Enzyme Reactions with Phenolic Compounds: Formation of Hydroxystyrenes through the Decarboxylation of 4-Hydroxycinnamic Acids by Aerobacter*


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A constitutive enzyme present in strains of Aerobacter has been found to decarboxylate phenylacrylic (cinnamic) acids possessing a 4-hydroxy group to the corresponding hydroxystyrenes nonoxidatively. Two such products, 4-hydroxystyrene (4-vinylphenol) and 3,4-dihydroxystyrene (4-vinylcatechol) from p-coumaric and caffeic acids, respectively, were purified and identified. Only 4-hydroxystyrene has been previously reported from natural sources as a rare constituent of some higher plants (1, 2).

The decarboxylative enzyme, tentatively called 4-hydroxy cinnamic decarboxylase on the basis of its specificity, has been extracted, and some properties of the intracellular and solubilized enzyme are described.

EXPERIMENTAL PROCEDURE

Chlorogenic acid was isolated from green coffee beans (3). Caffeic and p-coumaric acids (4) were synthesized from protocatechuic acid (5) and 4-hydroxybenzaldehyde, respectively, and recrystallized from water. Protocatechuic, dimethoxycinnamic, piperonylacyclic, and 4-amino cinnamic acids were also synthesized. 4-Methoxystyrene and 3,4-dimethoxystyrene were prepared by decarboxylation of the corresponding cinnamic acids (6). o- and m-Coumaric, ferulic, isoferulic, and sinapic acids, 7-hydroxycoumarin, 4-hydroxypropionophene, and the 4-hydroxy compounds of benzoic, phenylacetic, phenylpropionic, and identified. Only 4-hydroxystyrene has been previously reported from natural sources as a rare constituent of some higher plants (1, 2).

The decarboxylative enzyme, tentatively called 4-hydroxy cinnamic decarboxylase on the basis of its specificity, has been extracted, and some properties of the intracellular and solubilized enzyme are described.

* A preliminary account of this work was presented before the American Society of Biological Chemists in Atlantic City, New Jersey, April 1961.
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maximal centrifugal force of 144,000 x g for 1 hour did not remove any significant activity.

Assay—Changes in the absorption curve of dilutions of culture supernatants or of whole culture suspensions gave preliminary indications of caffeic acid decomposition by cultures grown with 0.1% caffeic acid. When a preliminary purification of culture mixture components was desired, an appropriate aliquot was chromatographed on paper, and the cut out ultraviolet fluorescent spots were eluted in 5 ml of 70% ethanol and measured spectrophotometrically. Chromatography of caffeic acid in n-butanol-acetic acid-water (4:1:2.2; RF = 0.8) and elution in this manner yielded approximately 80% recovery.

The routine enzyme assay mixture contained 10 nmoles of phenolic acid and 27 nmoles of potassium phosphate buffer, pH 6.1, in 0.4 ml at room temperature, to which was added 0.2 ml of a concentrated cell suspension or cell-free extract in 0.02 M phosphate, pH 6.1, at zero time. Aliquots of 5 µl were withdrawn at intervals and either diluted directly to 5.0 ml with cold water and measured spectrophotometrically or spotted on paper and measured after chromatography and elution as described above. Thymol, which had no detectable effect on enzyme activity, was added during long incubations to reduce bacterial contamination.

Dry weight of cells was determined after 16 hours in a 60° vacuum oven. Nitrogen content of the cells used in most of the experiments was 11.8% of the dry weight, determined by a micro-Kjeldahl method. The quantity of cells used in an experiment was determined by measuring turbidity in an Evelyn colorimeter with a No. 515 green filter.

Nuclear magnetic resonance spectrometry was performed as described in the "Addendum:"

RESULTS

Decarboxylation of Caffeic Acid—When washed A. aerogenes B-2614 cells were incubated with caffeic acid, a new product was detected, either as a dark blue fluorescent spot on paper (254 nm peak ultraviolet lamp) or as a shift in the absorption spectrum of the supernatant, shown in Fig. 2A. The product had a prominent peak at 258 nm. When chlorogenic acid (5-caffooylquinic acid) was added to a cell suspension, caffeic acid appeared (detected by paper chromatograph) and then disappeared with the formation of the new product.

The decomposition of caffeic acid was accompanied by evolution of carbon dioxide. This was measured manometrically and titrimetrically in separate experiments. Cell suspensions reacting with caffeic acid in air in Warburg flasks showed a vigorous evolution of carbon dioxide, with minor oxygen consumption. When measured anaerobically, gas evolution approached 1 mole of carbon dioxide per mole of substrate (Fig. 3). No gas evolution occurred with cell suspensions heated for 10 minutes at 100°. Automatic titration at pH 6.2 (30°) indicated a decrease in acidity of the reaction solution. In air, enzyme became inactivated during the course of the reaction, but under nitrogen the enzyme was relatively stable. Per mole of substrate, 1.2 equivalents of acid were used up, suggesting decarboxylation. Nonoxidative decarboxylation of caffeic acid would yield 3,4-dihydroxystyrene.

\[
\begin{align*}
&\text{HO} \\
&\text{CH} = \text{CH}-\text{COOH} \quad \text{HO} \\
&\text{HO} - \text{CH} = \text{CH} - \text{COOH} - \text{HO} \\
&\text{CH} = \text{CH} + \text{CO}_2
\end{align*}
\]

As a further check on the extent of decarboxylation, 2 µmoles of caffeic acid-α-C¹⁴ (containing 0.027 µc) were added to the reaction mixture. Liberated carbon dioxide was trapped in potassium hydroxide solution, and its radioactivity was deter-
Decarboxylation of 4-Hydroxycinnamic Acids

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HOURS

FIG.

3. Gas evolution from 5 pmoles of sodium caffeate tipped into 8.3 mg, dry weight, of cells suspended in 140 pmoles of phosphate buffer, pH 6.1, in a nitrogen atmosphere at 30°. Total volume, 2.8 ml. Arrow designates addition of substrate.

<table>
<thead>
<tr>
<th>Substrate acid</th>
<th>Ring substitution</th>
<th>Gas evolution (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 min.</td>
</tr>
<tr>
<td>Cinnamic</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>o-Coumaric</td>
<td>2-Hydroxy</td>
<td>0</td>
</tr>
<tr>
<td>m-Coumaric</td>
<td>3-Hydroxy</td>
<td>0</td>
</tr>
<tr>
<td>p-Coumaric</td>
<td>4-Hydroxy</td>
<td>64</td>
</tr>
<tr>
<td>Caffeic</td>
<td>3,4-Dihydroxy</td>
<td>33</td>
</tr>
<tr>
<td>Ferulic</td>
<td>3-Methoxy-4-hydroxy</td>
<td>11</td>
</tr>
<tr>
<td>Isoferulic</td>
<td>3-Hydroxy-4-methoxy</td>
<td>0</td>
</tr>
<tr>
<td>Sinapic</td>
<td>3,5-Dimethoxy-4-hydroxy</td>
<td>0</td>
</tr>
</tbody>
</table>

mined in a Nuclear-Chicago Dynacon ionization chamber. No radioactive carbon dioxide (less than 1% of the total counts added) was found after completion of the reaction under either aerobic or anaerobic conditions.

Substrates for Reaction—Other compounds were also tested as possible substrates. The relative decarboxylation rates of several cinnamic acids are indicated in Table I. Enzyme activity requires a relatively unhindered 4-hydroxy group on the aromatic ring and an acrylic acid side chain. The following compounds showed no carbon dioxide production over a 30-minute period or longer: 3,4-dimethoxycinnamic acid, piperonyl-acrylic acid, 2,4-dihydroxycinnamic lactone (7-hydroxycoumarin), 4-hydroxypropiophenone, and cinnamaldehyde; several hydroxy acids (4-hydroxybenzoic, -phenylacetic, -phenylpropionic, -phenylactic, and -phenylpyruvic acids, 3,4-dihydroxy- benzoic acid, tyrosine, and 3,4-dihydroxyphénylaniline); and phthalic, fumaric, maleic, oxaloacetic, oxalic, and pyruvic acids. Maleic acid inhibited the decarboxylation of p-coumaric acid: 20 µmoles of maleic acid reduced the decarboxylation rate of 5 µmoles of p-coumaric acid by 75% in 0.05 M phosphate buffer, pH 6.1. 4-Hydroxybenzoeic, protocatechue, and fumaric acids were anaerobically decarboxylated, but only after a delay of 30 to 75 minutes, suggesting that enzyme formation was induced as reported in other bacteria (12).

The compounds listed in Table I also acted as substrates for the soluble enzyme preparation in approximately the same order of reactivity. The other compounds mentioned above were also tested with the soluble enzyme, and in addition, several other cinnamic acid derivatives (4-amino, 4-, 3-, and 2-nitro-, and 3-chloro- cinnamic acids), as well as cis- and trans-aconite, β-hydroxy-β-methyl-glutaric, mevalonic, urocanic, citraconic, meaconic, and itaconic acids. A cinnamic acid bearing a 4-hydroxy group on the ring appears to be an absolute requirement for activity by the soluble system as well as by whole cells. Additional substituents on the 4-hydroxybenzene ring interfere with the reaction, a 3-methoxy group (ferulic acid) more than a 3-hydroxy (caffeic acid); the 3,5-dimethoxy compound (sinapic acid) is inactive.

The cell-free enzyme inactivates in air but not under anaerobic conditions. The delayed activities against fumaric, p-hydroxybenzoic, and protocatechue acids noted with whole cell suspensions were not detected in the soluble system.

pH Optimum—The optimal pH (Fig. 4) was approximately 5.7 in either citrate or phosphate buffer when whole cells or cell-free extracts were used, with either p-coumaric or caffeic acid as substrate.

Identification of Product Substances

The product arising from enzymic decarboxylation of caffeic acid was used for the initial identification. Later it became evident that the product from p-coumaric acid had similar prop-
erties and, because it was more stable and more easily characterized, the latter was used for subsequent identification data.

**Product Characteristics**—Reaction solutions containing 1 to 2 
µmoles of p-coumaric, caffeic, or ferulic acid decarboxylation product per ml had pungent phenolic odors, each characteristic of the particular product. When the caffeic acid reaction mixture was chromatographed (Table II), a dark blue fluorescent product spot with the following properties of an o-dihydroxybenzene was obtained. Exposure of the paper to air caused the spot to darken within a few hours. Silver nitrate-ammonia spray resulted in an immediate blackening of the spot. Extracts in various aqueous and nonpolar solvents (exception: ethanol) showed a progressive decrease in absorption at 258 mp at room temperature. In sodium acetate solution, the 258 mp absorption peak showed a bathochromic shift of 7 mp upon addition of boric acid (13). As would be expected, p-coumaric and ferulic acid decarboxylation products did not show o-dihydroxybehavior.

The product compounds were not carboxylic acids, since they were easily extracted from slightly alkaline solutions with ether and did not show a hypsochromic shift in the absorption curve on the addition of sodium acetate solution (14).

**Large Scale Separation of Product from Caffeic Acid; Characteristics**—Caffeic acid (600 mg) was dissolved hot in 25 ml of 0.09 M tribasic potassium phosphate, and 4.8 ml of 1 M potassium dihydrogen phosphate were then added to a final pH of 5.8. A cold suspension (11 ml) containing 1.45 g, dry weight, of B-2614 cells, flushed with nitrogen gas, was added. Nitrogen flushing was continued during 4 hours of incubation at room temperature. The incubated suspension was centrifuged in the cold, the supernatant liquid was removed, and the cells were washed with 10 ml of water. A second 4-hour incubation of the combined supernatants followed, after a new batch of bacterial suspension had been added. Chromatography of the second incubation supernatant revealed only traces of caffeic acid and other fluorescent substances. The supernatant liquid was stored frozen.

The major component of the supernatant was purified by LKB Chromax paper column chromatography. A column, at 1° under a flow of nitrogen, was washed with 5% acetic acid (boiled to remove air). The supernatant was also made 4% in acetic acid (a precipitate was centrifuged off) and applied to the column. Effluent fractions were monitored by fluorescence of dried aliquots on paper and paper chromatography of the peak fractions. The latter were brought to pH 6 with sodium hydroxide and extracted with cold ether, and volume was reduced under vacuum.

Since the new product has not been described previously as a naturally occurring compound, its observed properties will be recorded in some detail. Difficulties were encountered in concentrating the unknown product, in that vacuum evaporation of the ether solution to dryness at 0° produced a white, insoluble residue (polymer). Vacuum drying, at room temperature, of the Chromax column peak fractions of unknown material (4% in acetic acid) gave a different absorption curve, with a single peak at 282 mp and a shape very similar to that of ethyl hydrocaffeate, a compound that would, from its structure, be expected to absorb like the polymer of 3,4-dihydroxyxystrene. When, in an attempt to protect against transformation of the compound, the peak fractions were lyophilized in a -11° cold room, with liquid nitrogen in the cold trap, the unaltered 282 mp peak com-

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>System*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.8</td>
</tr>
<tr>
<td>Caffeic product</td>
<td>0.9</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.94</td>
</tr>
</tbody>
</table>

* A = n-butanol-acetic acid-water (4:1:2.2); B = 4% acetic acid; C = chloroform-acetic acid-water (2:1:1, lower phase); D = isopropanol-5% ammonia (4:1).

**FIG. 5.** Absorption spectra of 95% ethanol solutions. Solid line, synthetic 4-hydroxystyrene, 5.0 µg per ml; O, crystalline product from p-coumaric acid decarboxylation. Absorbance of the product from p-coumaric acid was made equal to that of the synthetic 4-hydroxystyrene at 290 mp.

**Crystaline Product from p-Coumaric Acid; Characteristics**—p-Coumaric acid (580 mg) was dissolved and treated with B-2614 cells as described for caffeic acid. The clear cell supernatant solution was extracted and recrystallized by the procedure of Dale and Hennis (7) for 4-hydroxystyrene.
The crystals were thin, shiny plates, unstable except at very cold temperatures (7). Product m.p., 69-70° (Kofler stage); synthetic 4-hydroxystyrene m.p., 70.5-72°; mixed m.p., 68-71°. Addition of a 1% solution of aqueous ferric chloride gave a green solution. Concentrated sulfuric acid gave yellow, then reddish, particles and solution. Concentrated hydrochloric acid and turned the particles an intense light (cornflower) blue. The spectrum in 95% ethanol (Fig. 5) shows an ultraviolet peak at 259 rnl.c.

Addition of a 1% solution of aqueous ferric chloride gave a green solution. Concentrated hydrochloric acid turned the particles an intense light (cornflower) blue. The spectrum in 95% ethanol (Fig. 5) shows an ultraviolet peak at 259 rnl.c.

Biological Properties of Enzyme

Constitutive Nature—The activity of B-2614 per unit weight of dry cells was approximately the same whether it was grown in shake flasks or fermenters, on glucose or sucrose medium (extracellular invertase was absent), for short or long periods, and with 5-fold variation in the amount of sugar fed in fermenters. The multiplication (a) from a slant washing to a and with 5-fold variation in the amount of sugar fed in fermenters. The multiplication (a) from a slant washing to a

Discussion

This paper describes the formation of hydroxystyrenes from 4-hydroxycinnamic acids through the activity of a bacterial decarboxylase. The enzyme is constitutive in several strains of the common genus, Aerobacter. Such decarboxylation reactions are perhaps not uncommon, particularly by the microorganisms present in forest litter and other decomposing ligneous materials in which hydroxycinnamic acid derivatives are present. The extreme lability of the bacterial decarboxylation products to both polymerization and oxidation would be a simple explanation of their not having been discovered earlier.

Hydroxystyrenes and styrenes have been reported in plants. Schmid and Karrer (1) characterized a crystalline compound derived from poppy straw as 4-hydroxystyrene, although they considered this as possibly an artifact of the preparative procedure. More recently, Hattori and Imaseki (2) have crystallized a glycoside of 4-hydroxystyrene from Viburnum furcatum leaves. The leaves contain a glycoside that can liberate free hydroxystyrene (15). p-Coumaric acid or other hydroxycinnamic acid derivatives occur in poppy straw (1) and, in fact, in most higher plants (16). They may be parent compounds of hydroxystyrenes. In contrast, by balsamic species of Liquidambar, Myroxylon, and Styrax, cinnamic acid and styrene—not the hydroxylated compounds—have been reported (17-19). The parallel occurrence of cinnamic acids and their corresponding styrenes suggests decarboxylase action and implies that careful analysis of plant tissues rich in polysubstituted hydroxycinnamic acids, e.g., caffeic acid, may disclose the presence of less stable hydroxystyrenes.

The Aerobacter enzyme is an anaerobic decarboxylase. Non-oxidative decarboxylation of acyclic α,β-unsaturated acids has been reported (20, 21). More closely analogous to the presently described decarboxylation is the formation of styrene from cinnamic acid by molds (22, 23) and yeast (24). The bacterial decarboxylation of hydroxyxycinnamic acids described by Whiting and Carr (25) goes by a different, reductive route involving a hydroxyphenylpropionic acid intermediate and a product saturated in the side chain. Neither cinnamic acid nor 4-hydroxyphenylpropionic acid was attacked by the decarboxylase described in the present paper. Another type of decarboxylation occurs during the biosynthesis of the vinyl side chains of the I and II rings of protoporphyrin. A propionic acid side chain is decarboxylated to a vinyl group; but, unlike the vinyl formation described here, the porphyrin decarboxylation is reported to proceed by an oxidative, concerted mechanism with no acrylic acid type of intermediate (26).
SUMMARY

1. A constitutive nonoxidative decarboxylase from *Aerobacter* species is described whose substrates are cinnamic acids bearing a 4-hydroxy group. p-Coumaric, caffeic, and ferulic acids (4-hydroxy, 3,4-dihydroxy, and 4-hydroxy-3-methoxycinnamic acids, respectively) are attacked in decreasing order of rate by *Aerobacter aerogenes* NRRL B-2614.

2. The products from p-coumaric and caffeic acids were established as hydroxystyrenes (4-vinylphenol and 4-vinylacetol). The products were identified by their characteristic properties and by nuclear magnetic resonance spectra and, in the case of the product from p-coumaric acid, by synthesis. Absorption spectra and the RF values of the hydroxystyrenes in four solvent systems are given. The decarboxylation product from ferulic acid, presumably 2-methoxy-4-vinylphenol, was not specifically characterized.

3. A cell-free extract of the enzyme showed similarity of substrate specificity and pH optimum to the cell-bound enzyme.

4. The presence and position of the 12-line spectrum characteristic of aromatic ether linkages was established from the known resonance of the benzylic hydroxyl in the spectra of the products described above. The compound was 3,4-dihydroxystyrene.

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## ADDENDUM

### LEWIS, J. C., and LAND, R. E.

#### Nuclear Magnetic Resonance Spectrometry

Proton magnetic resonance (PMR) spectra were obtained with a Varian high resolution spectrometer system operating at 90.0 megacycles per second. All compounds were run as very dilute solutions in either diethyl ether or acetone, each containing tetramethylsilane as an internal reference.

The product obtained from caffeic acid was dissolved in ether. Although this is not a choice solvent for PMR because of the multiple proton signals, the low yield and great instability of the product prohibited transfer manipulations. The product from p-coumaric acid was dissolved in acetone, whose single resonance lies outside the field region of interest in this study.

#### Proton Magnetic Resonance Data

**3,4-Dimethoxystyrene and Product from Caffeic Acid**—Only a barely adequate spectrum of the reaction product in diethyl ether could be obtained at maximal spectrometer sensitivity after maximal concentration of the solution. That the reaction product was a substituted styrene was shown unmistakably by the presence and position of the 12-line spectrum characteristic of a substituted ethylene (27). The close agreement in position of resonance centers (approximate shielding values) and in spin-coupling constants for these lines from the reaction product and from 3,4-dimethoxystyrene can be seen in Table III.

The positions and splitting of aromatic peaks often can be used to establish the nature and location of substituents on aromatic rings (28). The similarity in shape and position of the reaction product aromatic multiplets to those of 3,4-dimethoxystyrene, together with the known shielding effect of aromatic ether linkages, suggests that the ring substituents were not altered by the reaction. The presence of a hydroxyl resonance, even though the bands from the reaction product were too weak to determine the number of hydroxy substituents, makes it most likely that the 3,4-dihydroxy structure of the parent acid was retained and that the compound was 3,4-dimethoxystyrene.

**4-Hydroxystyrene and Product from p-Coumaric Acid**—Values from the spectrum of the product in acetone are shown in Table III together with those of 4-methoxystyrene. Again the spectrum of a substituted styrene is immediately apparent. In this case the aromatic methoxyl region could be scanned without solvent interference, and no peaks were observed. The aromatic band is entirely typical of an asymmetric p-disubstituted benzene. Unknown product and synthetic 4-hydroxystyrene gave identical spectra.

### REFERENCES


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