The Metabolism of Coumarin by a Microorganism. II. The Reduction of *o*-Coumaric Acid to Melilotic Acid

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An enzyme partially purified from extracts of a microorganism (Arthrobacter sp.) has been shown, in the presence of NADH, to catalyze the reduction of *o*-coumaric acid to melilotic acid. Melilotic acid, isolated as a crystalline product from a reaction mixture consisting of enzyme, NADH, *o*-coumaric acid, and buffer, was characterized by comparison of the physical properties of the isolated material with those of an authentic specimen. The conversion of *o*-coumaric acid to melilotic acid was found to be stoichiometric; i.e., for every mole of *o*-coumaric acid reduced, one mole of NADH was oxidized and one mole of melilotic acid was formed. The reverse reaction could not be demonstrated. The enzyme is highly specific for *o*-coumaric acid and has a Michaelis-Menten constant of 1.73×10^{-5}. Inhibition studies indicated sensitivity to known sulfhydryl inhibitors, particularly *p*-chloromercuribenzoate.

In a previous communication (Levy and Weinstein, 1964), it had been shown that a bacterium (Arthrobacter sp.) isolated from soil and utilizing coumarin as its sole carbon source produced L-tyrosine in the culture medium. A study to find possible intermediates in this conversion demonstrated that *o*-coumaric acid could be utilized as efficiently by the organism as coumarin (Levy and Weinstein, 1964). Moreover, alterations in the ultraviolet spectrum of coumarin observed during enzymatic attack suggested that *o*-coumaric acid was an intermediate in the sequential degradation of coumarin (Levy and Weinstein, 1963, unpublished).

In the present paper, a highly specific enzyme, partially purified from extracts of Arthrobacter, is described which can reduce *o*-coumaric acid to melilotic acid in the presence of NADH. In keeping with the suggested rules of nomenclature in the Report of the Commission on Enzymes of the International Union of Biochemistry (1961), NADH: *o*-coumarate oxidoreductase is proposed for its name.

MATERIALS

Coumarin and *p*-nitrocinnamic, *o*-nitrocinnamic, and *m*-nitrocinnamic acids were purchased from Eastman Organic Chemicals Company. Cinnamic acid, *p*-coumaric acid, *o*-coumaric acid, and dihydrocoumarin were obtained from K and K Laboratories, Inc. Caffeic, ferulic, and sinapic acids, as well as 7-hydroxycoumarin, were supplied by Mann Research Laboratories, Inc. Melilotic acid was prepared by heating (80°) dihydrocoumarin in 5 N NaOH for one hour. The mixture was reduced in volume by vacuum distillation, acidified with HCl, and extracted three times with ether. The combined ether extracts were evaporated and the melilotic acid crystallized out. This material, recrystallized twice from water, had a melting point of 87–88°. Alkaline hydrolysis of 7-hydroxycoumarin gave umbellic acid. NADH and NAD were purchased from Pabst Laboratories.

METHODS

The organism, cultured as described previously with coumarin as the sole carbon source, was collected by high-speed centrifugation and stored at −20°. Frozen cells could be stored for as long as 3 months with no appreciable loss in enzyme activity. Generally, 2 g (wet weight) of cells were collected per liter of culture medium.

Assay for NADH: o-Coumarate Oxidoreductase.—The enzyme was assayed spectrophotometrically, by the decrease in optical density of NADH at 340 mμ. One unit of activity is defined as the oxidation of one micromole of NADH per 5 minutes at 27° at *p*H 7.3. Protein was determined by the method of Lowry *et al.* (1951).

Paper Chromatography.—All paper chromatographic work was performed on Whatmann 3 MM paper employing the following solvents: (1) Methyl ethyl ketone–water–diethylamine (921:77:2); (2) 1-propanol–15 N ammonium hydroxide (7:3); (3) 20% aqueous KCl; (4) isopropanol–water–15 N ammonium hydroxide (8:1:1).

Enzyme Purification.—STEP I.—Ten grams (wet weight) of cells were suspended in 40 ml of a solution consisting of 0.5 M sucrose, 0.05 M phosphate buffer *p*H 7.3, and 0.001 M cysteine. The mixture was sonicated for 15 minutes (Biosonik, Bronwill Scientific) at 0°, and then clarified by centrifugation at 75,000 × *g* for 20 minutes. All subsequent steps in the purification procedure were carried out at 4°.

STEP II.—To 315 ml of the crude extract prepared in 40-ml batches, 63 ml of a 2% solution of protamine sulfate in 0.05 M phosphate buffer, *p*H 7.3, was added slowly with constant stirring. After standing for one-half hour, the mixture was centrifuged at 75,000 × *g* for 30 minutes and the relatively clear supernatant solution retained.

STEP III.—The enzyme solution (355 ml) obtained from Step II was fractionated by the addition of 74.5 g of solid $(NH_4)_2SO_4$. After one hour, the protein precipitate (0–30%) was removed by centrifugation at 75,000 × *g* for 20 minutes and discarded.

STEP IV.—Three hundred ml of aged calcium phosphate gel (25 mg/ml) was added to 300 ml of the enzyme solution obtained in Step III and the mixture kept at 4° for 30 minutes. After centrifugation (75,000 × g for 20 minutes) all the enzyme activity was in the supernatant solution.

STEP V.—Ninety-six grams of solid ammonium sulfate were added to 550 ml of the enzyme solution which was then stirred for an hour and centrifuged for 20 minutes at 75,000 × *g*. The precipitated protein, which was redissolved in 275 ml of 0.05 M phosphate buffer, *p*H 7.3, contained essentially all the activity. Because of the stability of the enzyme at this stage, even after 4 weeks of storage at −20°, all studies, unless stated otherwise, employed this preparation.

STEP VI.—Fifty ml of the enzyme obtained in Step V was dialyzed for $1^1/_2$ hours against two changes (4 liters) of 0.005 M phosphate buffer, *p*H 7.3. The dialyzed material was then adsorbed on a DEAE-cellulose column (15 × 1 cm). Protein was eluted by a gradient method in which the mixing chamber contained, initially, 300 ml of 0.05 M phosphate buffer (*p*H 7.3) and the reservoir contained 500 ml of 0.1 M phosphate buffer, *p*H 7.3. Both solutions also contained 10^{-3} M cysteine. The eluate was collected in 10-ml fractions, and the fractions contained in tubes 26 through 28, having essentially all the activity, were combined. This preparation retained approximately 50% of its original activity after a week of storage at −20°.

The purification steps are summarized in Table I.

TABLE I
SUMMARY OF PURIFICATION PROCEDURE

Step Fraction	Protein (mg/ml)	Activity (units/ml)	Specific Activity (units/mg)
I. Crude extract	15.3	2.2	0.14
II. Protamine sulfate	11.6	2.2	0.19
III. Amm. sulf. 0–30% sup.	6.1	2.0	0.33
IV. Calcium phosphate gel.	3.1	2.4	0.77
V. Amm. sulf. 0–25% ppt.	1.9	1.8	0.96
VI. DEAE-cellulose	0.04	0.12	2.8

Comments on Purification Procedure.—Considerably higher degrees of purification were obtained, at times, through chromatography on various Sephadex gels, particularly DEAE-Sephadex. Because of the extreme lability of these preparations and their general lack of reproducibility, they were not included in the routine purification procedure.

Although it was found necessary to dialyze preparations prior to chromatography on DEAE-cellulose columns, dialysis for more than 2 or 3 hours often caused considerable loss in enzyme activity. Attempts to restore this activity with a variety of cofactors administered singly or in combination proved unsuccessful. Nor were boiled crude extracts more effective.

The need for NADH as a cofactor for the enzyme was discovered when attempts at fractionation of crude extracts with ammonium sulfate resulted in total loss of enzyme activity. This could be restored, however, upon the addition of NADH.

Isolation and Characterization of Melilotic Acid.—Preliminary experiments with reaction mixtures consisting of NADH, *o*-coumaric acid, enzyme, and phosphate buffer had established that *o*-coumaric acid was reduced to a product which, upon paper chromatography, had the same R_F as authentic melilotic acid in four different solvent systems (methyl ethyl ketone–water–diethylamine (R_F = 0.28), 1-propanol–ammonium hydroxide (R_F = 0.70), 20% KCl (R_F = 0.82) and isopropanol–ammonium hydroxide–water (R_F = 0.60)).

To establish beyond doubt that the product of the reaction was melilotic acid, a reaction mixture on a much larger scale was employed. This mixture, in a 500-ml distillation flask, consisted of 606 μmoles (100 mg) of *o*-coumaric acid, 757 μmoles (500 mg) of NADH, 190 mg of enzyme protein (100 ml), 5 mmoles of phosphate buffer, *p*H 7.3, and 100 ml of water. The flask was kept at 30° for 6 hours and, when *o*-coumaric acid could no longer be detected spectrophotometrically, was immersed in a boiling water bath for 20 minutes.

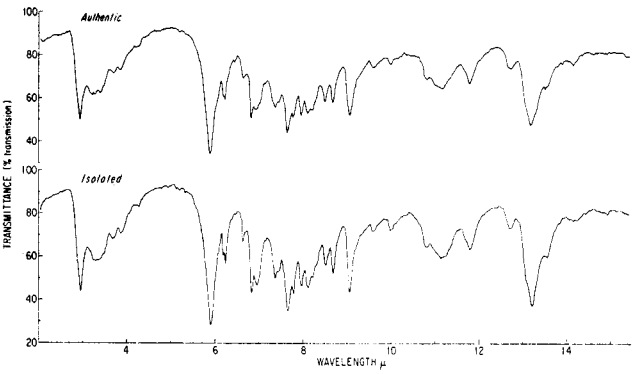


FIG. 1.—Comparison of the infrared absorption spectra of the isolated compound and of an authentic sample of melilotic acid; 0.2% in KBr, 1 to 2 mm thick uncorrected for the absorption of KBr.

Denatured protein was removed by centrifugation and the supernatant solution was reduced in volume to approximately 50 ml and acidified to *p*H 1. The product of the reaction was extracted five times with equal volumes of diethyl ether and the combined extracts (250 ml) were taken to dryness on a water bath at 40°. The residue, consisting of melilotic acid and trace amounts of *o*-coumaric acid, was shaken three times with warm (30–35°) 30-ml portions of chloroform (*o*-coumaric acid is insoluble in chloroform). The combined chloroform extracts were filtered, reduced in volume to 10 ml and placed in a refrigerator overnight. The crystalline material which separated out was collected by centrifugation and recrystallized from chloroform. It was then dissolved in warm water (15 ml) and heated (60–70°) with a small amount of charcoal (Darco-G-60 Atlas Powder Co.). After filtration through a sintered glass funnel, the clear filtrate was placed in the cold overnight. The crystalline product was collected by centrifugation and dried in a vacuum desiccator over concentrated H_2SO_4. Yield: 22.2 mg of colorless prisms, mp 87–88°. No depression was observed in a mixed melting point determination with authentic melilotic acid. The isolated compound cochromatographed with an authentic sample of melilotic acid in two solvent systems, giving a single spot in each system (20% KCl [R_F = 0.80] and isopropanol–ammonium hydroxide–water [R_F = 0.60]). The ultraviolet absorption spectra of both the authentic and isolated compounds were identical when compared in acid and alkaline solutions. Finally, the infrared absorption spectra of both compounds were identical (Fig. 1). Based on the evidence presented above, the isolated product of the reduction of *o*-coumaric acid is considered to be melilotic acid (*o*-hydroxyphenylpropionic acid).

Stoichiometry.—Two methods were used to study the stoichiometry of the reduction of *o*-coumaric acid.

A. The reaction mixtures were prepared by introducing into each of two 50-ml distillation flasks 0.4 mg of enzyme protein (0.25 ml), 4.5 μmoles of NADH (1.5 ml), 5.1 μmoles of *o*-coumaric acid (1.5 ml), and 580 μmoles of phosphate buffer, *p*H 7.3 (11.75 ml). Both vessels were incubated in a water bath at 27°. The oxidation of NADH was followed spectrophotometrically, in aliquots of the reaction mixtures, by recording the decrease in optical density at 340 mμ. At the end of 5 and 10 minutes, respectively, both reactions were stopped by immersion of the flasks in a boiling water bath. The reaction mixtures were reduced to a small volume and chromatographed (overnight) on Whatmann 3 MM paper using isopropanol–water-

TABLE II

STOICHIOMETRY OF THE ENZYMATIC REDUCTION OF *o*-COUMARIC ACID[a]

Reaction Time (min)	*o*-Coumaric Acid Present Initially (μmoles)	*o*-Coumaric Acid Recovered (μmoles)	*o*-Coumaric Acid Reduced (μmoles)	NADH Oxidized (μmoles)	Melilotic Acid Recovered (μmoles)
5	5.10	4.32	0.78	0.68	0.73
10	5.10	3.92	1.18	1.10	1.17

[a] See text for experimental conditions.

TABLE III

RELATIONSHIP BETWEEN *o*-COUMARIC ACID AND NADH[a]

Experiment	Optical Density Change at 340 mμ (*p*H 7.3)	NADH Oxidized (mμmoles/ml)	Optical Density Change at 322 mμ (*p*H 1.0)	*o*-Coumaric Acid Reduced (mμmoles/ml)
1	−0.130	21.0	−0.187	21.2
2	−0.130	21.0	−0.192	21.8

[a] See text for experimental details.

ammonium hydroxide as solvent. The residual *o*-coumaric acid, as well as the newly formed melilotic acid, were eluted with water and their amounts determined, spectrophotometrically, using the following molecular extinction coefficients: for *o*-coumaric acid: $\epsilon_{pH\ 7.3}^{308\ m\mu} = 7400$; for melilotic acid: $\epsilon_{pH\ 7.3}^{270\ m\mu} = 2000$. (Chromatographic experiments with known amounts of *o*-coumaric acid revealed that approximately 95% of the acid could be recovered from the paper, whereas melilotic acid recovery was somewhat more complete, varying between 98 and 100%.) The amount of *o*-coumaric acid utilized represents the difference between the amount of the acid present initially and the amount recovered after the specific incubation period (Table II). It is apparent that, for every mole of *o*-coumaric acid reduced, one mole of NADH is oxidized and one mole of melilotic acid is formed. In the absence of enzyme, or with boiled enzyme, no reaction occurred.

B. A reaction mixture consisting of the following was placed in a 3-ml cuvet: 0.16 of enzyme protein (0.1 ml); 230 mμmoles of *o*-coumaric acid (0.1 ml); 453 mμmoles of NADH (0.2 ml); and 130 μmoles of phosphate buffer, *p*H 7.3 (2.6 ml). Simultaneously, a reference cuvet was prepared containing the entire reaction mixture with the exception of NADH. Both cuvets were placed in the cell holder of a Beckman DK-2A Ratio Recording Spectrophotometer and allowed to equilibrate for 15 seconds, and the decrease in optical density at 340 mμ was recorded. At the end of one minute the reaction was stopped with 0.15 ml of 2 N HCl. To determine the amount of *o*-coumaric remaining in the sample cuvet, a second reference mixture was prepared which differed from the original reaction mixture in that it did not contain *o*-coumaric acid but did contain NADH and, in addition, 0.15 ml of 2N HCl. By balancing this second reference cuvet against the sample it was possible to determine the decrease in optical density of *o*-coumaric acid and thus the amount of acid reduced. For *o*-coumaric acid: $\epsilon_{0.1\ N\ HCl}^{322\ m\mu} = 8800$. In Table III are summarized the results of two experiments using this method. It is indicated quite clearly that for every mole of *o*-coumaric acid reduced, one mole of NADH is oxidized.

Specificity.—The enzyme was examined for its activity against a number of substrates structurally related to *o*-coumaric acid. The activity of the enzyme toward *o*-coumaric acid is considered to be 100%, so that the activities of the enzyme toward other substrates (Table IV) are expressed as a percentage of that activity. Examination of Table IV reveals that the enzyme is quite specific for *o*-coumaric acid.

TABLE IV

SPECIFICITY OF NADH : *o*-COUMARATE OXIDOREDUCTASE[a]

Compound	Activity (%)
Cinnamic acid (*trans*-benzenepropenoic acid)	0.4
o-Coumaric acid (*o*-hydroxycinnamic acid)	100
p-Coumaric acid (*p*-hydroxycinnamic acid)	1.4
o-Nitrocinnamic acid	0
m-Nitrocinnamic acid	0
p-Nitrocinnamic acid	0
Umbellic acid (2,4-dihydroxycinnamic acid)	1.6
Caffeic acid (3,4-dihydroxycinnamic acid)	0.4
Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	0.6
Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid)	0
Coumarin (*o-cis*-coumarinic acid lactone)	0

[a] The reaction mixture consisting of 250 Mμmoles of substrate (0.1 ml), 450 mμmoles of NADH (0.1 ml), 0.16 mg of enzyme protein (0.1 ml), and 135 μmoles of phosphate buffer, *p*H 7.3 (2.7 ml), was placed in a 3-ml cuvet. The reference cuvet contained the entire reaction mixture with the exception of NADH. The oxidation of NADH was followed by the decrease in optical density at 340 mμ, over a 5-minute period.

Specificity of NADH as the Enzyme Cofactor.—Substitution of an equimolar amount of NADPH for NADH in a reaction mixture resulted in a 70% decrease in the rate at which *o*-coumaric acid was reduced.

Reversibility of the Reaction.—Repeated attempts to demonstrate the reverse reaction, i.e., the conversion of melilotic acid to *o*-coumaric acid, were unsuccessful. The enzyme, melilotic acid, NAD, and buffer were incubated under varying conditions and for varying lengths of time, but in no case could reduction of NAD be shown, nor was there any indication of oxidation of melilotic acid.

Inhibition Studies. Although a number of well-known inhibitors were examined for their ability to block the reduction of *o*-coumaric acid, the only effective compounds appeared to be those known for their effects on sulfhydryl groups (Table V). Thus, for example, *p*-chloromercuribenzoate at a concentration of 3.3×10^{-4} M completely inhibited the reaction.

Michaelis-Menten Constant.—The K_m for the enzyme-substrate complex was determined using the Lineweaver-Burk method of analysis (Lineweaver and

TABLE V
EFFECT OF VARIOUS INHIBITORS ON NADH : o-COUMARATE OXIDOREDUCTASE ACTIVITY[a]

Inhibitor	Concentration (M)	% Inhibition
Potassium cyanide	3×10^{-3}	0
Sodium azide	3×10^{-3}	0
Sodium fluoride	3×10^{-3}	0
Sodium arsenate	3×10^{-3}	0
Iodoacetate	3.3×10^{-3}	29
Iodoacetate	6.7×10^{-4}	0
Iodoacetamide	3.3×10^{-3}	15
Iodoacetamide	6.7×10^{-4}	0
N-Ethyl maleimide	3×10^{-3}	63
p-Chloromercuribenzoate	3×10^{-3}	100
p-Chloromercuribenzoate	3.3×10^{-4}	37

[a] The reaction mixture, prepared in a 3-ml cuvet, consisted of 0.16 mg of enzyme protein (0.1 ml), 300 mμmoles of *o*-coumaric acid (0.1 ml), 450 mμmoles of NADH (0.2 ml), 0.1 ml of inhibitor (having the concentration in the cuvet as shown), and 125 μmoles of phosphate buffer, *p*H 7.3. A reference cuvet contained the entire reaction mixture with the exception of NADH. The reaction was followed over a 5-minute interval at 27° by recording the decrease in optical density at 340 mμ. Enzyme activity in the absence of inhibitor is considered to be 100%, so that

$$\% \text{ inhibition} = \frac{100 - \text{enzyme activity in presence of inhibitor} \times 100}{100}$$

Burk, 1934). This value was found to be 1.73×10^{-5} M.

pH Optima.—The enzyme exhibits optimal activity over a rather wide range of *p*H between 6.8 and 8.5 (Fig. 2).

Metal Requirements.—The 20-fold purified preparation was used to study metal requirements of the enzyme. At the present stage of purification, no requirement could be shown for Mg^{++}, Mn^{++}, or Ca^{++}.

DISCUSSION

The degradation of coumarin has been studied both in plant and animal systems. Kosuge and Conn (1959) have observed the rapid conversion of coumarin to melilotic acid in sweet clover. They postulate that coumarin is reduced to dihydrocoumarin, the pyrone ring of which is then opened in a second step to form melilotic acid (Kosuge and Conn, 1962). They have, in fact, partially purified an enzyme from sweet clover (dihydrocoumarin hydrolase) which catalyzes this ring-opening reaction (Kosuge and Conn, 1962). On the other hand, evidence obtained from the isolation of various metabolites in the urine of the rat (Both *et al.*, 1959) and of the rabbit (Both *et al.*, 1959; Furuya, 1958) both fed on a diet of coumarin have suggested that *o*-coumaric acid is an intermediate between coumarin and melilotic acid. In neither of these studies, however, has direct enzymological evidence of this transformation been offered. Thus, it appears that there are two possible pathways for the conversion of coumarin to melilotic acid; one via the postulated intermediate of dihydrocoumarin, and the other via *o*-coumaric acid. In our own work, degradative studies of coumarin with crude extracts of Arthrobacter sp. have given rise to a compound whose ultraviolet spectrum closely resembles that of *o*-coumaric acid. Moreover, repeated attempts to demonstrate the enzymatic conversion of dihydrocoumarin to melilotic acid with various enzyme preparations obtained from this organism have been unsuccessful.

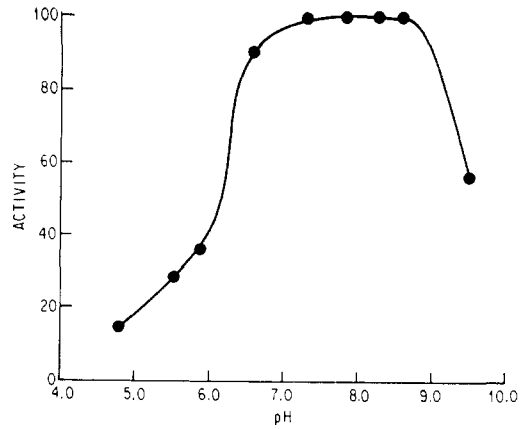
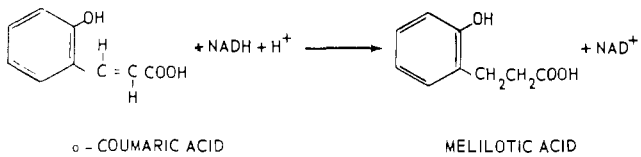


FIG. 2.—*p*H optima curve of NADH–*o*-coumarate oxidoreductase. The reaction mixtures were buffered from *p*H 4.8 to *p*H 8.0 with 0.1 M phosphate buffer and from *p*H 8.5 to *p*H 9.5 with 0.1 M Tris-HCl buffer.

In the present study, NADH: *o*-coumarate oxidoreductase partially purified from bacterial extracts has been shown to catalyze the conversion of *o*-coumaric acid to melilotic acid in accordance with the equation given.



It should be stressed that the enzyme, highly specific for *o*-coumaric acid, was obtained from an organism grown on coumarin as the only carbon source. This, together with the fact that enzymatic hydrolysis of dihydrocoumarin could not be demonstrated in this organism, strongly suggests that *o*-coumaric acid is an intermediate in the conversion of coumarin to melilotic acid. This conversion is presently under investigation.

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