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E. ENZYMIC STUDIES ON THE MECHANISM OF DOUBLE HYDROXYLATION

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The mechanism of single hydroxylation reactions involving both aromatic and aliphatic compounds has been discussed in detail by Dr. Udenfriend and Dr. Kaufman in the preceding presentations. In addition to monohydroxylated compounds, however, many dihydroxylated derivatives occur in nature. For example, recent studies in several laboratories have provided evidence for the presence of significant quantities of conjugated catechol derivatives in normal human urine (3, 5), but the metabolic pathway by which these compounds are formed is not clear. It might, therefore, be appropriate on this occasion to compare various routes for the synthesis of these *ortho*-dihydroxyaromatic compounds, or catechol derivatives, in nature.

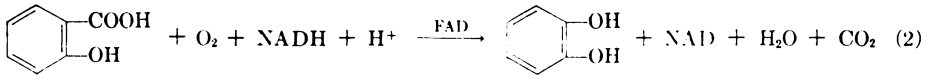
According to our present knowledge, catechol derivatives may be formed by at least four different mechanisms, namely 1) by two successive monooxygenase reactions, 2) by the further hydroxylation of a monohydroxylated compound or its derivatives, 3) by dehydrogenation of dihydrodiol compounds, and 4) by the incorporation of two atoms of oxygen followed by reduction. I would like to review briefly these four pathways, with particular attention to the nature of the enzymes involved.

Catechol derivatives are produced by two successive single hydroxylation reactions. As discussed by Dr. Udenfriend, dihydroxyphenylalanine is synthesized in this way. Phenylalanine is hydroxylated to tyrosine by phenylalanine monooxygenase, and the resulting tyrosine is further hydroxylated at the *ortho*-position by another monooxygenase to form the end product, dopa (9). The overall stoichiometry is shown in equation 1, in which "Sub" stands for substrate.

$$\text{Sub}\begin{matrix}\diagup\text{H}\\\diagdown\text{H}\end{matrix} + 2O_2 + 2\text{NADPH} + 2\text{H}^+ \rightarrow \text{Sub}\begin{matrix}\diagup\text{OH}\\\diagdown\text{OH}\end{matrix} + 2\text{NADP} + 2H_2O \qquad (1)$$

In this case two moles of molecular oxygen and two moles of reducing agent, such as NADPH, are utilized per mole of product formed. Both oxygen atoms inserted into the molecule are derived from molecular oxygen, but not from the same molecule of oxygen.

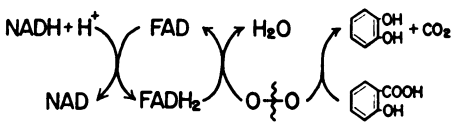
Salicylate hydroxylase, an enzyme which forms catechol from salicylic acid in the presence of NADH, has recently been purified from *Pseudomonas sp.* in our laboratory. The reaction proceeds by equation 2.



(2)

The purified enzyme is homogeneous upon electrophoresis and ultracentrifugation, although it has not yet been obtained in a crystalline form (7, 13). The molecular weight is estimated to be about 57,000, and one mole of FAD is bound per mole of enzyme protein. The overall reaction may be considered as the sum of two reactions, namely, 1) the reduction of FAD by NADH, and 2) the subsequent reduction of oxygen by $FADH_2$ and the substrate (equation 3).

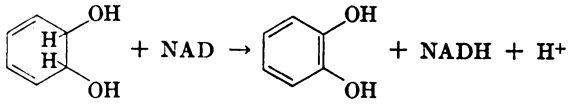
THE MECHANISM OF SALICYLATE HYDROXYLASE



(3)

Despite considerable effort we have been unable to resolve these two steps. These two reactions are tightly coupled, therefore, and presumably are catalyzed by a single enzyme. In this regard salicylate hydroxylase is different from phenylalanine hydroxylase studied by Kaufman (8) and from diketocamphane lactonase described by Conrad *et al.* (4). In the latter two reactions, the reduction of a pteridine coenzyme by NADPH and of FMN by NADH, respectively, are catalyzed by enzymes which can be separated from the hydroxylating enzyme.

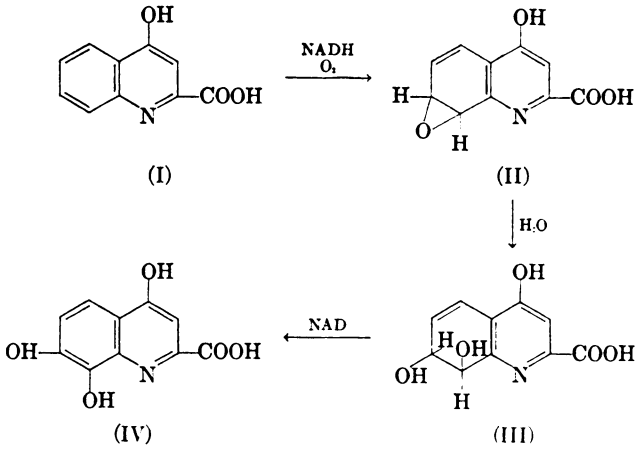
An enzyme which catalyzes the irreversible oxidation of *cis* and *trans* 5,6-dihydroxycyclohexa-1,3-diene, is ubiquitously found in animal tissues and microorganisms, and has been purified from rabbit liver in our laboratory (1). The product was identified as catechol (equation 4).



(4)

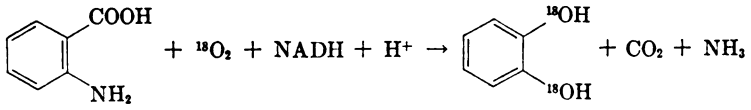
Both *trans* and *cis* glycols are oxidized at about the same rate, and the K_m values are 1.4×10^{-3} M for *trans* 5,6-dihydroxycyclohexa-1,3-diene and 8.6×10^{-3} M for the *cis* isomer. Either NADP or NAD can serve as an electron acceptor. Although the physiologic significance of this dehydrogenase remains to be elucidated, a similar enzyme is involved in the formation of 7,8-dihydroxykynurenic acid from kynurenic acid.

In this case, the formation of catechol derivatives occurs by the successive action of a monooxygenase, a hydrase, and a dehydrogenase. When kynurenic acid (I) is converted to 7,8-dihydroxykynurenic acid (IV), the primary reaction is catalyzed by a monooxygenase which requires NADH and oxygen. The primary product was tentatively identified as a 7,8-epoxide (II), which is hydrated by a hydrase to give a vicinal glycol (III). The latter compound is then dehydrogenated by an NAD-linked dehydrogenase to form 7,8-dihydroxykynurenic acid (12). The reaction sequence is shown in equation 5.

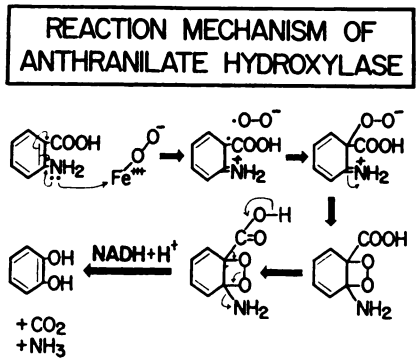


In this case, one of the oxygen atoms of dihydroxykynurenic acid was assumed to be derived from molecular oxygen while the other was derived from water. Similarly Booth *et al.* (2) have shown that an epoxide of naphthalene is formed in the presence of NADPH, oxygen, and rat liver microsomes, and that the epoxide is further converted to a dihydrodiol of naphthalene. Although various other dihydrodiols and epoxides occur in nature, the mechanism of their formation is not understood at present.

Anthranilate hydroxylase, which catalyzes the formation of catechol from anthranilic acid, is an example of the fourth group of double hydroxylation pathways. The enzyme has also been partially purified from cells of a *Pseudomonad* in our laboratory (11). During the conversion of one mole of anthranilic acid to catechol, one mole each of oxygen and NADH is used, and one mole each of CO_2 and ammonia is produced (equation 6).

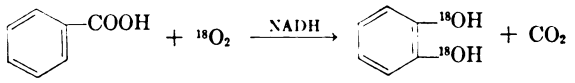


Both atoms of oxygen in catechol are exclusively derived from molecular oxygen, as shown by experiments with O^{18} and H_2O^{18}. Since one mole each of oxygen and NADH is utilized for the formation of one mole of catechol, the reaction apparently involves the direct incorporation of 2 atoms of molecular oxygen into the substrate rather than two successive single hydroxylation reactions. A reasonable mechanism for this reaction is given in equation 7. Since ferrous ion is essential for the reaction, the activation of oxygen may involve the formation of perferryl ion as has been suggested previously (6). Two oxygen atoms, presumably in the same molecule, may add to the double bond between C-1 and C-2 of anthranilate by the depicted series of reactions. The loss of ammonia and CO_2 and the reductive cleavage of the unstable cyclic peroxide might then proceed in a concerted manner.



(7)

Recent experiments (10) in our laboratory have shown that another enzyme of *Pseudomonas* forms catechol from benzoic acid. Interestingly, reduced pyridine nucleotides are essential for the reaction, but only in catalytic amounts. The overall reaction simply involves the insertion of two atoms of oxygen into the substrate molecule and the loss of CO_2 (equation 8). By the use of O^{18}, both atoms of oxygen were shown to be derived from molecular oxygen. The role of NADH in this reaction has not yet been elucidated.



(8)

In summary, I have briefly reviewed four routes of enzymic synthesis of catechol derivatives. Obviously there would be other types of reactions, by which catechol derivatives are formed in nature. Unfortunately most monooxygenases are localized in the particulate fraction of cells or are unstable during purification. Since crystalline enzymes have not been available for use in studying the mechanism of these reactions, progress has been delayed in understanding the intimate mechanism of hydroxylation. Nevertheless, it is clear that catechol derivatives are synthesized in nature by several different pathways, and it is interesting to speculate about the evolution of these pathways in specific cases.

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