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D. COENZYMES AND HYDROXYLASES: ASCORBATE AND DOPAMINE-β-HYDROXYLASE; TETRAHYDROPTERIDINES AND PHENYLALANINE AND TYROSINE HYDROXYLASES

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If one took any aromatic compound from a bottle on a laboratory shelf and added it to an extract of any tissue, one would almost certainly be able to detect the hydroxylated derivative in the mixture after a short aerobic incubation. This is another way of saying that the metabolism of aromatic compounds almost invariably involves hydroxylation. It is not surprising, therefore, that in the transformation of an aromatic amino acid, tyrosine, to an aromatic hormone, norepinephrine (NE), several hydroxylation reactions are involved as key steps. The steps I am referring to, of course, are the conversion of tyrosine to dopa and the subsequent conversion of dopamine to NE. If one starts further back in this biosynthetic pathway, then still another hydroxylation reaction comes into the picture, *i.e.*, the conversion of phenylalanine to tyrosine.

I would like to start with some statements that are so general that they would apply to all hydroxylation reactions of this type. Later, I will go into detail on some of the distinct features of some of the individual hydroxylating enzymes involved in the NE biosynthetic pathway. The general reaction catalyzed by the hydroxylases under consideration is shown in equation 1 where RH stands for the substrate to be hydroxylated, ROH for the hydroxylated product and XH_2 for an electron donor.

$$RH + O_2 + XH_2 \rightarrow ROH + H_2O + X \quad (1)$$

Enzymes that catalyze this kind of reaction have been called mixed-function oxidases by Mason (27) and monooxygenases by Hayaishi (9). They are characterized by a requirement for an external electron donor, and, when examined, it has been shown that the oxygen in the hydroxylated product comes from the atmosphere and not from water. This has been demonstrated experimentally for the reactions catalyzed by both phenylalanine hydroxylase and dopamine-β-hydroxylase (20).

At least three different kinds of electron donor have been reported to function as coenzymes or co-reactants in different hydroxylating systems: TPNH, tetrahydropteridines and ascorbate. As far as the enzymes in the NE pathway are concerned, only the last two need be discussed. A tetrahydropteridine is the coenzyme in the systems that hydroxylate phenylalanine (13, 16) and tyrosine (1, 18, 28), and ascorbate (26) is the coenzyme for dopamine-β-hydroxylase.

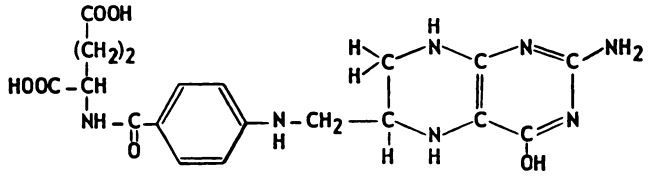
I mentioned earlier that one could visualize NE biosynthesis as starting with the amino acid, phenylalanine. Even if this reaction, the conversion of phenylalanine to tyrosine, bore no relationship to NE, it would still have to be mentioned in this context because most of what we know about the cofactor role of

pteridines in hydroxylation reactions has been learned from this enzyme reaction. In the discussion that follows, therefore, I will be talking about pteridines and the phenylalanine-hydroxylating system. There is good evidence, however, that pteridines function in the same way in the enzymatic conversion of tyrosine to dopa (1, 18, 28).

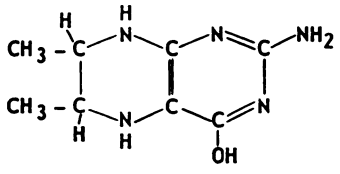
The discovery that a new cofactor was required for the hydroxylation of phenylalanine came out of attempts to purify the enzyme system that catalyzed the reaction. It was known that TPNH and at least two enzymes were needed for the conversion of phenylalanine to tyrosine. We had purified these enzymes from rat and sheep liver extracts (11). On the basis of balance studies, the overall reaction catalyzed by these enzymes was formulated (11) as shown in equation 2.

$$\text{phenylalanine} + O_2 + \text{TPNH} + H^+ \rightarrow \text{tyrosine} + H_2O + \text{TPN}^+ \qquad (2)$$

It can be seen that the reaction fits into the general equation for mixed-function oxidases. If TPNH or either of the two enzymes were omitted, no reaction could be detected. During the course of further purification of the two enzymes, it became clear that another essential component was being removed. This component proved to be a non-protein, moderately heat stable, dializable factor. It was isolated from boiled extracts of rat liver on the basis of its ability to stimulate tyrosine formation in the presence of TPNH and the purified rat and sheep liver enzymes (13). When some of the properties of the purified material suggested that it might be a pteridine, various pteridines, including many folic acid derivatives, were tested. Tetrahydrofolate had good cofactor activity, whereas 5-formyltetrahydrofolate, 7,8-dihydrofolate and folate were inactive (12). Since there was evidence that the cofactor did not contain the typical *para*-aminobenzoic acid-glutamic side chain of folic acid, it seemed most likely that it was an unconjugated pteridine. Several of these were prepared and tested and proved to have much greater activity than tetrahydrofolate (21). A comparison of the



TETRAHYDROFOLIC ACID



2-AMINO-4-HYDROXY-6,7-DIMETHYL-TETRAHYDROPTERIDINE

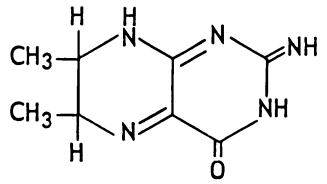
structure of tetrahydrofolate and one of these more active, unconjugated pteridines, is shown. In much of our work, we have used either the 6,7-dimethyl compound, or the still more active 6-methyl derivative. It was found that, in the presence of one of these tetrahydropteridines, tyrosine formation was still completely dependent on the presence of the rat liver enzyme and oxygen; the sheep liver enzyme and TPNH were no longer essential (14).

If phenylalanine and the tetrahydropteridine were incubated aerobically in the presence of only the rat liver enzyme, tyrosine was formed in amounts approximately equal to the amount of tetrahydropteridine added, *i.e.*, the pteridine functioned stoichiometrically. When the sheep liver enzyme and TPNH were also added, the only difference was that the pteridine could function catalytically. These results proved that the rat liver enzyme was the hydroxylating enzyme, and that in the hydroxylation reaction the tetrahydropteridine was consumed. In the presence of sheep liver enzyme and TPNH, the pteridine could somehow be converted back to the tetrahydro level so that it could function again. The fact that TPNH was needed for the catalytic functioning of the pteridine indicated that "consumption" of the pteridine involved its oxidation. These results led to the following formulation of the conversion of phenylalanine to tyrosine.

$$\text{tetrahydropteridine} + O_2 + \text{phenylalanine} \xrightarrow[\text{enzyme}]{\text{rat liver}} \text{oxidized pteridine} + \text{tyrosine} + H_2O \quad (3)$$

$$\text{oxidized pteridine} + \text{TPNH} + H^+ \xrightarrow[\text{enzyme}]{\text{sheep liver}} \text{tetrahydropteridine} + \text{TPN}^+ \quad (4)$$

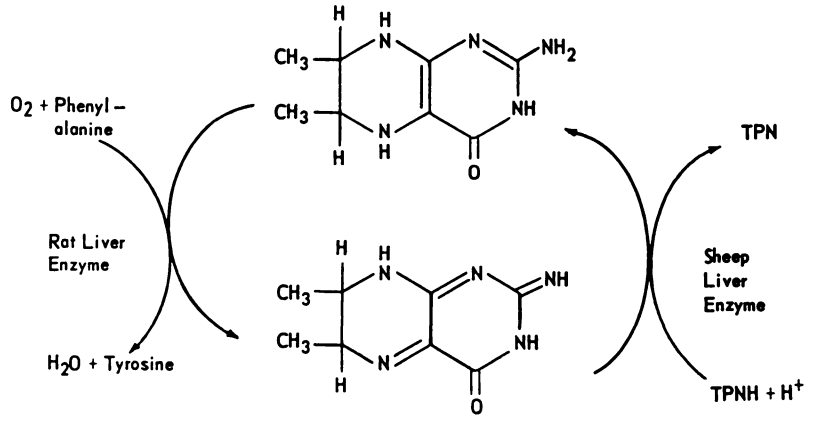
The oxidized pteridine shown in equations 3 and 4 proved to be a new type of



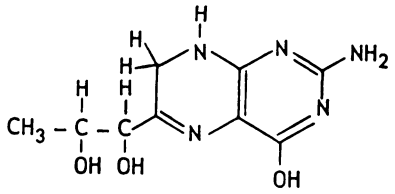
"QUINONOID" TAUTOMER OF A DIHYDROPTERIDINE

dihydropteridine (17) with a quinonoid structure. It is a strong oxidizing agent, capable of oxidizing a variety of reducing compounds, including TPNH, ascorbate and mercaptans. At high concentrations, therefore, these compounds can replace the sheep liver enzyme. The reduction of the quinonoid dihydropteridine catalyzed by sheep liver enzyme, on the other hand, is specific for TPNH. The reactions catalyzed by the rat and sheep liver enzymes are shown (p. 64).

It should be emphasized that these pteridine transformations were established through the use of model tetrahydropteridines in place of the natural cofactor. In fact, the situation was somewhat anomalous in that the general role of pteridines in the phenylalanine-hydroxylating system had been put on a firm basis long before the structure of the cofactor had been elucidated.



Recently, the cofactor from rat liver was obtained in pure form; structural studies proved that it is 7,8-dihydrobiopterin (16). It is an unconjugated pteri-

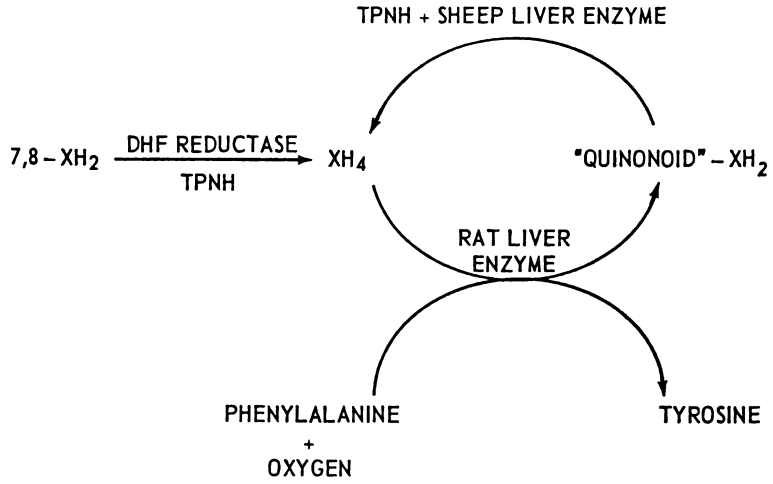


DIHYDROBIOPTERIN

dine with a rather simple hydroxy-alkyl substituent at carbon 6 of the pteridine ring. This class of compound had been known for years before the conjugated pteridine, folic acid, had been discovered. They occur as pigments in the skin of insects, amphibia and fish, and in the eyes and wings of insects (32). The demonstration that the phenylalanine-hydroxylation cofactor is an unconjugated pteridine, however, established the first metabolic role for this class of pteridines.

As far as we can tell, the cofactor functions in the hydroxylase system in the same way as do the synthetic tetrahydropteridines. The only difference is that the cofactor, as isolated, being in the 7,8-dihydro configuration, is not active in the hydroxylating system and must first be reduced to the active, tetrahydro form. The enzyme that catalyzes this initial reductive reaction is dihydrofolate reductase (16, 19), an enzyme with an established role in one-carbon metabolism (10). Once this initial reaction occurs, dihydrofolate-reductase plays no further role in the hydroxylation reaction as the cofactor then shuttles back and forth between the tetrahydro and the quinonoid-dihydro forms.

There is still one facet of this initial reduction of the cofactor that is not completely understood. It was reported several years ago (15) that, in contrast to the



activity of synthetic *dl*-tetrahydrofolate,[1] *l*-tetrahydrofolate, the product of the reduction of dihydrofolate catalyzed by dihydrofolate reductase, is essentially inactive in the phenylalanine-hydroxylating system. This finding suggested that only the *d*-isomer of tetrahydrofolate could function as a cofactor in this system.

In view of these results, the demonstration that dihydrofolate reductase catalyzed the initial reduction of the cofactor was somewhat surprising. It indicated that *l*-tetrahydrobiopterin, the presumed product of this enzymatic reduction, was active in the hydroxylating system, whereas *l*-tetrahydrofolate was not. An alternative possibility was that *l*-tetrahydrobiopterin, itself, was inactive but that it could be converted to an active compound by some mechanism that led to racemization.

We recently obtained evidence that appears to support this racemization theory. A protein that markedly stimulates the hydroxylation reaction has been purified from rat liver. Significantly, it stimulates the reaction much more in the presence of *l*-tetrahydrobiopterin than in the presence of chemically reduced, *dl*-tetrahydrobiopterin. In the presence of TPN, this protein can catalyze the oxidation of the cofactor to sepia pteridine, *i.e.*, it can catalyze the oxidation of the side-chain hydroxyl group of dihydrobiopterin to the corresponding keto group. If this oxidation occurred with *l*-tetrahydrobiopterin, it could lead to its racemization. It is not yet certain, however, that this is the mechanism by which this protein stimulates the hydroxylation reaction.

Although these pteridine transformations have so far been established only for the phenylalanine-hydroxylating system, there is good reason to believe that

[1] Tetrahydrofolate prepared by chemical reduction of dihydrofolate is a mixture of the diastereoisomers *l*-5,6,7,8-tetrahydropteroyl-L-glutamic acid and *d*-5,6,7,8-tetrahydropteroyl-L-glutamic acid. It will be designated as *dl*-L-tetrahydrofolate, or simply as *dl*-tetrahydrofolate, where the uncapitalized letters refer to the configuration at carbon 6 of the pteridine ring. Tetrahydrofolate prepared by enzymatic reduction of dihydrofolate will be referred to as *l*-tetrahydrofolate.

they will prove to be generally true in other oxidative systems in which unconjugated pteridines function as cofactors. Thus, in both the glyceryl-ether cleaving system (30) and in the adrenal tyrosine-hydroxylating system (1, 18, 28), the preliminary evidence indicates that the same transformations take place.

The second hydroxylating step in the NE biosynthetic pathway involves the conversion of dopamine to NE. The enzyme catalyzing this reaction, dopamine-β-hydroxylase, was extensively purified from bovine adrenal particles and shown to require ascorbate and a dicarboxylic acid, such as fumarate (26). The mechanism by which fumarate stimulates the reaction is not known. From balance studies, the conversion of dopamine to NE was formulated as shown in equation 5.

$$\text{dopamine} + \text{ascorbate} + O_2 \rightarrow l\text{-norepinephrine} + \text{dehydroascorbate} + \text{water} \quad (5)$$

It can be seen that this reaction, like the conversion of phenylalanine to tyrosine, fits into the general equation for mixed-function oxidases. The oxygen in the hydroxylated product in this system, too, is derived from molecular oxygen and not from water (20).

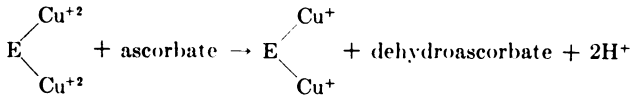
In addition to dopamine, phenylethylamine (25), tyramine (29) and epinine (2) can also serve as substrates for the enzyme.

Recently, dopamine-β-hydroxylase was obtained in essentially pure form and shown to be a copper protein (4). The enzyme has a molecular weight of approximately 290,000 (assuming a specific volume of 0.720) and contains between 0.65 and 1.0 μg of copper per mg of protein (4). This corresponds to 4 to 7 moles of copper per mole of protein. Part of the copper is present as Cu^{+} and part as Cu^{+2}. Although the amount of Cu^{+} varies from one enzyme preparation to another, the amount of Cu^{+2} is relatively constant and is equal to about 2 moles per mole of enzyme.

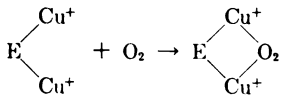
In an attempt to identify the first reaction in the sequence that leads to hydroxylation of the substrate, experiments were carried out with stoichiometric amounts of the enzyme. It was found that in the absence of substrate, the enzyme could oxidize rapidly an approximately equivalent amount of ascorbate. One of the products of this reaction was shown to be dehydroascorbate; the other was a reduced form of the enzyme (4). It was also shown that the group on the enzyme that accepted the electrons from the ascorbate was the copper, *i.e.*, copper was reduced from the cupric to the cuprous state. Furthermore, in experiments in which the excess ascorbate was removed with ascorbate oxidase, it was shown that this reduced form of the enzyme was an intermediate in the hydroxylation reaction. It could react with the substrate to form an amount of hydroxylated product that was approximately equal to the amount of enzyme originally present. Concomitantly, part of the protein-bound copper that had been reduced by the treatment with ascorbate was reoxidized during the hydroxylation of the substrate. The amount of copper reoxidized was equivalent to the amount of product formed (4). These changes in valence have also been confirmed with electron paramagnetic resonance techniques (6). Recently, Goldstein *et al.* (7, 8) have detected similar changes in the oxidation state of copper in impure prepara-

tions of dopamine-β-hydroxylase. No data were presented, however, which related these valence changes to the amount of enzyme used, the amount of ascorbate oxidized, or the amount of hydroxylated product formed.

On the basis of our results, the hydroxylation reaction can be formulated as shown in equations 6, 7 and 8 where RH stands for substrate and ROH for hydroxylated product.



(6)



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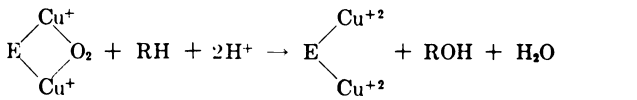

(8)

Reaction 7, which depicts the combination of O_2 with the cuprous ions in the reduced enzyme, was included in the scheme because this is one of the classical roles for metals in oxidative enzymes (31). In the case of dopamine-β-hydroxylase there is also experimental evidence in favor of this idea. Recently, it was found that this system is sensitive to CO as an inhibitor; in the presence of a mixture of 40% CO, 4% O_2, 56% N_2, the hydroxylase was inhibited 77% and this inhibition was not reversed by light (5). Since the mechanism of the CO inhibition almost certainly involves competition with O_2 for the copper in the active site of the enzyme, these results strongly support the occurrence of reaction 7 as part of the sequence leading to hydroxylation. Because CO has a much higher affinity for cuprous than for cupric complexes (3), the sensitivity of the hydroxylase to CO also supports the idea that monovalent copper plays an essential role in the catalytic action of this enzyme.

The light-insensitive inhibition of dopamine-β-hydroxylase by CO is reminiscent of the effects of this poison on phenolase, a copper-enzyme that is also inhibited by CO (22, 24) in a light-insensitive manner. Contrary to the older ideas (24), the recent evidence strongly indicates that the copper in this plant enzyme is all in the cuprous state (23).

Reactions 6, 7 and 8, of course, still do not answer all the questions that can be raised about the mechanism of the hydroxylation reaction. One of the most compelling ones, perhaps, is whether the oxygen molecule is reduced prior to or subsequent to the transfer of the oxygen to the substrate molecule.

The demonstration that the first step in the reaction catalyzed by dopamine-β-hydroxylase is the reduction of the enzyme by ascorbate also raises the question of whether tetrahydropteridines function in a similar manner in the phenylalanine- and tyrosine-hydroxylating systems. Although this seems to be likely, the answer must wait for future experiments.

In closing, it may be of interest to note that this work on dopamine-β-hydroxylase fulfills a prediction made by Warburg (31) 16 years ago when he wrote,

"If I am correct in my opinion that the haemocyanins have been evolved from oxygen transporting enzymes, then animal cells should also have enzymes which are capable of transporting oxygen by means of copper. In the future, a look-out should be kept for animal cells the CO inhibited respiration of which is only partly light sensitive. A respiration partly non-light sensitive would indicate that copper proteins are involved." Dopamine-β-hydroxylase is the first enzyme isolated from animal tissues to fit these criteria.

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