The Metabolism of Coumarin by a Microorganism

V. MELILOTATE HYDROXYLASE

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SUMMARY

Melilotate hydroxylase partially purified from extracts of Arthrobacter species catalyzes the stoichiometric conversion of melilotic acid to 2,3-dihydroxyphenylpropionic acid. The hydroxylation is shown to require both reduced nicotinamide adenine dinucleotide and atmospheric oxygen. A partial resolution of the enzyme is achieved with ammonium sulfate. The preparation so treated is almost devoid of enzyme activity, but activity can be completely restored with flavin adenine dinucleotide.

The role of melilotate hydroxylase in the general metabolism of coumarin is discussed.

In earlier studies the degradation of coumarin by Arthrobacter species has been shown to proceed through the intermediates o-coumaric acid (o-hydroxy-trans-cinnamic acid) and melilotic acid (o-hydroxyphenylpropionic acid) (1, 2). In this metabolic pathway the reduction of o-coumaric acid to melilotic acid by the enzyme reduced nicotinamide adenine dinucleotide:o-coumarate oxidoreductase has been described (1).

It was noted that culture filtrates of the bacterium grown on coumarin or o-coumaric acid contained an ultraviolet-absorbing material which gave a positive reaction with a reagent specific for o-dihydroxyphenols (2). This material, now identified as 2,3 dihydroxyphenylpropionic acid, arises from melilotic acid by enzymatic hydroxylation. The enzyme catalyzing this reaction has been partially purified from extracts of Arthrobacter and has been called melilotate hydroxylase. The present studies on this hydroxylase indicate a requirement for NADH and atmospheric oxygen and suggest a role for flavin adenine dinucleotide as the prosthetic group.

EXPERIMENTAL PROCEDURE

Materials

NAD, NADH, and NADPH were purchased from P-L Biochemicals. FAD, FMN, and riboflavin were obtained from Mann. Melilotic acid was synthesized as described previously (1). 2,4-Dihydroxyphenylpropionic acid was synthesized by the method of Langley and Adams (3). 2,3-Dihydroxyphenylpropionic acid was prepared from 2,3-dimethoxyphenylpropionic acid by catalytic hydrogenation followed by demethylation of the reduced product with 40% HBr. m-Hydroxyphenylpropionic acid was obtained from m-methoxyphenylpropionic acid after demethylation with 40% HBr. All other aromatic compounds were purchased either from K and K Laboratories or from Aldrich Research Chemicals, Inc. Alcohol dehydrogenase was obtained as the crystalline enzyme from Worthington.

Methods

Thin layer chromatography was performed according to Randerath (5), on plates (20 x 20 cm) of Silica Gel-G (Brinkmann Instruments) with a gel thickness of about 250 μ. All paper chromatography was carried out on Whatman No. 3MM paper, with the use of ascending techniques. The solvents used both for thin layer and for paper chromatography were those described by Coulson and Evans (6): benzene-glacial acetic acid-water (20:5:1), 1-butanol-ethanol-borate buffer (9.5 g of sodium tetraborate per liter of water) (1:1:1). A third solvent, isopropyl alcohol-water-glacial acetic acid (8:1:0.2), was also employed at times. 2,3-Dihydroxyphenylpropionic acid was visualized with Evans' nitrite-molybdate reagent (6). The concentration of 2,3-dihydroxyphenylpropionic acid was measured by the procedure of Arnow (7) for catechols and catechol acids. Reducing agents other than the pyridine nucleotides caused some inhibition of color development with this procedure at concentrations above 350 μmoles per ml. Consequently, in cofactor specificity studies the concentration of each reducing agent was kept at 300 μmoles per ml. Protein was determined by the method of Lowry et al. (8).

Standard Assay for Melilolate Hydroxylase

Under standard conditions, the reaction mixture contained, in a final volume of 3 ml, melilotic acid, 250 μmoles; NADH, 300 μmoles; phosphate buffer, pH 7.3, 10 μmoles; and enzyme, 2.5 units (Step 4; specific activity, 197). Incubation was conducted for 1 min at 30°, and enzyme activity was assayed spectrophotometrically by recording the decrease in absorbance of NADH at 340 μ. The spectrophotometer was a Beckman DK-2A ratio recording instrument equipped with a constant temperature device. A unit of enzyme activity is equivalent to a decrease of 0.1 optical density unit in 5 min at 30°.
Metabolism of Coumarin by a Microorganism. V

Preparation of Enzyme

Growth of Cells The organism, *Arthrobacter* species, was cultured as described elsewhere (9), except that melilotic acid (1 g per liter) was used instead of coumarin as the carbon source. After 16 to 20 hours of growth, the cells were harvested by centrifugation and stored at -20° until used.

All of the following operations were carried out at 0° unless stated otherwise.

Step 1: Extraction—The cells (20 g, wet weight) were thawed and uniformly suspended in 80 ml of cold 0.05 M phosphate buffer, pH 7.3, containing 0.001 M cysteine and 0.5 M succrose. The suspension was divided into two equal portions and each portion was subjected to sonic oscillation in a Bronwill 20-Kc oscillator for 15 min. Cellular debris was removed by centrifugation at 75,000 × g for 30 min, and the precipitate was discarded. This procedure yielded 60 ml of a yellowish solution containing 3.7 mg of protein per ml.

Step 2: Protamine Sulfate Fractionation—To 50 ml of the crude extract, 14 ml of a 2% suspension of protamine sulfate in 0.05 M phosphate buffer, pH 7.3, were added slowly, with stirring. After 20 min of stirring, the mixture was clarified by centrifugation at 75,000 × g for 30 min, and the precipitate was discarded. This procedure yielded 60 ml of a yellowish solution containing 3.7 mg of protein per ml.

Step 3: Adsorption and Elution from Calcium Phosphate Gel—Calcium phosphate gel (100 ml; 20.2 mg dry weight per ml) was added to 50 ml of the enzyme solution obtained from the previous step. The mixture was stirred for 30 min and centrifuged, and the supernatant solution was discarded. The adsorbed enzyme was eluted by suspending the gel in 50 ml of a solution containing 0.1 M phosphate buffer at pH 7.3, 0.5 M succrose, and 0.001 M cysteine, and stirring for 30 min. After centrifugation, the gel was discarded and the supernatant fluid was found to have 0.62 mg of protein per ml. The eluate could be stored at -20° for 3 months with no significant loss of activity.

Step 4: DEAE-cellulose Column Chromatography—The enzyme solution (20 ml) obtained from the previous step was diluted with an equal volume of water and applied to a DEAE-cellulose column (0.9 cm × 10 cm) which had been equilibrated with 0.05 M phosphate buffer at pH 7.3. After all of the enzyme had been adsorbed, the column was washed with water and the enzyme was eluted with 0.1 M phosphate buffer at pH 7.3. The effluent was collected in 1-ml fractions, and the fractions, numbered 5 through 10, contained almost all of the activity. The fractions were pooled and used as the source of enzyme for most of the studies reported here. When stored at -20°, the enzyme at this stage was stable for about 1 week. The purification procedure is summarized in Table I.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Units</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>50</td>
<td>840</td>
<td>3000</td>
<td>3.6</td>
<td>100</td>
</tr>
<tr>
<td>2. Protamine sulfate fractionation</td>
<td>60</td>
<td>220</td>
<td>2800</td>
<td>12.7</td>
<td>93</td>
</tr>
<tr>
<td>3. Ca₃(PO₄)₂ gel eluate</td>
<td>50</td>
<td>31</td>
<td>1230</td>
<td>39.9</td>
<td>44</td>
</tr>
<tr>
<td>4. DEAE-cellulose fractionation</td>
<td>12</td>
<td>3.6</td>
<td>710</td>
<td>197</td>
<td>24</td>
</tr>
</tbody>
</table>

Inactivation of Enzyme

It had been shown in an earlier study that melilotic acid, formed enzymatically by the reduction of o-coumaric acid, could be recovered almost quantitatively from reaction mixtures when incubated with crude extracts of *Arthrobacter* species. The addition of NADH to the incubation mixture caused both a rapid disappearance of melilotic acid and an oxidation of NADH. In the present study, the disappearance of melilotic acid has been shown to be linear up to a 4-fold increase in the concentration of enzyme (Fig. 1). In the absence of enzyme, or in the presence of boiled enzyme, there was neither disappearance of melilotic acid nor oxidation of NADH. Concomitant with the disappearance of melilotic acid, a substance appeared in the reaction mixture which gave a positive test with Evans' reagent for o-dihydroxyphenols (2). A material with similar properties was also present on chromatograms of culture filtrates of *Arthrobacter* when the organism was grown either on coumarin or on o-coumaric acid. The substances obtained from both the reaction mixture and the culture media had similar mobilities in three different solvent systems. These were as follows: benzene-glacial acetic acid-water (Rf 0.26);

![Fig. 1](http://example.com/fig1.png)

**Fig. 1.** Effect of increasing enzyme concentration on the rate of oxidation of NADH. The reaction mixture, in a final volume of 3 ml, consisted of 250 μmoles of melilotic acid, 350 μmoles of NADH, 10 μmoles of phosphate buffer (pH 7.3), and various amounts of enzyme (Step 1; specific activity, 3.6) as shown. Incubation was conducted for 1 min at 30° and enzyme activity was assayed by recording the decrease in absorbance of NADH at 340 μm.
1-butanol-ethanol-borate buffer ($R_F$ 0.46); and isopropyl alcohol-water-acetic acid ($R_F$ 0.78). The $R_F$ values found by Coulson and Evans (6) for 2,3-dihydroxyphenylpropionic acid in the first two solvents are in good agreement with those presented above.

Isolation and Characterization of 2,3-Dihydroxyphenylpropionic Acid—More definitive identification of the compound was made by the isolation and characterization of the product of the reaction between enzyme, melilotic acid, NADH, and oxygen.

To a flask containing 100 mg of melilotic acid dissolved in 50 ml of water, the following substances were added: 1 g of NADH; 25 ml of 0.02 M phosphate buffer, pH 7.3; and 30 ml of enzyme, 730 units (Step 3; specific activity, 39.9). The flask, incubated aerobically in a water bath at 30°, was shaken gently, and at 4-hour intervals small aliquots of the reaction mixture were examined for the formation of o-dihydroxyphenol. When, at the end of 24 hours, no further increase in the amount of o-dihydroxyphenol was found, the vessel containing the incubation mixture was immersed in a boiling water bath for 10 min. After centrifugation to clarify the solution, the volume was reduced to about 25 ml by vacuum distillation, and acidified with a few drops of concentrated HCl. The resulting flocculent precipitate was removed by centrifugation, and the clear solution was shaken with equal volumes of ether until the o-dihydroxyphenol could no longer be detected in the aqueous phase. The ether extracts were combined and evaporated to dryness. The residue was dissolved in about 10 ml of ethanol and filtered, and the filtrate was applied to 15 thin layer chromatography plates coated with Silica Gel-G. After development in the benzene-acetic acid-water solvent, the plates were dried and examined under ultraviolet light. Two ultraviolet-absorbing bands were seen, one having migrated the same distance as melilotic acid ($R_F$ 0.56); the other, the same distance as 2,3-dihydroxyphenylpropionic acid ($R_F$ 0.26). The sections of silica gel containing the latter band were removed from the plates and combined, and the adsorbed material was eluted with 50 ml of hot water. The water was carefully decanted and the eluting process was repeated three times. The aqueous eluates (200 ml) were combined and filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in warm benzene (10 ml) and refrigerated for a few hours. Crystals were collected by filtration, washed with small amounts of cold water, and dried in a vacuum desiccator over concentrated H$_2$SO$_4$. Yield, 20.6 mg of pale gray needles (m.p. 124-125°). The mixture of isolated material and an authentic specimen of 2,3-dihydroxyphenylpropionic acid melted at 123-125°. The infrared spectra of the isolated and authentic materials were similar (Fig. 2).

Comparison of the molecular extinction coefficients of the authentic (pH 7.3, $\epsilon_{max}$ 1910) and isolated compounds (pH 7.3, $\epsilon_{max}$ 1840) indicated that they are essentially the same. Finally, cochromatography of the isolated and authentic compounds gave a single spot in each of three different solvent systems: benzene-acetic acid-water ($R_F$ 0.26); isopropyl alcohol-water-acetic acid ($R_F$ 0.79); and 1-butanol-ethanol-borate buffer (0.46).

On the basis of the evidence presented above, the isolated compound is considered to be 2,3-dihydroxyphenylpropionic acid.

FAD, Possible Prosthetic Group of Enzyme—Preliminary attempts at purification of the enzyme from crude extracts by fractionation with ammonium sulfate resulted in large losses of activity. Prolonged dialysis to remove what were thought to be the inhibitory effects of ammonium sulfate only served to increase these losses. Since the methods by which the decrease in activity was brought about have been used for the demonstration of flavins as prosthetic groups in other systems (10, 11), riboflavin, FMN, and FAD were added to the ammonium sulfate-treated and dialyzed enzyme to determine whether enzyme activity could be restored. It is apparent from Table II that both riboflavin and FMN were only slightly effective, whereas FAD, at the same concentration, resulted in almost complete restoration of enzyme activity.

Several other lines of evidence lend some support to the possibility that FAD may be the prosthetic group of the enzyme. It was found, for example, that addition of FAD to reaction mix-

![Fig. 2. Comparison of the infrared spectra of the isolated and authentic samples of 2,3 dihydroxyphenylpropionic acid. The concentration of both samples was 0.2% in KBr, and the spectra are uncorrected for the absorption of KBr.](http://www.jbc.org/fig)
The reaction mixtures contained, in a final volume of 3 ml, phosphate buffer, pH 7.3, 10 μmoles; melilotic acid, 250 μmoles; NADH, 300 μmoles; and enzyme, 6 units (crude extract; specific activity, 3.6) in Experiment 1, 0.1 ml (inactivated crude extract;* 15.2 mg of protein per ml) in all other experiments. When any of the flavins were added to the reaction mixture, the same volume (3 ml) was maintained by slight alterations in the amount of buffer. Incubation was conducted for 1 min at 30°. Enzyme activity was measured by recording the decrease in absorbance of NADH at 340 μm.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>100</td>
</tr>
<tr>
<td>2. Inactivated crude extract</td>
<td>15</td>
</tr>
<tr>
<td>2a. Plus riboflavin (3.3 X 10^-6 M)</td>
<td>15</td>
</tr>
<tr>
<td>Plus riboflavin (6.6 X 10^-6 M)</td>
<td>18</td>
</tr>
<tr>
<td>2b. Plus FMN (3.3 X 10^-6 M)</td>
<td>19</td>
</tr>
<tr>
<td>Plus FMN (6.6 X 10^-6 M)</td>
<td>27</td>
</tr>
<tr>
<td>2c. Plus FAD (3.3 X 10^-6 M)</td>
<td>83</td>
</tr>
<tr>
<td>Plus FAD (6.6 X 10^-6 M)</td>
<td>97</td>
</tr>
</tbody>
</table>

* This enzyme, obtained from a crude extract, was prepared as described under "Inactivation of Enzyme."

### Table III
**Formation of 2,3-dihydroxyphenylpropionic acid under aerobic and anaerobic conditions**

Into each of three tubes fitted with a side arm, the following substances were placed: melilotic acid, 1.2 μmoles; NADH, 1.4 μmoles; and phosphate buffer, pH 7.3, 20 μmoles. The lip of each tube was sealed with a self-sealing rubber cap 1 cm thick, and the tubes were alternately flushed with helium gas and evacuated with a mechanical pump. After this process was repeated three times for a total of 2 hours, 0.3 ml of enzyme (12 units; Step 4; specific activity, 133) was injected into the reaction mixture through the cap of each tube. The final volume of each reaction mixture was 3 ml. Incubation was performed in a water bath at 30°, and at the time intervals indicated the reaction was stopped by the injection of 0.2 ml of 6 N HCl. Small aliquots of the incubation mixtures were taken for 2,3-dihydroxyphenylpropionic acid analysis. Another set of three tubes containing identical reaction mixtures was treated in the same manner as described above except that, after injection of the enzyme, the caps were removed and the tubes were placed in the water bath. The reaction was stopped by the addition of 6 N HCl, and 2,3-dihydroxyphenylpropionic acid was determined. All values are averages of duplicate determinations. Since 2,3-dihydroxyphenylpropionic acid was not detected in control samples at zero time, no correction was necessary for the values obtained at the later time periods.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>2,3-Dihydroxyphenylpropionic acid formed</th>
<th>μmoles/3 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0.590</td>
<td>0.720</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.095</td>
<td>0.130</td>
</tr>
</tbody>
</table>

### Figure 3
The oxidation of NADH by melilotate hydroxylase under aerobic and anaerobic conditions. Each reaction mixture contained, in the main compartment of a Thunberg quartz cuvette, 350 μmoles of melilotic acid, 450 μmoles of NADH, and 10 μmoles of phosphate buffer, pH 7.3. In each side bulb, 5 units of enzyme (Step 4; specific activity, 133) were placed. Both cuvettes were alternately flushed with helium gas and evacuated for 30 min. The enzyme solution was then tipped into the main compartment of each cuvette, bringing the final volume of each reaction mixture to 3 ml. In the aerobic system (Curve A), immediately after tipping in the enzyme, air was admitted into the cuvette and the oxidation of NADH was followed by recording the decrease in absorbance at 340 μm.

In the anaerobic system (Curve B), NADH oxidation was recorded for 10 min after tipping in the enzyme. Air was then admitted into the cuvette (arrow, Curve B) and the reaction was followed for an additional 5 min. Both reactions were studied at 30°.

### Table II
**Effect of flavins on restoration of enzyme activity**

Both reactions were studied at 30°. As might be anticipated, the oxidation of NADH under aerobic conditions paralleled the initial rate in the aerobic system (Fig. 3A). Immediately after tipping in the enzyme, air was admitted into the cuvette and the oxidation of NADH was followed by recording the decrease in absorbance at 340 μm. In the anaerobic system (Curves B), NADH oxidation was recorded for 10 min after tipping in the enzyme. Air was then admitted into the cuvette (arrow, Curve B) and the reaction was followed for an additional 5 min. Both reactions were studied at 30°.

### Oxygen Requirement
The oxygen requirement of the hydroxylase reaction was suggested by relating both the amount of 2,3-dihydroxyphenylpropionic acid formed and the rate of NADH oxidized to the presence or absence of air. In the presence of atmospheric oxygen, the formation of 2,3-dihydroxyphenylpropionic acid was increased 6-fold over that formed in the anaerobic system (Table III).

### Stoichiometry
The stoichiometry of the reaction was established as detailed in Table IV. It was found, at the end of 10 min, that for every mole of oxygen utilized, approximately 1 mole of melilotic acid and 1 mole of NADH were oxidized.
Concomitantly, 1 mole of 2,3-dihydroxyphenylpropionic acid and 1 mole of NAD were formed. The reaction can be depicted as follows.

\[
\begin{align*}
\text{MELILOTIC ACID} & \quad + O_2 + \text{NAD} + H^+ \quad \rightarrow \\
\text{2,3 DIHYDROXYPHENYLPROPIONIC ACID} & \quad + \text{NAD}^+ + H_2O
\end{align*}
\]

Cofactor Specificity—A number of enzymes similar to melilotate hydroxylase utilize reducing agents other than the pyridine nucleotides (14−17). In an attempt to determine the specificity of NADH as a cofactor of the hydroxylation, ascorbic acid, cysteine, glutathione, mercaptoethanol, 2-amino-4-hydroxy-6,7-dimethylytetrahydropyridine, and NADPH were substituted individually for NADH in reaction mixtures.

In no case when a reducing agent other than a pyridine nucleotide was present was there any evidence for the formation of 2,3-dihydroxyphenylpropionic acid (Table V). The amount of 2,3-dihydroxyphenylpropionic acid formed in the presence of NADPH was only about one-third of that found when NADH was used as the cofactor. The Michaelis constant (Km) for NADH, determined by the method of Lineweaver and Burk (18), is 9.1 × 10⁻⁸ M.

The addition of any of the non-pyridine nucleotide-reducing agents to reaction mixtures containing NADH had no effect on either the rate of oxidation of NADH or on the formation of 2,3-dihydroxyphenylpropionic acid.

Substrate Specificity—When tested with a number of compounds structurally related to melilotic acid, melilotate hydroxylase exhibited a remarkably high degree of specificity. Enzyme activity was assayed under standard conditions except that each substrate studied was substituted for melilotic acid, on a molar basis. Of the 22 aromatic substrates examined, enzyme activity was found only with m-hydroxyphenylpropionic acid (20%) and with phenylpropionic acid (1 to 2%). (The percentages indicate the extent to which NADH was oxidized in the presence of each substrate relative to that found with melilotic acid (100%).) The Michaelis constant (Km) for melilotic acid, determined by the method of Lineweaver and Burk (18), is 8.6 × 10⁻⁴ M.

Reversibility of Reaction—Repeated attempts to demonstrate the reversibility of the hydroxylation were unsuccessful. A typical reaction mixture contained, in a final volume of 3 ml, 2,3-dihydroxyphenylpropionic acid, 510 μmoles; NAD, 500 μmoles; phosphate buffer, pH 7.3, 10 μmoles; and enzyme, 12 units (Step 4; specific activity, 66.6). A control sample prepared in the same manner but containing boiled enzyme in place of the enzyme solution was incubated simultaneously.

At the end of 1 hour at 30°, there was no evidence in either mixture for the disappearance of 2,3-dihydroxyphenylpropionic acid or for the reduction of NAD.

Effect of pH—The variation of activity with pH for melilotate hydroxylase is shown in Fig. 4. Optimal activity was observed between pH 7 and 7.5. On the acid and alkaline sides of this region, a rapid decline in activity was noted.

Inhibitors—The incubation conditions and the method of assay were the same as in the standard assay system except that inhibitors were included in the reaction mixtures. The following substances were found to be inhibitory: p chloromercu-
The present work and the studies which preceded it (1, 2) suggest the following sequence of reactions in which coumarin is metabolized by *Arthrobacter*. Coumarin, hydrolyzed enzymatically between the oxygen and carbonyl carbon atoms of the pyrone ring, gives rise to o-trans-coumaric acid (2). (Since coumarin is, in fact, the lactone of cis-coumarinic acid shown in brackets in Scheme 1, a cis-trans isomerization presumably occurred about the double bond in the side chain of the aromatic ring.) The enzyme NADH:o-coumarate oxidoreductase reduces o trans coumaric acid to melilotic acid (1), which is then oxidized to 2,3-dihydroxyphenylpropionic acid by melilolate hydroxylase in the presence of atmospheric oxygen and NADH. The sequence is depicted in Scheme 1.

Kosuge and Conn (19) have shown that, in sweet clover, in contrast to the bacterial system, the initial attack on coumarin is a reduction to dihydrocoumarin. It is the subsequent hydrolysis of dihydrocoumarin by dihydrocoumarin hydrolase which gives rise to melilotic acid. There has been no report, to the best of our knowledge, of the hydroxylation of melilotic acid in higher plants. This type of reaction, however, may be fairly ubiquitous among bacteria, since a number of workers have reported the accumulation of both melilotic acid and 2,3-dihydroxyphenylpropionic acid in the culture media of organisms grown on cinnamic acid (20, 21). Presumably, the hydroxylation is mediated by an enzyme similar to or identical with melilolate hydroxylase.

Melilolate hydroxylase can be classified, according to the definition of Mason (22), as a mixed function oxidase, in that it requires both atmospheric oxygen and a reducing agent, NADH. Although the need for atmospheric oxygen was not definitively established by 18O incorporation studies, the results of the comparative study between aerobic and anaerobic systems clearly show a dependence of 2,3-dihydroxyphenylpropionic acid synthesis on atmospheric oxygen. Indeed, the fact that any 2,3-dihydroxyphenylpropionic acid was synthesized anaerobically probably means that not all the dissolved oxygen was completely removed from solution.

With respect to both its cofactor and to its substrate, the enzyme exhibits rather stringent specificity requirements. In no case, for example, could a reducing agent other than a pyridine nucleotide affect either the rate of hydroxylation of melilotic acid or the amount of 2,3-dihydroxyphenylpropionic acid formed. Similarly, with respect to the substrate, any deviation from the basic structure of melilotic acid resulted, with few exceptions, in the elimination of enzyme activity.

The flavin requirement of the enzyme, established after partial resolution of the hydroxylase with ammonium sulfate, appears specific for FAD. Other flavin requiring enzymes active in aromatic hydroxylations have been described recently. Salicylate hydroxylase, which catalyzes the stoichiometric conver-
sion of salicylic acid to catechol, also requires FAD (23). FMN has been shown to be a component of NADH oxidase, an enzyme which catalyzes the hydroxylation of diketocamphane (24–26). FMN in this system mediates the electron transfer through which NADH keeps the iron of the enzyme in the reduced or active state.

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