxidase and fumarase, enzymes associated with the tricarboxylic acid cycle (13). Hillarp *et al.* (64, 65, 66, 69, 70) have analyzed these granules and obtained, as per cent of wet weight, 68.5% water, 6.7% catecholamines, 4.5% adenosine phosphates, 11.5% protein and 7% lipid. The adenosine phosphates are primarily present as adenosine triphosphate (ATP) in the beef adrenal gland, although in other species more may be present as adenosine di- and monophosphates (ADP, AMP) (20, 60). The amount of ATP present in the medullary granules of the cow is extremely high, 302 μg/g protein (12). For comparison, liver mitochondria contain 23 μg/g protein and rabbit platelets 46 μg/g protein. It has been estimated (21, 64, 66) that the number of negative charges present as adenosine nucleotides is equivalent to the number of positive charges present as catecholamines [0.55 M catecholamines, 0.13 M adenosine phosphate (66)]. Although the concentration of catecholamines is close to 0.6 M, Hagen and Barnett have calculated (60) that if they are present as an ATP-catecholamine complex, the total concentration in the granules would be approximately isotonic with body fluids. After depletion of catecholamines with reserpine, however, the amount of amines lost from the fowl adrenal gland is greater than the loss of nucleotides (20).

The only enzymes, in addition to Dopamine-β-hydroxylase, that have been found in the granules are an ATPase and an ADP transphosphorylase (65, 66). A hemochromogen (156) similar to that present in microsomes has also been found. About 70% of the protein present is soluble (65, 66) and approximately 70% of this soluble protein has an isoelectric point at about pH 4 (65). This protein has been purified by Smith *et al.* (152) and shown to consist primarily of

two components with molecular weights of 39,800 and 19,400. Since the tryptic digests of both proteins give similar fingerprints, they suggested that the heavier protein may be a dimer of the lighter one. These proteins can bind catecholamines in the presence of ATP and Mg^{++} and can inhibit Dopamine-β-hydroxylase (152). They are present in a concentration of approximately 10^{-3} M in the adrenal vesicles. The adrenal vesicles also have been shown to contain a small amount of Dopamine (37).

Similar vesicles have been isolated from other tissues with sympathetic innervation (42, 44, 45, 82, 137, 138, 144, 145) and from the brain (6, 138, 168). These particles were found to contain ATP (145). Unlike the adrenal vesicles, however, which contain all the catecholamines present in the adrenal gland, vesicles isolated from the splanchnic nerve contained only 30 to 40% of the norepinephrine and none of the Dopamine present (145). The vesicles from the cat brain contained most of the norepinephrine but only small amounts of Dopamine (162). Whether or not this is an artifact of isolation is not known. Recently, similar particles have been isolated from the heart, brain, salivary glands, vas deferens and pineal of the rat (138). Dopamine was not associated with the particles isolated from these tissues.

There is also histologic evidence that these particles are in neuronal axons in the pineal, vas deferens, carotid body and cardiac muscle of the rat (140). The size of these vesicles varies from tissue to tissue, but the histologic structure appears similar. Evidence that these vesicles contain catecholamines in the rat has been obtained *in vivo*; tritiated norepinephrine was injected and grains were detected in the region of the vesicles after histologic section and autoradiographic study of the pineal (171). The granular elements, but not the vesicles, decrease in quantity after administration of reserpine (33).

Synthesis of catecholamines in tissues other than the adrenal

In view of the presence of norepinephrine-containing vesicles in all tissues with sympathetic innervation so far studied, as well as the brain, it is not surprising that these organs also appear to have the capacity to synthesize norepinephrine from tyrosine. Goodall and Kirshner were able to repeat their work with adrenal medullary slices using canine and bovine sympathetic nervous tissue (55). Both ^{14}C-tyrosine and ^{14}C-DOPA were converted to norepinephrine. Cholinergic fibers from the vagus did not form significant amounts of norepinephrine from either precursor. Creveling and Udenfriend (162) demonstrated the conversion of ^{14}C-Dopamine to ^{14}C-norepinephrine in brain homogenates of the rat, dog, cow and sheep. The most active areas were comparable in activity to the adrenal medulla, although the adrenal medulla has over 1000 times the catecholamine content of those areas of the brain. When ^{14}C-labeled tyrosine was injected directly into one side of a cat brain *in vivo* (114), a significant amount of labeled DOPA and Dopamine, as well as smaller amounts of norepinephrine were formed; the conversion was greatest in the hypothalamus. The isolated perfused heart of the rabbit (121), dog (25, 26) or guinea pig (155) also can synthesize norepinephrine from tyrosine. Suggestive evidence has been obtained that Dopamine-β-hydrox-

ylase is associated with the vesicular elements that can be isolated from cardiac tissue (137).

Rate-limiting step of norepinephrine synthesis

A complete understanding of how a metabolic pathway is regulated in the cell requires a knowledge of the rate-limiting step in that pathway. Usually it is at this "pacemaker" step that controls are exerted. This is no less true for physiological regulating agents such as hormones than it is for drugs designed to control the formation or breakdown of a metabolite. In the logical search for drugs that might inhibit norepinephrine biosynthesis (and might therefore be of therapeutic value in the lowering of arterial blood pressure), knowledge of the identity of the rate-limiting step in the tyrosine-to-norepinephrine pathway is essential.

The identification of these "pacemaker" steps in any interlocking sequence of enzyme-catalyzed reactions has proved to be a difficult task. Even in such well-studied metabolic pathways as glycolysis and respiration (100), the rate-limiting step is still a subject for speculation. In most cases, including that of norepinephrine biosynthesis, more information is needed on rates of degradation of intermediates, precursor and intermediate pool sizes, and possible compartmentalization of enzymes and substrates. Furthermore, it should be emphasized that the rate-limiting step for the same process may vary from one tissue to another and, obviously, it need not be an enzyme-catalyzed transformation. Thus, glucose metabolism in muscle is limited by the rate of its entry into the cell, whereas in liver, it is some other step that is rate-limiting. Indeed, even for the same tissue, the rate-limiting step for a metabolic pathway might not be the same in all species. For these reasons, it is an unwarranted over-simplification to speak of *the* rate-limiting step in norepinephrine biosynthesis, as if all tissues constituted a homogeneous continuum; the topic can be discussed only with reference to a specific organ in a specific animal.

Within these limitations, some tentative conclusions can be reached about the rate-limiting step in norepinephrine biosynthesis. In 1956, before much was known about the individual enzymes involved, Blaschko (11) made the suggestion that the side-chain hydroxylation of Dopamine was the rate-limiting step in norepinephrine formation. This suggestion has not been supported by recent evidence. On the contrary, there are many indications that in the adrenal medulla and heart, the rate-limiting step may be the first one, the hydroxylation of tyrosine to form DOPA catalyzed by the enzyme, tyrosine hydroxylase. Some of the evidence in favor of this conclusion follows: a) In perfused calf adrenals (141) and in beef-adrenal slices (54), Dopamine is a much better precursor of norepinephrine than is tyrosine. b) In slices of adrenal medulla, the activity of tyrosine hydroxylase is 4 to 20 mμmol/g/hour, whereas the activities of Dopamine-β-hydroxylase and DOPA decarboxylase are greater than 10,000 mμmol/g/hour (125). (It is worth noting that estimates of the rate of catecholamine synthesis in the adrenal gland *in vivo* are close to this figure for the activity of tyrosine hydroxylase. See next section.) c) In the isolated heart of the guinea pig, the Km for the conversion of tyrosine to norepinephrine is the same as for the conversion of tyrosine to DOPA as catalyzed by tyrosine hydroxylase (125).

An additional argument that has been cited in favor of tyrosine hydroxylation being the rate-limiting step in norepinephrine synthesis is that the concentration of tyrosine in tissues is about 10^{-4} M, whereas DOPA and Dopamine are not normally detectable (125). This argument would certainly be suggestive, since in a linear sequence of reactions only the substrate for the rate-limiting step would be expected to accumulate to any extent; the data, however, serve not only to strengthen the case for tyrosine hydroxylation being the limiting step in the adrenal tissues of most animals, but also to weaken it for other tissues.

In most animals studied, it has been found that the amount of Dopamine is either very low or not detectable (148). In the adrenal gland of the sheep, however, the concentration of Dopamine is quite high (148). Furthermore, it is known that in the brain there are high levels of DOPA and Dopamine (for review see 22) and that the catecholamines of sympathetic nerve cells are almost exclusively Dopamine and norepinephrine (44, 144, 145). In addition, when ^{14}C-tyrosine is injected into the cat brain (114), most of the radioactivity is found in DOPA and Dopamine and only small amounts in norepinephrine.

The significance of these tissue and species variations in catecholamine concentrations cannot be fully evaluated at this time. The situation is complicated by the possibility of compartmentalization of the enzymes involved in pressor amine synthesis—thus, DOPA decarboxylase is soluble (74), whereas tyrosine hydroxylase (124) and Dopamine-β-hydroxylase (95, 108) appear to be particle-bound. There is also evidence that Dopamine may be present in separate cells such as the storage granules found in the lung (46), liver (5), and intestines (5, 7) of ruminants. Adding to the difficulty in interpretation of the data is the possibility that this compound may have other functions than just to serve as a precursor of norepinephrine (8). In spite of these complications, however, it is clear that the concentration of Dopamine in tissues shows wide fluctuations and the pattern is not always the one that would be expected if tyrosine-hydroxylation were the rate-limiting step in norepinephrine synthesis.

In summary, in the adrenal medulla, and to a lesser extent in the heart, there is evidence in support of the conclusion that the slowest enzymatic step in norepinephrine biosynthesis is the conversion of tyrosine to DOPA. In brain and nerve tissue (and also in sheep adrenal tissue) this conclusion is not consistent with all the facts and more information is needed.

It should be added that conceptually, the possibility that norepinephrine synthesis is limited by the hydroxylation of tyrosine is an attractive one—it would be in agreement with all the data available from bacterial systems in which it has been found that the first step in a biosynthetic pathway is the one for which microorganisms have evolved control mechanisms (83).

The rate of norepinephrine synthesis in vivo

As already mentioned, the observed rate of tyrosine hydroxylation in adrenal slices is of the same order of magnitude as the estimated rate of norepinephrine synthesis in the whole organ. This estimate is based on a determination of the half-life of norepinephrine in adrenal glands. Analogous data are available for several tissues in which norepinephrine synthesis is known to occur.

Udenfriend and Zaltzman-Nirenberg have determined that the half-life in the guinea pig brain is approximately 4 hours (164); this corresponds to the synthesis of 0.03 to 0.04 μg/g/hour as compared to a value of 0.05 to 0.20 μg/g/hour for the heart (half-life 12 to 13 hours) (110, 118, 154, 155), and 1.0 μg/g/hour for the rat and rabbit adrenal (161, 163) (half-life 300 hours). Total synthesis by the guinea pig brain would then be 2.4 μg per 24 hours and by the adrenal gland of the rat or the guinea pig, 1.8 μg/24 hours (164). These results indicate that adrenalectomy should not have too significant an effect on catecholamine synthesis in the whole animal (150).

In patients with pheochromocytomas, the half-life for tumor norepinephrine has been estimated to be of the order of 8 to 12 hours (149). It is noteworthy that in some of these tumors (115, 167) Dopamine may be the predominant amine formed. In this instance there may be a dissociation in the normal control mechanisms that determine enzyme levels and rates of synthesis.

Embryonic development of Dopamine-β-hydroxylase

The neural crest tissue that appears early in morphogenesis gives rise to the sympathetic ganglia and adrenal medullary tissue (76). This tissue which migrates from the neural tube early in embryonic life also gives rise to the melanophores, another tissue that has a specialized tyrosine metabolism (170). Other cells that arise from the neural crest, but migrate from the neural tube at later stages of development do not apparently develop into cells with a predominant tyrosine metabolism (169, 170). It would be of interest, therefore, to investigate the time and order of appearance of the various intermediates and enzymes necessary for norepinephrine biosynthesis and to study the factors that determine which course their tyrosine metabolism will take.

In the frog (24), both in whole embryos and in neural crest tissue, Dopamine appears at an earlier stage than norepinephrine. Epinephrine appears much later than either of its precursors and was not found in the neural crest tissue that has not migrated from the area of the neural tube. The sequence of appearance of intermediates on the norepinephrine biosynthetic pathway also has been studied in chick embryos with the use of ^{3}H-DOPA (19). In homogenates of chicks less than 30 hours old, only ^{3}H-Dopamine was formed. At 3 days, norepinephrine could be synthesized, and by 48 days, ^{3}H-epinephrine also could be recovered from the incubation medium.

Karki *et al.* (85) have shown that rats, 2 days before birth, have essentially no norepinephrine in their brain tissue. It did not reach the adult level until six weeks after birth. Similar findings were obtained with the rabbit. The guinea pig, however, is born with high catecholamine levels. Since rats and rabbits are essentially helpless at birth and guinea pigs are almost self-sufficient, these findings of biochemical immaturity agree with the behavioral immaturity of these animals. This also correlates with the electrical activity (EEG) of the brains of these animals (28, 84, 142), and with the development of other enzymes (56, 71, 85, 92, 123, 139). Myelin synthesis also occurs during these early stages of development in the rat (166).

These results imply that the enzymes involved in norepinephrine synthesis

appear during development in the same sequence as they operate in the norepinephrine biosynthetic pathway in adult tissue. They also suggest that behavioral maturity may be related to the development of pathways mediated by catecholamines. The pathway for serotonin synthesis appears to develop at the same time that the catecholamine pathway develops (85). These findings may have implications in phenylpyruvic oligophrenia in the light of recent experiments by Woolley and van der Hoeven (172) who demonstrated that a behavioral defect caused by feeding large amounts of phenylalanine to newborn mice could be reversed by feeding serotonin derivatives.

DOPAMINE-β-HYDROXYLASE

There are many aspects of norepinephrine biosynthesis that can be, and have been, profitably studied at different levels of cellular organization—from the whole animal to intact organs, and finally to slices, homogenates and extracts of these organs. Indeed, some aspects of the problem, such as the physiological regulation of norepinephrine formation, can be studied only in systems with some residue of structural integrity. When one comes to a study of the enzymatic mechanisms involved in norepinephrine biosynthesis, however, there are obvious advantages to the use of purified enzyme preparations.

Before discussing the details of the enzymatic conversion of Dopamine to norepinephrine, some general aspects of hydroxylation reactions must be considered. Since this subject has been reviewed recently (88), only a brief outline will be presented here.

If one defines hydroxylation as the conversion of a $-\overset{|}{\underset{|}{CH}}-$ group to a $-\overset{|}{\underset{|}{COH}}-$ group, there are two distinct types of biological hydroxylation reaction—the aerobic and the anaerobic. The aerobic hydroxylation reaction is characterized by a requirement for oxygen and an external electron donor. Enzymes which show these characteristics have been classified by Mason as "mixed function oxidases" (113). The requirement for oxygen is specific, no reaction occurring anaerobically in the presence of other oxidizing agents such as oxidation-reduction dyes. In those systems in which the reaction has been studied with ^{18}O, it has been shown that the hydroxyl-oxygen atom is derived from atmospheric oxygen.

These criteria serve to distinguish this type of hydroxylation from the more familiar, anaerobic type, which may be illustrated by the conversion of succinate to malate or by the conversion of butyrate to β-hydroxybutyrate. These "hydroxylations" are actually hydration reactions in which the hydroxyl-oxygen is derived from water as shown in equations (1) and (2). They require an electron acceptor, which may be oxygen but may also be a redox dye—*i.e.*, these hydroxylation reactions can take place anaerobically.

$$-CH_2-CH_2- + A \rightarrow -CH=CH- + AH_2 \quad (1)$$

$$-CH=CH- + H_2O \rightarrow -CHOH-CH_2- \quad (2)$$

It should be clear that in the presence of catalytic amounts of the hydrogen ac-

ceptor, A, this kind of hydroxylation reaction also may show a requirement for oxygen, which will serve to reoxidize the reduced form of the hydrogen acceptor. According to the terminology used in this review, therefore, the mere demonstration that a hydroxylation reaction is dependent on oxygen is not sufficient evidence for classifying it as an aerobic hydroxylation reaction.

It is apparent that the two types of hydroxylation reaction can be distinguished by their requirements—an electron acceptor for the hydration type and an electron donor and oxygen for the aerobic type.

This requirement for oxygen and an electron donor for mixed-function oxidases may appear to be puzzling at first glance, since one does not usually think of an oxidative reaction as requiring a reducing agent. As has already been pointed out (88), however, every oxidative reaction involves a reducing agent, and this is ordinarily the component being oxidized; the only unique feature about aerobic hydroxylation reactions is that they require an *external* reducing agent. The reason for this requirement is apparent if one considers that in this type of hydroxylation reaction both atoms of an oxygen molecule are reduced to the level of hydroxyl groups and the reduction therefore requires the input of 4 electrons, one electron pair from the substrate to be hydroxylated and the other pair from an external reducing agent as shown in equation 3.

$$4\epsilon + :\ddot{O}:\ddot{O}: \rightarrow 2[:\ddot{O}:]^- \qquad (3)$$

The general equation (107, 108) that describes aerobic hydroxylation reactions is (where RH stands for the substrate and ROH for the hydroxylated product):

$$RH + O_2 + 2H^+ + 2\epsilon \rightarrow ROH + H_2O \qquad (4)$$

Because, as already mentioned, general aspects of reaction mechanism often can be delineated by the requirements shown by the enzyme system, much of the early work on Dopamine-β-hydroxylase was concerned with attempts to define and redefine the cofactor requirements of the reaction with greater and greater precision.

Properties of crude preparations of Dopamine-β-hydroxylase

In experiments performed with adrenal homogenates or acetone powders of beef adrenals (95, 96, 128), it was found that oxygen, ATP and Mg^{++} stimulated the conversion of Dopamine to norepinephrine. More recently it has been demonstrated (23, 97) that the Mg^{++} is required for the uptake of catecholamines by the adrenal vesicles. EDTA inhibits the uptake of the precursor, hydroxytyramine, but does not inhibit the enzymatic conversion to norepinephrine if the vesicles are intact. If the vesicles are disrupted by lysis, however, EDTA does inhibit the conversion of Dopamine to norepinephrine. The presumption is that the enzyme, Dopamine-β-hydroxylase, is bound to the inner surface of the vesicular membrane or some site inaccessible to EDTA and not capable of reacting with the substrate until the substrate enters the vesicle. Reserpine (97) also will inhibit the conversion of Dopamine to norepinephrine *in vitro* by in-

hibiting uptake of the precursor by the vesicle. Since the precursors of Dopamine, tyrosine and DOPA, do not appear to be able to enter the granule (97), it is not surprising that the enzymes that act upon them, tyrosine hydroxylase (16, 125) and DOPA decarboxylase (75), are not as intimately associated with the vesicles as is Dopamine-β-hydroxylase. Any amine that is taken up by the granule and is a substrate for Dopamine-β-hydroxylase will then be hydroxylated (26, 49, 97, 122). The activity of the enzyme does not appear to be necessary for uptake of the amines (23, 122), but is necessary for storage of such amines as tyramine. Dopamine, however, can be stored without β-hydroxylation (23). Those granules that store large amounts of Dopamine (5, 7, 46) probably do not contain Dopamine-β-hydroxylase; the presence of these granules in ruminant tissues may account for the large amount of Dopamine found in bovine splenic nerves (144).

Assay of Dopamine-β-hydroxylase

Many methods for the determination of norepinephrine have been developed but only a few of them can be used for the assay of crude enzyme preparations. Paper chromatography cannot be used since the amine, 2,4,5-trihydroxyphenylethylamine, is not only isomeric but also isographic with norepinephrine (146, 147). The compound, 6-hydroxy-Dopamine, can be formed from Dopamine, both by auto-oxidation *in vitro* by tissue homogenates (146, 147), even after they are boiled, or by the ascorbic acid-EDTA system (160). It can also be formed *in vivo* from Dopamine. It is likely that any system containing either flavin nucleotides, which can undergo cyclic oxidation and reduction to form H_2O_2, or ascorbic acid and Fe^{++}, might produce this compound from Dopamine. The presence of such systems could explain the finding of a soluble, iron-dependent, quinacrine-inhibited norepinephrine-synthesizing system in adrenal homogenates (79). An assay dependent upon the separation of norepinephrine from Dopamine on ion exchange resins was developed by Goodall and Kirshner (54). With the use of this assay they were able to demonstrate an oxygen requirement for the crude adrenal medullary enzyme. A more rapid assay procedure was developed by Levin *et al.* (108). These investigators took advantage of the unusual lability of the side-chain of norepinephrine to oxidation with periodate. Because of the presence of adjacent amino and hydroxyl groups, the carbon side-chain of norepinephrine is more susceptible to oxidation than that of Dopamine. Dopamine labeled with ^{14}C in the terminal position of the side-chain was used, and the norepinephrine produced was treated with periodate. The radioactive formaldehyde liberated during the periodate treatment was trapped as the dimedon complex and counted. The purified enzyme can be assayed by a fluorometric determination of the norepinephrine formed (108). Of several fluorometric methods described for this determination, the trihydroxyindole method has proved most convenient (43).

A variation of the periodate assay has been developed (134) that is dependent upon the ultraviolet absorption of the *p*-hydroxybenzaldehyde formed after periodate oxidation of octopamine. (Octopamine is the hydroxylated product formed when tyramine is used as substrate for Dopamine-β-hydroxylase.)

STUDIES WITH PURIFIED DOPAMINE-β-HYDROXYLASE FROM BOVINE ADRENAL MEDULLA

Purification of the enzyme present in bovine adrenals was complicated by its tenacious attachment to the vesicle membrane. It was solubilized from the adrenal medullary particles with the neutral detergent isooctylphenoxypolyethoxyethanol (cutscum) and purified by standard techniques (108).

Dependencies and stoichiometry: requirement for a reducing agent

From what has been mentioned previously about hydroxylation reactions, it could be anticipated that the conversion of Dopamine to norepinephrine would require either an electron acceptor or an electron donor. The first indication that Dopamine-β-hydroxylase is a mixed-function oxidase was the demonstration of a requirement for ascorbate (108). It was found that this reducing agent could stimulate the hydroxylation reaction even in adrenal particles, and the requirement for ascorbate became more pronounced as the enzyme was purified. With the purified enzyme, the Km for ascorbate was found to be about 6×10^{-4} M.

With the identification of ascorbate as an essential component of the hydroxylating system, it was possible to determine the stoichiometry of the reaction. It was found (108) that equimolar amounts of oxygen, Dopamine and ascorbate were consumed and an equivalent amount of norepinephrine was produced. The norepinephrine synthesized was later shown to be all of the hormonally active, L- configuration (107). From these results, the hydroxylation reaction was formulated as shown in equation 5.

$$\text{Dopamine} + \text{ascorbate} + O_2 \rightarrow \text{L-norepinephrine} + \text{dehydroascorbate} + H_2O \quad (5)$$

This formulation is in agreement with the general equation for aerobic hydroxylation reactions already discussed (see equation 4). It is of interest that the reaction catalyzed by Dopamine-β-hydroxylase is the first one in which it has been demonstrated that ascorbate is stoichiometrically consumed.

The specificity of the ascorbate requirement is shown in Table 1. In these early studies (108), only ascorbate, and its analogues, D-ascorbate, isoascorbate, and glucoascorbate showed high activity; reduced pteridines, such as tetrahydrofolate and 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, previously shown to have high cofactor activity for the phenylalanine hydroxylating system (87, 91), showed slight activity. More recent studies with essentially pure Dopamine-β-hydroxylase have demonstrated that the reduced form of the dye, 2,6-dichlorophenolindophenol, is highly active as a substitute for ascorbate (51).

It may be noted in Table 1 that, in the absence of any added electron donor, some hydroxylation of Dopamine still occurs. Since the enzyme is presumably still functioning as a mixed-function oxidase under these conditions, it was assumed that some other reducing agent was acting as a substitute for the ascorbate. It was shown that in the absence of ascorbate, the catechol grouping of the substrate, Dopamine, can function as a reducing agent. In this case, one

TABLE 1

Specificity of ascorbate requirement for Dopamine-β-hydroxylase activity

Added Compound	μmol	Norepinephrine Formed (μmol/ml)
Ascorbate	6	0.37
Isoascorbate	6	0.38
Glucoascorbate	6	0.31
D-Ascorbate	6	0.35
Alloxan	6	0.08
Dihydroxymaleate	5.5	0.04
Fe^{++}	0.75–6.0	0.02–0.03
Ascorbate + Fe^{++}	6 + 0.75	0.01
Glutathione	6	0.00
DPNH	2	0.04
TPNH	2	0.06
Tetrahydrofolic acid	1	0.06
2 Amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine	1	0.07
None		0.04

Data taken from reference 108.

molecule of Dopamine is oxidized to an orthoquinone during the hydroxylation of the side-chain of another molecule of Dopamine to norepinephrine (107).

$$2 \text{ Dopamine} + O_2 \rightarrow \text{Dopamine-quinone} + H_2O + \text{norepinephrine} \quad (6)$$

When Dopamine fills this dual role of substrate and electron donor, the hydroxylation reaction can be coupled to the oxidation of DPNH (107). This is possible because DPNH can react non-enzymatically (173) with the Dopamine-quinone formed in the reaction—according to equation 7.

$$\text{Dopamine-quinone} + \text{DPNH} + H^+ \rightarrow \text{Dopamine} + DPN^+ \quad (7)$$

The hydroxylation reaction under these conditions is described by the sum of equations 6 and 7, which gives the net reaction shown in equation 8.

$$\text{Dopamine} + O_2 + \text{DPNH} + H^+ \rightarrow \text{norepinephrine} + DPN^+ + H_2O \quad (8)$$

It should be emphasized that reaction 8, which incidentally provides a quick and convenient assay (107) for purified Dopamine-β-hydroxylase, is not an indication that pyridine nucleotides function as cofactors in the reaction, but only that DPNH can reduce substrate molecules that have participated as electron donors in the hydroxylation of other substrate molecules.

These ideas were further verified and extended when it was found that, in contrast to Dopamine, substrates for the enzyme without the catechol group, such as phenylethylamine, show an absolute requirement for an added electron donor. With these substrates, pyrocatechol can function as a reducing agent but on a molar basis it is only one-tenth as potent as ascorbate (107).

Whether or not ascorbate functions as a cofactor for this enzyme *in vivo* is still uncertain. Several attempts have been made to make the hydroxylation reaction dependent on ascorbate by use of whole organ or adrenal particles from ascorbate-deficient guinea pigs (110). No ascorbate dependence could be demonstrated; however, the significance of these negative experiments is difficult to assess since there was enough ascorbate left in the preparations to account for the synthesis of all the norepinephrine formed. Furthermore, Dopamine was used as the substrate and, as already mentioned, it can support its own slow hydroxylation.

Recently, it has been shown (77) that an enzyme is present in animal tissues that can catalyze the conversion of dehydroascorbate to ascorbate. This enzyme would amplify the ability of small amounts of ascorbate to participate in the hydroxylation reaction, and could make the demonstration of an ascorbate requirement for Dopamine-β-hydroxylation *in vivo* more difficult.

It is significant that the two hydroxylation steps in the pathway for biosynthesis of norepinephrine utilize different electron-donating cofactors; the first step in the pathway, the ring-hydroxylation of tyrosine, involves a tetrahydropteridine (16, 125), whereas the side-chain hydroxylation of Dopamine involves, as just discussed, ascorbate (108). Moreover, there is essentially no overlap in specificity; ascorbate is inactive in the tyrosine hydroxylating system (16) and tetrahydropteridines have only slight activity in the Dopamine-hydroxylating system (108).

The requirement for an electron-donor and oxygen, as well as the stoichiometry of the hydroxylation reaction (equation 5), provided strong presumptive evidence that Dopamine-β-hydroxylase is a mixed-function oxidase. This point was established unequivocally when the hydroxylation reaction was studied in the presence of either ^{18}O-enriched water or ^{18}O-enriched oxygen. It was found that during the Dopamine-β-hydroxylase-catalyzed conversion of phenylethylamine to phenylethanolamine, the hydroxyl-oxygen atom in the synthesized phenylethanolamine was derived from molecular oxygen and not from the labeled water in the medium (89).

Activators

In early experiments with Dopamine-β-hydroxylase, α-ketoglutarate was employed in all reaction mixtures as a source of potential energy (108). It was later observed, with more highly purified preparations of the enzyme, that activity was diminished if α-ketoglutarate was omitted from the reaction mixture. Of the various dicarboxylic acids and related compounds tested, fumarate was found to be most active (Table 2). Fumarate also stimulates certain adrenal steroid hydroxylating systems (63, 158), an effect that has been partially explained by its function as a substrate for the generation of reduced pyridine nucleotides. In the Dopamine hydroxylating system, however, the stimulation by fumarate is not due to its serving as an electron donor since, as has already been discussed, this role is filled by ascorbate. Indeed, in the absence of ascorbate, Dopamine-hydroxylation was only slightly stimulated by fumarate (107).

TABLE 2

Specificity of fumarate requirement for Dopamine-β-hydroxylase activity

Addition	μmol	Relative Activity
Fumarate	10	100
Fumaric epoxide	10	66
α-Ketoglutarate	10	66
Malate	10	47
Maleate	10	39
Succinate	10	28
Dihydroxymaleate	4	27
Glutamate	10	20
Malonate	10	18
γ-Aminobutyrate	10	16
None		16
Aspartate	10	14
Ashed fumarate	(10)	9
Pyruvate	10	7
Glutamine	10	6

Data taken from reference 108.

Furthermore, TPNH and DPNH are completely inactive as substitutes for fumarate.

In view of this activating effect of fumarate in the Dopamine-β-hydroxylase system, a reinvestigation of the fumarate stimulation of steroid hydroxylation might be warranted. It is possible that certain dicarboxylic acids have a more general role as activators of hydroxylation reactions than hitherto has been suspected.

Little is known about the mechanism of action of fumarate in the Dopamine-hydroxylating system. Experiments with ^{14}C-labeled fumarate have failed to detect either its consumption or its stoichiometric conversion to any other compound during the hydroxylation reaction (90).

The fumarate stimulation of the hydroxylation reaction has been observed not only with the presumed physiological substrate, Dopamine, but also with phenylethylamine (107) and epinine (17). Perhaps of significance is the report that with tyramine as a substrate for Dopamine-β-hydroxylase, fumarate stimulates the hydroxylation reaction less than 2-fold (57, 143).

Although there is no direct evidence to support the idea, an attractive possibility is that fumarate stimulates the hydroxylation reaction by inducing an allosteric change (117) in the enzyme molecule; *i.e.*, fumarate could change the conformation of the enzyme from one with low, to one with high, catalytic activity. There is no clue to suggest whether fumarate or a related dicarboxylic acid regulates the activity of the enzyme *in vivo*.

ATP and catalase

The stimulation by ATP of the conversion of Dopamine to norepinephrine was first reported by Neri *et al.* (128) and later confirmed by Kirshner (96). In

contrast to the 5- to 10-fold stimulation of the reaction observed with crude adrenal extracts, the partially purified Dopamine-β-hydroxylase showed only a 1.5-fold stimulation by added ATP (108). Since this slight effect of ATP disappeared when initial rates of the hydroxylation reaction were measured (107), ATP was probably not intimately involved in the hydroxylation reaction. This suggestion was confirmed when it was found that in the presence of catalase ATP had no effect (107). This effect of catalase was shown to be due to a protection of the Dopamine-β-hydroxylase from inactivation. The inactivation occurs when the enzyme is incubated with either ascorbate or Dopamine. Since both of these substances are readily auto-oxidized to produce H_2O_2, it seems likely that H_2O_2 is the inactivating agent. Levin and Kaufman (107) showed that small amounts of H_2O_2 will inactivate the enzyme and that catalase will protect the enzyme from this inactivation.

A discussion of the possible mechanism of the protective effects of ATP and catalase will be postponed for a later section.

Substrate specificity

The first demonstration that adrenal Dopamine-β-hydroxylase was not specific for Dopamine was made by Levin and Kaufman (107), who showed that the enzyme catalyzed the conversion of β-phenylethylamine to β-phenylethanolamine. These workers presented convincing evidence that the same protein was responsible for the hydroxylation of these 2 substrates (107). Somewhat later, Goldstein and Contrera (52a) independently reported that the β-phenylethylamine was active as a substrate for the hydroxylase and also showed that 3-methoxy-Dopamine and *p*-hydroxy-α-methyl-phenyl-4-ethylamine (*p*-hydroxyamphetamine) could be hydroxylated in the presence of the enzyme.

Earlier studies with adrenal slices had demonstrated that tyramine could be hydroxylated to norsynephrine (134). These studies with slices did not prove that Dopamine-β-hydroxylase was the catalyst involved in the hydroxylation of tyramine. Support for this idea was provided in later experiments carried out with the partially purified enzyme (29, 52a), where it was found that tyramine not only is a substrate, but may actually be a better one than Dopamine. It should be noted, however, that the pH optimum for tyramine is 5.5, whereas that for Dopamine is 6.2 (29).

There have been two extensive studies (29, 52a) of the substrate specificity of Dopamine-β-hydroxylase purified from adrenals according to the method of Levin *et al.* (108). These have provided enough information to permit some generalizations about the structural basis for substrate activity (Table 3).

The basic requirements for activity as a substrate for Dopamine-β-hydroxylase are the benzene ring with a side-chain containing either 2 or 3 carbon atoms and a terminal amine. Hydroxyl substitutions on the ring in the *meta*- or *para*-position, or both, increase activity. Methylation of the *meta*-hydroxyl group decreases activity only slightly, whereas methylation of the *para*-hydroxyl decreases activity markedly. Methylation of the amine causes a 75% decrease in activity, whereas methylation of the *alpha* carbon has only a small effect. An

TABLE 3

Relative activity of substrates of Dopamine-β-hydroxylase (activity with p-tyramine was arbitrarily taken as 100%)

Substrate	Relative Activity
p-Tyramine	100
m-Tyramine	60–80
o-Tyramine	0
Dopamine	85–90
Epinine	25
N-Methyltyramine	25
N,N-Dimethyltyramine	0
α-Methyltyramine	60–70
α-Methyl-*m*-tyramine	50–70
α-Methyl-Dopamine	50–60
β-Methyltyramine	5–10
3-Methoxytyramine	50–70
3-Methoxy-α-methyltyramine	5–10
4-Methoxy-*m*-tyramine	+
3-Methoxy-6-hydroxytyramine	0
3,5-Dimethoxytyramine	25–30
3,4,5-Trimethoxy-β-phenethylamine (mescaline)	2–5
p-Amino-β-phenethylamine	0
β-Phenethylamine	10–15
p-Methoxyphenethylamine	2–5
3,4-Dimethoxyphenethylamine	2–5
γ-Phenylpropylamine	+
p-Hydroxy-γ-phenylpropylamine	++
p-Hydroxy-β-phenethanol	0
p-Hydroxy-β-phenyl acetic acid	0
Ethylbenzene	0
p-Hydroxyphenylethane	0
p-Tyrosine	0
p-Tyrosine ethyl ester	0
N-Acetyltyramine	0
Tryptamine	0
5-Hydroxytryptamine (serotonin)	0

Data taken from reference 29.

ortho-hydroxyl or the formation of a tertiary amine eliminates activity, as does a carboxyl group on the α-carbon (29, 52a).

The broad specificity of the enzyme has some implications with regard to possible alternate routes of pressor amine biosynthesis. The activity of tyramine (29, 52a) as a substrate would be consistent with the pathway: tyrosine→tyramine→norsynephrine→norepinephrine. Another pathway involving tyramine as an intermediate is suggested by the recent finding of a nonspecific aromatic hydroxylase in liver microsomes that will form Dopamine from tyramine *in vitro* (3). That either of these pathways is of significance under normal circumstances is doubtful, since tyramine has been shown to be converted primarily to octopamine in the isolated mammalian heart (26, 121) as well as in the salivary

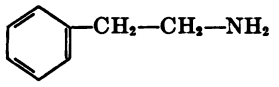
gland of the rat after administration of tritiated tyramine *in vivo* (49). In these experiments only small amounts of radioactive norepinephrine were found. Octopamine has been found in normal mammalian urine (133) and tyramine in both urine and mammalian tissues (153).

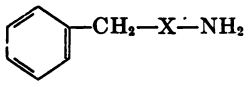
The activity of epinine (N-methyl-Dopamine) as a substrate for Dopamine-β-hydroxylase (17, 29, 52a) would be consistent with another alternate biosynthetic pathway for epinephrine: tyrosine→DOPA→Dopamine→epinine→epinephrine. The conversion of Dopamine to epinine has been reported to be catalyzed by a nonspecific N-methyl transferase (2). It has been claimed also that epinine can be converted to epinephrine *in vivo* (52a). It is possible that, as previously suggested (17), this pathway may be of significance in some tissues in which there is a relatively large amount of epinephrine as compared with norepinephrine (39, 73).

Finally, it should be mentioned that many sympathomimetic drugs such as amphetamine (29, 52a), are good substrates for Dopamine-β-hydroxylase and, in many cases, their hydroxylated products are also well-known drugs with high activity. It may be, as has been suggested (29), that at least some of the activity of the parent drug is due to the hydroxylated product.

Analogues as inhibitors

Since many of the analogues are good substrates it is not surprising that they are competitive inhibitors of the conversion of Dopamine to norepinephrine (29, 52a). Their order of activity as substrates is similar to their activity as competitive inhibitors (29). In an attempt to find better inhibitors of the enzyme, isosteres were synthesized that were as close as possible to the parent molecules, except that a nitrogen or oxygen atom was substituted for the α-carbon atom (30, 72, 102, 129, 143).





These compounds are potent inhibitors of the enzyme. Substitutions on the ring, the terminal nitrogen, the hydrazine nitrogen, or the β-carbon affected inhibitory activity in the same way that they affected the activity of the phenylethylamine compounds as substrates (30). The type of inhibition caused by these isosteres varied with the conditions under which they were tested. If the inhibitor was preincubated with the enzyme, the inhibition was apparently noncompetitive with respect to substrate. If the substrate and inhibitor were added to the enzyme at the same time, the inhibition was competitive (or partially competitive) (30, 143). The enzyme could be reactivated after preincubation with the benzyloxyamine inhibitor by prolonged dialysis (143). This indicated the inhibitor was tightly bound to the enzyme by a non-covalent link. The enzyme was inhibited 98 % when it was preincubated with 10^{-5} M *p*-hydroxybenzyloxyamine. Without preincubation the enzyme was inhibited only 40 %.

The Km for the hydroxylation of Dopamine at pH 5.5 is 6×10^{-3} M and that

for tyramine calculated from the data of Creveling *et al.* (30) is 8×10^{-4} M. The Ki (assuming that the inhibition is competitive and that fumarate was present) for benzyloxyamine is about 6.6×10^{-6} M (30). Although this suggests that the benzyloxyamine analogues might be inhibitors of Dopamine-β-hydroxylase when administered *in vivo*, this has not proven to be true. They are extremely toxic (102, 129) in the doses that must be administered. One method of demonstrating inhibition *in vivo* has been first to deplete the catecholamines present [with the use of an agent such as metaraminol (32, 129)], and then to administer the inhibitor and measure the resynthesis of norepinephrine. Another method has been to observe the rise in brain catecholamines after the administration of an inhibitor of monoamine oxidase (102). To date, no competitive inhibitor of Dopamine-β-hydroxylase has been uncovered that lowers the catecholamine content of tissues solely on the basis of its inhibition of this enzyme. Those that have been effective also have other properties, such as (129): 1) depleting amines already present; 2) replacing other amines in the storage granule; and 3) competing with other amines for either transport mechanisms or receptor sites. Thus, the decrease in norepinephrine in the brain observed after administration of *m*-hydroxy-N-methylbenzylhydrazine (102) was probably due to release of norepinephrine rather than to inhibition of Dopamine-β-hydroxylase.

The failure to observe inhibition of the synthesis of norepinephrine in heart tissue by these potent inhibitors of the enzyme would support the idea that in this organ, the rate-limiting step in norepinephrine synthesis is not normally the β-hydroxylation of Dopamine.

Metal content

It had been known for some time that Dopamine-β-hydroxylase could be inhibited by metal-chelating agents (53, 57, 97, 108). Only recently, however, was the enzyme obtained in a pure state and the metal characterized by direct analysis (50). The metal is copper and it is present in a concentration of 0.65 to 1.0 μg per mg enzyme (4 to 7 moles of copper per mole of protein). It can be removed by treatment with concentrated potassium cyanide. This copper-free enzyme is inactive but can be reactivated by addition of Cu^{++}. The amount of reactivation achieved is only 40% of the activity of the starting material. That the enzyme cannot be fully reactivated is probably due to the drastic treatment used to remove all the copper. None of the other metals tried, including Fe^{++}, Mo^{+6} (as the molybdate anion) and Co^{++}, could replace copper in reactivating the enzyme at concentrations for which copper gave maximal stimulation. The copper is present in both the cuprous and cupric form (50). Although the Cu^{+} content varies, the Cu^{++} content is constant in different preparations of the enzyme and is equal to 2 moles per mole of enzyme. Many copper proteins contain copper in both the cuprous and cupric state (15, 18, 135, 136). Although most proteins that contain cupric copper have some absorption in the red region of the spectrum, the highly purified Dopamine-β-hydroxylase is colorless (50).

The enzymic activity is inhibited by carbon monoxide (CO) (51). There are only three other enzymes that are known to be inhibited by CO, and two of

these, phenolase (93, 101) and cytochrome oxidase (58, 132), both contain copper. The third is a complex adrenal microsomal system that hydroxylates progesterone (40). Cytochrome oxidase, however, contains a heme pigment that can combine with CO (132) and the same appears to be true of the microsomal system (40). There is no evidence for a heme pigment in the purified Dopamine-β-hydroxylase and only trace amounts of iron are present in the best preparations (50). Phenolase and Dopamine-β-hydroxylase, therefore, appear to be the only colorless, copper-containing, CO-inhibited enzymes found to date. In contrast to Dopamine-β-hydroxylase, all the copper in phenolase appears to be in the cuprous state (94).

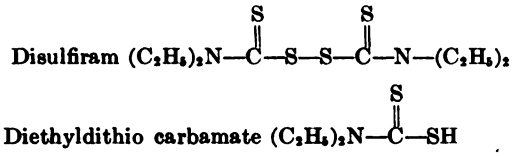
The demonstration that Dopamine-β-hydroxylase is a copper-protein provides a possible explanation for the previously mentioned protection of the enzyme by catalase. The situation is reminiscent of another copper enzyme, ascorbate oxidase, which also is inactivated by small amounts of H_2O_2 and is protected by catalase (99). The production of H_2O_2 in this case is due to a reaction catalyzed by the small amount of Cu^+ present in the enzyme (135). Most of the copper in ascorbate oxidase is present as Cu^{++} and catalyzes the reaction:

$$\text{Ascorbic acid} + \tfrac{1}{2}O_2 \rightarrow \text{dehydroascorbic acid} + H_2O \qquad (9)$$

H_2O_2 causes significant inactivation of ascorbate oxidase only when the enzyme is functioning, *i.e.*, catalyzing the oxidation of ascorbate (135). It has been proposed that the H_2O_2 reacts with the active site of ascorbate oxidase only when the active copper is in the cuprous form (135). Whether or not this is true for Dopamine-β-hydroxylase will require further investigation.

Inhibition by chelating agents

The first demonstration that Dopamine-β-hydroxylase was sensitive to inhibition by certain chelating agents was described in the report that cyanide is a potent inhibitor (108). Since then it has been shown that the enzyme can be completely inhibited by the following chelating agents: diethyldithio carbamate (2×10^{-6} M), and 2,9-dimethyl *o*-phenanthroline (5×10^{-6} M) (57). The enzyme is inhibited also by 8-hydroxyquinoline, 2,2′-bipyridine, EDTA, bathocuproine sulfonate, cuprizone, and disulfiram (50, 53, 57, 97), as well as by excess copper (57).



Recently it was demonstrated that disulfiram (Antabuse) will lower norepinephrine levels *in vivo* and inhibit the conversion of ^{14}C-Dopamine to norepinephrine. It was suggested that this agent, which can be reduced to the potent copper-complexing agent, diethyldithio-carbamate, inhibits Dopamine-β-hydroxylase *in vivo* (52, 53). The compound does not inhibit uptake of ^{3}H-norepinephrine (52,

122) and there is actually an increase in the Dopamine levels in the heart and spleen of animals treated with disulfiram (52, 122). There was a 50 % decrease in the endogenous norepinephrine content in the hearts of rats that had been given 400 mg disulfiram per kg 18 hours previously. After administration of ^{3}H-tyramine to rats treated with disulfiram, formation of octopamine was markedly inhibited. In these animals the ^{3}H-octopamine was found in the particulate fraction, the same fraction that takes up the ^{3}H-norepinephrine.

When diethyldithio-carbamate is added to a highly purified sample of the enzyme, a yellow complex is formed. The diethyldithio carbamate binds very tightly to the enzyme, and even after prolonged dialysis the protein remains yellow (50). This tight binding to the enzyme probably accounts for its high potency in inhibiting Dopamine-β-hydroxylase *in vivo*.

Disulfiram is the first inhibitor of the activity of the enzyme effective *in vivo*. It is already proving to be a useful tool in the study of the storage, release and biosynthesis of norepinephrine.

Properties of the purified protein

The enzyme has been obtained in a highly purified state and catalyzes the hydroxylation of about 1000 moles of Dopamine per mole of enzyme per minute at 25°C (50). The enzyme has a $s_{20,w}$ of 8.9 in 0.1 M NaCl + 0.005 M potassium phosphate, pH 6.8. The molecular weight is 290,900 ($\bar{v}$ assumed to be 0.720). It was homogeneous on disc and starch gel electrophoresis at pH 8.8 and 6.8. The $E_{280}^{1\%}$ is 9. The enzyme has a 280/260 ratio of 1.6 and no absorption in the visible region (50).

In early work with the soluble enzyme (108) it was noted that the enzymatic activity increased many-fold during purification. This indicates either that an inhibitor is present in the intact medulla and that this inhibitor is gradually removed during purification, or that during purification the enzyme molecule changes to an altered form with higher catalytic activity. Smith *et al.* (152) have noted that the small proteins they isolate from adrenal vesicles can inhibit Dopamine-β-hydroxylase. Whether this is the major cause of enzyme inhibition in crude preparations or whether other substances are responsible for the inhibition has not been determined.

MECHANISM OF THE REACTION CATALYZED BY DOPAMINE-β-HYDROXYLASE

The earliest suggestion (11) about the mechanism of the side-chain hydroxylation of Dopamine postulated a dehydrogenation-hydration sequence of reactions similar to those involved in the conversion of succinate to malate (see equations 1 and 2). More recently, there was proposed an elaboration of the hydration theory involving the essential participation of the catechol grouping of the Dopamine molecule in the hydroxylation reaction (146). Any hydration theory for the hydroxylation reaction was made unlikely by the finding that an electron-donor, such as ascorbate (108), is an essential component of the Dopamine-β-hydroxylating system. As already mentioned, the hydration mechanism requires, not an electron donor, but an electron acceptor. The demonstration

(151) that the α hydrogen atoms of Dopamine were not rendered labile during its conversion to norepinephrine provided additional evidence against any theory of hydroxylation that involves *alpha-beta*-dehydrogenation as an obligatory step. The specific idea that the catechol grouping of Dopamine is intimately involved in the hydroxylation reaction (146) was made untenable by the finding that phenylethylamine was a good substrate for the enzyme (107). Finally, all variations of the dehydrogenation-hydration theory were unequivocally ruled out when Kaufman *et al.* proved that the hydroxyl-oxygen atom is derived from molecular oxygen (89). These ^{18}O experiments firmly established Dopamine-β-hydroxylase as a mixed-function oxidase or an aerobic hydroxylase.[5]

There are many possible mechanisms that can be proposed for an aerobic hydroxylation reaction and these have been discussed extensively by Mason (113) and Kaufman (88).

An important clue to the mechanism of action of Dopamine-β-hydroxylase was provided by the observation (90) that the purified enzyme, in the absence of substrate, can catalyze the oxidation of catechol, a weak substitute for ascorbate (107). It was shown that this catechol-oxidase activity, which for convenience will be called the "substrate-less" reaction, and the hydroxylating activity, were associated with the same protein molecules (90). Additional evidence that the substrate-less reaction is related to the hydroxylation reaction was provided by the observation that it too is stimulated by fumarate (90). More recently it has been found that the essentially pure enzyme will also catalyze the oxidation of reduced 2,6-dichlorophenolindophenol (51). In contrast, ascorbate, the presumed physiological electron-donating cofactor for the hydroxylation reaction, is not oxidized by catalytic amounts of the enzyme unless substrate is present.[6]

The demonstration that Dopamine-β-hydroxylase could catalyze the oxidation of certain electron donors, in the absence of substrate, is of importance because the substrate-less reaction probably reflects the first reaction in the sequence that ultimately leads to side-chain hydroxylation. It suggests that this first reaction involves an interaction between enzyme, electron donor and oxygen.

When large amounts of enzyme became available, it was possible to confirm and extend these observations. It has been demonstrated recently (50) that a stoichiometric amount of enzyme will oxidize an equivalent amount of ascorbate. This oxidation occurs both aerobically and anaerobically and dehydro-

[5] Another enzyme system that can catalyze the conversion of Dopamine to norepinephrine has been partially purified from bananas (150a). This enzyme differs in many respects from the adrenal Dopamine-β-hydroxylase, *e.g.*, the product of the reaction is racemic norepinephrine; in addition, ascorbate, rather than serving as a cofactor, is a potent inhibitor. Although the enzyme from bananas does show a requirement for oxygen, this requirement alone, as already indicated, is not sufficient to rule out a hydration type of hydroxylation mechanism.

[6] It is not clear why the enzyme can catalyze the oxidation of reductants such as catechol whereas it can oxidize only an equivalent amount of ascorbate. One possibility is that catechol, in contrast to ascorbate, could reduce the postulated oxygenated intermediate to liberate free enzyme; the latter would then be available for another cycle of reduction and oxidation. An alternate possibility is that catechol alters the enzyme molecule in such a way that the reduced enzyme intermediate becomes easily reoxidized by molecular oxygen.

ascorbic acid was identified as the product of the reaction under anaerobic conditions. Thus, the enzyme could accept two electrons in the absence of oxygen according to equation 10.

$$E + \text{ascorbate} \rightarrow E^- + 2H^+ + \text{dehydroascorbate} \quad (10)$$

Since contaminant enzymes and metals can catalyze the oxidation of ascorbate, it was necessary to prove that this reduced enzyme was an intermediate in the hydroxylation reaction.

The reduced enzyme was prepared aerobically by incubation of the enzyme with ascorbate, and excess, unreacted ascorbate was destroyed by addition of a small amount of ascorbate oxidase. Substrate (phenylethylamine) was then added, and after a few minutes the reaction was stopped by heat-denaturation of the enzyme. It was found that phenylethanolamine was formed in amounts equivalent to the amount of enzyme present. This experiment proved that the oxidation of ascorbate is related to the hydroxylation reaction and that the reduced enzyme is an intermediate. It also proved that the hydroxylation reaction can be separated into two partial reactions: the first, shown in equation 10, is the reduction of the enzyme by ascorbate, and the second, shown in equation 11, is the aerobic utilization of the reduced-enzyme intermediate to form the hydroxylated product (where RH stands for the substrate and ROH for the hydroxylated product):

$$E^- + O_2 + 2H^+ + RH \rightarrow ROH + E + H_2O \quad (11)$$

Reactions 10 and 11 still leave unanswered certain crucial questions about the mechanism of the hydroxylation reaction: What group on the enzyme accepts the electrons from ascorbate in equation 10? What is the nature of the actual hydroxylating agent in equation 11?

To consider the latter question first, we can visualize equation 11 as the sum of two other partial reactions shown as equations 12 and 13.

$$E^- + O_2 \rightarrow EO_2^- \quad (12)$$

$$EO_2^- + RH + 2H^+ \rightarrow E + ROH + H_2O \quad (13)$$

According to this scheme, the hydroxylating agent would be an enzyme-bound, reduced form of oxygen (EO_2^-) at the reduction level of a peroxide.[7] To see if such an oxygenated intermediate could be detected, the enzyme was reduced by ascorbate in the presence of oxygen, the oxygen was removed, and the substrate was added anaerobically. Under these conditions, no hydroxylated product was found. This result suggests that if an oxygenated intermediate exists, it is present

[7] To explain certain kinetic peculiarities of the Dopamine-β-hydroxylase-catalyzed conversion of epinine to epinephrine, a hydroperoxide of the substrate was postulated as an intermediate in the reaction (17). Although no additional evidence has been gathered to support such a postulate, it can still be regarded as a possibility. Instead of transferring electrons to an enzyme-bound O_2 molecule, as proposed in equation 12, the reduced enzyme intermediate could transfer electrons to an enzyme-bound hydroperoxide of the substrate, reducing it to the hydroxylated product and water.

in very small amounts, perhaps because the equilibrium of reaction 12 lies far to the left.

The failure of the experiment also could be an indication that electron transfer from the reduced enzyme intermediate to the bound oxygen does not take place as a discrete step, but rather that the flow of electrons to oxygen and the transfer of oxygen to the substrate (*i.e.*, the hydroxylation step) occur simultaneously. In either case, the oxygen transferred to the substrate can be visualized as an oxygen atom with 6 electrons, as has been suggested by Hamilton (62).

The question of which group on the enzyme is reduced by ascorbate (equation 10) was examined recently. In view of the fact that copper has been shown to undergo reversible oxidation and reduction in some (18, 58, 127) enzyme systems (but see 81), it seemed likely that copper might play a similar role in the hydroxylation reaction catalyzed by Dopamine-β-hydroxylase.

The oxidation state of the enzyme-bound copper was studied with the use of chelating agent, 2,2′-biquinoline, which reacts with Cu^+. The complications and limitations of this method have been discussed (48, 136). The determination was made with 1) isolated enzyme, 2) the reduced enzyme intermediate, and 3) the reduced enzyme intermediate after the addition of phenylethylamine (50).

The enzyme preparation used for this experiment contained 3.6 moles of total copper per mole of enzyme, and since, as can be seen in Table 4, 33 % of the copper existed as cuprous ion, the cupric content was about 2 moles per mole of enzyme. In the reduced intermediate (*i.e.*, after reduction of the enzyme by ascorbate) about 70% of the copper reacted as cuprous ion and the amount of ascorbate oxidized was one-half of the amount of copper reduced, in other words, the reaction was stoichiometric. After substrate was added to the reduced intermediate, 50 % of the copper reacted as cuprous ion with the biquinoline reagent, an indication that approximately 60 % of the copper that had been reduced by the ascorbate treatment had been reoxidized during the hydroxylation of the phenylethylamine. The amount of copper oxidized was

TABLE 4

Reduction and oxidation of copper during catalysis

Enzyme Treatment	Cu^+	$\Delta Cu^+/2$	Ascorbate Oxidized	Phenethanolamine Formed
	mμmol/mμmol enzyme			
Isolated enzyme (total Cu = 3.60)	1.20			—
Enzyme after AA and AO (reduced enzyme)	2.60	+0.70	0.72	—
Reduced enzyme after PEA	1.80	−0.40	—	0.46

The enzyme was treated sequentially with ascorbate, ascorbate oxidase and phenylethylamine. The reaction mixture contained, in μmoles: potassium phosphate buffer, pH 6.8, 50; succinic acid, 50; ascorbic acid (AA), 0.020; phenylethylamine (PEA), 0.010; ascorbate oxidase (AO), 1 μl (14 units) (14); and 1 mg of enzyme in a volume of 0.40 ml. Copper was determined with the biquinoline reagent before and after the addition of ascorbic acid and ascorbic acid oxidase and after the addition of phenylethylamine.

Data taken from reference 50.

equivalent to the amount of phenylethanolamine formed. On the basis of these results, more detailed versions of equations 10 and 11 can be written:

$$E(Cu^{++})_2 + 2e \rightarrow E(Cu^{+})_2 \quad (14)$$

$$E(Cu^{+})_2 + O_2 + 2H^{+} + RH \rightarrow E(Cu^{++})_2 + ROH + H_2O \quad (15)$$

In addition to this postulated role of accepting electrons from ascorbate and transferring them to oxygen, the enzyme-bound copper could have other functions in the reaction catalyzed by Dopamine-β-hydroxylase. In other copper-proteins, two additional roles have been proposed for the metal. One is related to the ability of copper to bind oxygen (80, 98, 112), a role that does not appear to involve a change in the valence of the copper (127). This is the function of copper in hemocyanin, and, although it may not be its physiological role, it is interesting that this protein possesses catechol oxidase activity (9). It also has been suggested that copper plays an important part in binding substrate to an enzyme surface (18, 120). Charge transfer complexes have been observed (120) between enzyme-bound copper and substrate and it has been proposed that this is the function of the cuprous copper present in several proteins, but no direct evidence for this proposal has been obtained (18).

Some of these auxiliary roles for copper are implicit in the already postulated oxidation-reduction role for the metal. Thus, in order for copper to function as an electron acceptor from ascorbate and an electron donor to oxygen, it seems reasonable that the copper takes part in the binding of these two components to the enzyme surface. Whether the substrate to be hydroxylated is also bound at the same, or at a nearby, site on the enzyme is not known.

Studies with the purified hydroxylating enzyme have led to a delineation of some of the factors involved in the catalytic activity of the enzyme: ascorbate as an electron-donating cofactor, copper as part of the active site, and fumarate as a modulator of the activity. Identification of the metal has already contributed to an understanding of how some inhibitors of the enzyme function. The other factors shown to be required for the activity of the enzyme *in vitro* provide useful clues to how the activity of this enzyme might be regulated *in vivo*.

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