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Biosynthesis of *p*-Hydroxybenzoate from *p*-Coumarate and *p*-Coumaroyl-Coenzyme A in Cell-Free Extracts of *Lithospermum erythrorhizon* Cell Cultures[1]

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The enzymatic formation of *p*-hydroxybenzoate from *p*-coumarate in cell-free extracts of cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. was investigated. *p*-Coumaroyl-coenzyme A (*p*-coumaroyl-CoA) is the activated intermediate in this biosynthetic reaction. It is formed by an ATP-, Mg^{2+}-, and CoA-dependent 4-hydroxycinnamate:CoA ligase reaction. *p*-Coumaroyl-CoA is oxidized and cleaved to *p*-hydroxybenzoyl-CoA and acetyl-CoA in a thioclastic reaction in which NAD is an essential cofactor. These CoA esters are rapidly hydrolyzed to acetate and *p*-hydroxybenzoate, probably by thioesterases. The enzymes involved in the formation of *p*-hydroxybenzoate are soluble. *p*-Hydroxybenzaldehyde is not an intermediate in this conversion, and *S*-adenosylmethionine and uridine-5′-diphosphoglucose do not enhance formation of *p*-hydroxybenzoate in our system.

Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae) are capable of producing large amounts of the red naphthoquinone pigment shikonin (Tabata and Fujita, 1985). Feeding experiments have shown that cinnamate and *p*-hydroxybenzoate are intermediates in the biosynthetic pathway leading to shikonin (Schmid and Zenk, 1971; Inouye et al., 1979), whereby *p*-hydroxybenzoate is derived from cinnamate, presumably via *p*-coumarate. The direct formation of *p*-hydroxybenzoate from chorismate, as observed in bacteria, does not take place in *Lithospermum* (Heide et al., 1989).

Several attempts have been made to clarify the biosynthesis of C_6-C_1 compounds, such as *p*-hydroxybenzoate, from phenylpropanoid precursors. Zenk (1965) showed by in vivo feeding experiments that vanillin is derived from ferulic acid in *Vanilla planifolia* and that free vanillic acid is apparently not an intermediate in this reaction. He proposed a reaction mechanism analogous to the β-oxidation of fatty acids, followed by reduction of vanilloyl-CoA. Similar results were obtained from in vivo feeding experiments in other plants (El-Basyouni et al., 1964).

The β-oxidation hypothesis for the biosynthesis of hydroxybenzoic acids (Fig. 1) was given additional support after it was found that C_1 and C_2 of phenylpropanoid precursors are removed as acetate or acetyl-CoA (Vollmer et al., 1965). However, most of the subsequent in vitro studies in cell-free plant extracts (Alibert and Ranjeva, 1971; Kindl and Ruis, 1971; Alibert et al., 1972; Hagel and Kindl, 1975; French et al., 1976; Löffelhardt and Kindl, 1976; Yazaki et al., 1991; Schnitzler et al., 1992) showed that the enzymatic conversion of phenylpropanoid precursors to benzoic acids was not dependent on addition of ATP and CoA, which contradicts the β-oxidation mechanism.

Three independent in vitro studies carried out with potato tubers (French et al., 1976) and cell cultures of *Lithospermum* (Yazaki et al., 1991) and carrot (Schnitzler et al., 1992) presented evidence that *p*-hydroxybenzaldehyde is an intermediate in the biosynthesis of *p*-hydroxybenzoate. This aldehyde may be formed from *p*-coumarate or *p*-coumaroyl-CoA in a retro-aldol reaction (Fig. 1), and its enzymatic oxidation to *p*-hydroxybenzoate was shown in all three plant systems (French et al., 1976; Yazaki et al., 1991; Schnitzler et al., 1992). Thus, an alternative biosynthetic mechanism for the formation of *p*-hydroxybenzoate was proposed (Fig. 1) that is analogous to the formation of vanillin and vanillic acid from ferulic acid by the bacterium *Pseudomonas acidovorans* (Toms and Wood, 1970).

Recently, Funk and Brodelius (1990a, 1990b, 1992) postulated a different mechanism for the formation of vanillate in *V. planifolia*. They proposed a methylation of the 4-hydroxy group of the C_6-C_3 precursors, followed probably by a UDPG-dependent formation of glucosides as activated intermediates, which then undergo side chain degradation and demethylation to 4-hydroxybenzoic acids.

A recent study on the biosynthesis of the benzoyl moiety of cocaine proved that it is formed via the (*R*)- rather than the (*S*)-3-hydroxy-3-phenylpropanoic acid, but that study did not address the exact reaction mechanism (Bjorklund and Leete, 1992).

In the present study, we improved the assay system for the in vitro formation of *p*-hydroxybenzoate from *p*-coumarate and showed that this reaction proceeds via *p*-coumaroyl-CoA and *p*-hydroxybenzoyl-CoA, i.e. in a manner similar to the β-oxidation of fatty acids.

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Abbreviations: 4CL, 4-hydroxycinnamate:CoA ligase; CS, citrate synthase; MDCA, 3,4-(methylenedioxy)-cinnamic acid; SAM, *S*-adenosylmethionine; UDPG, uridine-5′-diphosphoglucose.

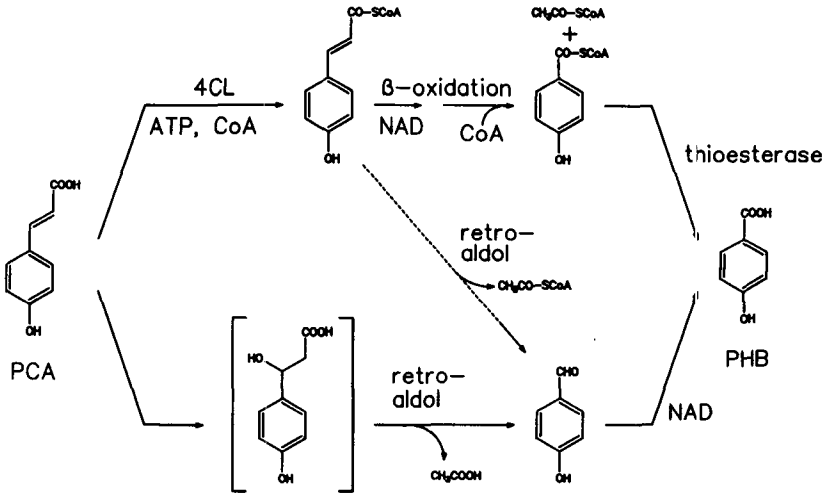

Figure 1. Proposed pathways for the biosynthesis of *p*-hydroxybenzoic acid. Upper pathway, β-oxidation mechanism; lower pathway: retro-aldol mechanism.

MATERIALS AND METHODS

Chemicals and Enzymes

L-[U-^{14}C]Tyr, sodium [2-^{14}C]acetate, and Phe ammonia-lyase from *Rhodotorula glutinis* were from Sigma (Deisenhofen, Germany); *p*-[carboxyl-^{14}C]hydroxybenzoic acid was from American Radiolabeled Chemicals (St. Louis, MO); catalase, CS, and acetyl-CoA synthase were from Böhringer (Mannheim, Germany); Polyamid SC6 was from Macherey & Nagel (Langerwehe, Germany); Multosphere RP18 was from Chromatographie Service (Düren, Germany); and Perisorb RP18 was from Merck (Darmstadt, Germany).

HPLC Methods

All separations were performed on a Multosphere RP18 5-μm column (250 × 4 mm) with a Perisorb RP18 30-μm precolumn (40 × 4 mm). The flow rate was 1 mL/min. ^{14}C-labeled compounds could be detected on line with a radioactive flow detector (HPLC radioactivity monitor LB 507 A with YG-150U4 flow cell, Berthold GmbH & Co., Wildbad, Germany).

Assay Analysis

For the analysis of free hydroxybenzoic acids, hydroxybenzaldehydes, and cinnamic acids, an isocratic solvent system was used: methanol:water:formic acid (20:79:1, v/v/v).

For the analysis of free acids and the corresponding CoA esters, different gradients with increasing concentrations of both acetonitrile (0–25%, v/v) and acetic acid (0–20%, v/v) in 1.5% (w/v) aqueous phosphoric acid were used.

The analysis of citrate and acetyl-CoA was performed by ion pair chromatography by a linear gradient of methanol (0–50%, v/v) in 2.5 mM aqueous KPi buffer (pH 6.8) containing 5 mM tetrabutylammonium hydrogen sulfate.

Purification of p-[U-^{14}C]Coumaroyl-CoA

The purification of *p*-[U-^{14}C]coumaroyl-CoA was performed by a linear gradient of acetonitrile (0–25%, v/v) in 1% (v/v) aqueous TFA.

Synthesis of *p*-Coumaroyl-CoA and *p*-Hydroxybenzoyl-CoA

p-Coumaroyl-CoA and *p*-hydroxybenzoyl-CoA were synthesized according to the method described by Stöckigt and Zenk (1975). The CoA esters were purified over Polyamid SC6 by elution with increasing concentrations of ammonia (0.025–0.075%, w/v) in methanol. Solvents were evaporated and the residues were dissolved in water. *p*-Coumaroyl-CoA was identified and quantified by UV spectroscopy and HPLC (see above) using authentic reference substance.

For *p*-hydroxybenzoyl-CoA, an alkaline esterolysis was performed using the method of Webster et al. (1974). The UV spectra before and after hydrolysis corresponded to the literature data (Webster et al., 1974). Quantification was achieved by complete alkaline hydrolysis followed by HPLC analysis of the free *p*-hydroxybenzoic acid.

Synthesis of *p*-[U-^{14}C]Coumaric Acid

p-[U-^{14}C]Coumaric acid was synthesized enzymatically from 50 μCi of L-[U-^{14}C]Tyr (512 Ci/mol) and 0.2 unit of Phe ammonia-lyase (grade I, Sigma) from *R. glutinis* in 1 mL of Tris-HCl buffer (0.1 M, pH 8.5). The assay was incubated at 30°C for 1 h and stopped by adding phosphoric acid to a final concentration of 0.1% (w/v). The *p*-[U-^{14}C]coumaric acid could be obtained in pure form by extraction with ethyl acetate (4 × 4 mL). The organic layers were combined and the solvent was evaporated. The residue was dissolved in 1 mL of water. The yield of *p*-[U-^{14}C]coumaric acid was 47 μCi (94%). Identity and purity were confirmed by HPLC (see above) with UV and/or radioactivity detection.

Synthesis of *p*-[^{14}C]Coumaroyl-CoA

p-[U-^{14}C]Coumaroyl-CoA was synthesized in the manner described for unlabeled *p*-coumaroyl-CoA (see above).

Synthesis of [^{14}C]Citrate and [^{14}C]Acetyl-CoA

[^{14}C]Citrate and [^{14}C]acetyl-CoA were enzymatically synthesized from sodium [2-^{14}C]acetate using CS and/or acetyl-CoA synthase.

(a) The assay for [^{14}C]citrate synthesis contained, in a final volume of 1 mL, 140 mM Tris-HCl (pH 8.4), 3 μCi of sodium [2-^{14}C]acetate (40–60 Ci/mol), 3.5 mM $MgCl_2$, 2.9 mM CoA, 2.9 mM ATP, 10.6 mM oxalacetic acid, 3.9 units of CS, and 0.1 unit of acetyl-CoA synthase. The reaction was performed at 30°C for 1 h and stopped by adding 100 μL of 1.5 M TCA.

(b) The assay for [^{14}C]acetyl-CoA synthesis contained the compounds mentioned above, but no oxalacetic acid or CS was added.

The identity of the products was confirmed by HPLC with UV and radioactivity detection using authentic unlabeled reference substances.

Identification of [^{14}C]Acetyl-CoA

Identification of [^{14}C]acetyl-CoA was performed by enzymatic conversion of the sample material to [^{14}C]citrate by CS reaction followed by HPLC analysis using an authentic reference substance. The assay contained Tris, $MgCl_2$, oxalacetic acid, and CS in the concentrations given above in a final volume of 100 μL. The reaction was performed at 30°C for 1 h and stopped by adding 10 μL of 1.5 M TCA.

Identification of *p*-[^{14}C]Hydroxybenzoyl-CoA

Identification of *p*-[^{14}C]hydroxybenzoyl-CoA was performed by alkaline hydrolysis according to the method of Webster et al. (1974) followed by HPLC analysis of free *p*-[^{14}C]hydroxybenzoic acid using an authentic reference substance.

Cell Culture

Cell cultures of *Lithospermum erythrorhizon* Sieb. et Zucc., strain M 18, were cultured in LS liquid medium (Linsmaier and Skoog, 1965) supplemented with 10^{-6} M IAA and 10^{-5} M kinetin. The medium (50 mL in a 300-mL Erlenmeyer flask) was agitated at 100 rpm on a rotary shaker at 25°C in the dark. Under these conditions, the cells produce no shikonin. Cells were subcultivated at intervals of 2 weeks. For enzyme induction, cells were transferred to fresh medium 7 d after inoculation and further incubated for 18 to 36 h prior to harvest.

Enzyme Extration and Assays

Cells (7.5 g fresh weight) were harvested and suspended in 10 mL of 0.1 M KPi buffer (pH 6.5) containing 10 mM DTT and 0.5 g of polyvinylpolypyrrolidone. They were ground in a mortar for 10 min and centrifuged at 20,000*g* for 10 min. The supernatant was desalted over a Sephadex G-25 column equilibrated with Tris-HCl buffer (0.2 M, pH 7.8) containing 2 mM DTT.

Unless described otherwise, enzyme activities were measured as follows:

The assay for *p*-hydroxybenzoate formation from *p*-coumarate contained, in a final volume of 100 μL of Tris-HCl (0.2 M, pH 7.8, containing 2 mM DTT and 20 mM sodium ascorbate): 0.4 to 30 mM unlabeled *p*-coumarate or 155,000 dpm of *p*-[U-^{14}C]coumarate (512 Ci/mol), 500 nmol of ATP, 500 nmol of $MgSO_4$, 50 nmol of CoA, 100 nmol of NAD, and 70 μL of cell-free extract (60–120 μg of protein).

The reaction was started by adding cell-free extract and the assays were incubated at 30°C for 20 min, unless described otherwise. The reaction was stopped by adding 10 of μL 1.5 M TCA. After centrifugation at 10,000*g* for 10 min, the supernatant was analyzed by HPLC using UV and radioactivity detection.

The formation of *p*-coumaroyl-CoA from *p*-coumarate (4CL reaction) was measured in an assay containing unlabeled *p*-coumarate or *p*-[U-^{14}C]coumarate, ATP, $MgSO_4$, CoA, and cell-free extract at the concentrations and using the conditions mentioned above.

The assay for in vitro formation of *p*-hydroxybenzoate from *p*-coumaroyl-CoA contained 20,000 dpm (4 Ci/mol) or 120,000 dpm (100 Ci/mol) of *p*-[U-^{14}C]coumaroyl-CoA and 100 nmol of NAD; all other conditions were as mentioned above.

Protein concentration was determined according to the method of Bradford (1976).

RESULTS

Improvement of Assay Conditions for the Enzymatic Formation of *p*-Hydroxybenzoate

Previous studies on the enzymatic formation of *p*-hydroxybenzoate were carried out using unlabeled *p*-coumarate as substrate (French et al., 1976; Yazaki et al., 1991; Schnitzler et al., 1992). To increase the sensitivity of the experiments, we synthesized *p*-[U-^{14}C]coumarate from [U-^{14}C]Tyr, using an enzyme preparation with Tyr ammonia-lyase activity (see "Materials and Methods").

When cell-free extracts of *L. erythrorhizon* were incubated with this labeled substrate, however, *p*-[U-^{14}C]coumarate was quickly decomposed to an unidentified polar product. The decomposition, which had been observed previously by Yazaki et al. (1991), is enzyme-catalyzed and substrate-specific for *trans*- rather than *cis*-*p*-coumarate (data not shown); it can be diminished by working under a nitrogen atmosphere. An earlier attempt to identify this polar product failed due to its instability (Yazaki et al., 1991). Catalase, sodium dithionite, and varying concentrations of sodium ascorbate in the assay reduced the decomposition of *p*-coumarate in the nonradioactive test (Fig. 2). Catalase and dithionite also reduced the formation of *p*-hydroxybenzoate, whereas ascorbate did not. Addition of ascorbate to a final concentration of 20 mM largely suppressed the observed decomposition to a polar product and enhanced the formation of *p*-hydroxybenzoate in the radioactive assay (Fig. 2).

Enzyme activity for *p*-hydroxybenzoate formation could be enhanced about 5-fold by transferring cells to fresh medium 18 to 36 h prior to harvest (data not shown). This method was used in the later experiments for the detection of intermediates between *p*-coumaroyl-CoA and *p*-hydroxybenzoate (see below).

Formation of *p*-[^{14}C]Hydroxybenzoate from *p*-[U-^{14}C]Coumarate

When *p*-[U-^{14}C]coumarate was incubated with cell-free extracts in the presence of ascorbate, ATP, CoA, Mg^{2+}, and NAD and analyzed by HPLC, a time-dependent formation

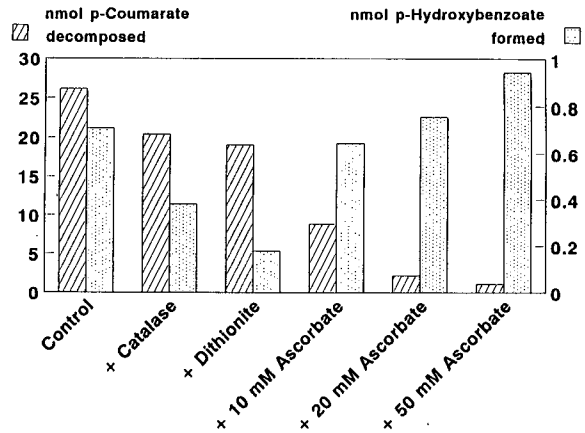

Figure 2. Decomposition of *p*-coumarate and formation of *p*-hydroxybenzoate under different assay conditions. Control, 40 nmol of *p*-coumarate were incubated for 1 h in the presence of the cofactors ATP, Mg^{2+}, CoA, and NAD and the cell-free extract (see "Materials and Methods"). The consumption of *p*-coumarate and the formation of *p*-hydroxybenzoate was measured by HPLC. Catalase (65 kilounits/mL), dithionite (50 mM), and ascorbate (10, 20, and 50 mM) were added to the assays.

of *p*-[^{14}C]hydroxybenzoate with *trans*-*p*-coumarate, but not *cis*-*p*-coumarate, as substrate was observed (data not shown). ATP, Mg^{2+}, CoA, and NAD are essential cofactors (Table I). The small amount of *p*-[^{14}C]hydroxybenzoate formed in the assay without CoA might be due to endogenous CoA in the enzyme preparation.

Under these improved assay conditions, the formation of *p*-hydroxybenzaldehyde in the absence of NAD, which had been reported by French et al. (1976), Yazaki et al. (1991), and Schnitzler et al. (1992), was not observed.

MDCA, an inhibitor of 4CL (Funk and Brodelius, 1990a), reduced the formation of *p*-hydroxybenzoate from *p*-coumarate (Table I), indicating that 4CL activity may be involved in this reaction.

UDPG and SAM were proposed to be cofactors in the formation of *p*-hydroxybenzoic acids from cinnamic acids in *V. planifolia* (Funk and Brodelius, 1992). In our assay, however, neither UDPG nor SAM increased *p*-hydroxybenzoate formation (Table I). The reduction of *p*-hydroxybenzoate formation in the presence of UDPG is due probably to UDPG-dependent glucosylation by UDPG:*p*-hydroxybenzoate glucosyltransferase (Bechthold et al., 1991). Hence, there is no indication that the reaction sequence leading from *p*-coumarate to *p*-hydroxybenzoate involves a methylation using SAM or a glycosylation using UDPG.

Formation of *p*-Hydroxybenzoate from *p*-Coumaroyl-CoA

It appears that the formation of *p*-hydroxybenzoate from *p*-coumarate proceeds via *p*-coumaroyl-CoA. To verify this hypothesis, we synthesized the CoA esters of unlabeled *p*-coumarate and of *p*-[U-^{14}C]coumarate (see "Materials and Methods") and tested these in our cell-free system.

Under identical assay conditions, i.e. in presence of ATP, CoA, Mg^{2+}, NAD, and ascorbate, the formation of *p*-hydroxybenzoate proceeded 3 times faster from *p*-coumaroyl-CoA than from free *p*-coumarate (0.79 compared to 0.26 nmol min^{-1} mg^{-1} protein in a nonradioactive assay).

In the radioactive assay, the formation of *p*-[^{14}C]hydroxybenzoate from *p*-[U-^{14}C]coumaroyl-CoA was linear with time for approximately 20 min (data not shown) in an assay containing ascorbate and NAD. As expected, the reaction was dependent on NAD (Table II). Addition of ATP, CoA and Mg^{2+} enhanced the formation of *p*-hydroxybenzoate by 14%. This effect might be due to a replenishment of the *p*-coumaroyl-CoA pool by the 4CL reaction, using free *p*-coumarate formed by the hydrolysis of *p*-coumaroyl-CoA (see below). Addition of CoA enhanced *p*-hydroxybenzoate formation only slightly. With NADP instead of NAD, product formation was reduced to 10% of the control.

The predominant metabolism of *p*-[^{14}C]coumaroyl-CoA, however, was not the formation of *p*-[^{14}C]hydroxybenzoate but rather the enzymatic hydrolysis to free *p*-[^{14}C]coumarate. In active enzyme preparations, up to 50% hydrolysis was

Table I. *Formation of p-[^{14}C]hydroxybenzoate from p-[U-^{14}C]coumarate and dependency on cofactors and inhibitors*

In the control assay, *p*-[U-^{14}C]coumarate (155,000 dpm) was incubated for 20 min at 30°C with ascorbate, ATP, Mg^{2+}, CoA, NAD, and cell-free extract (see "Materials and Methods"). Data are mean values ± SE of three replicates.

Assay	Formation of *p*-[^{14}C]Hydroxybenzoate	
	dpm	%
Control	19,020 ± 810	100
Heat-denatured extract	<300	<1.6
−NAD	<300	<1.6
−ATP	<300	<1.6
$-Mg^{2+}$	<300	<1.6
+CoA	1,000 ± 160	5
+10 nmol MDCA	13,940 ± 1,690	73
+40 nmol MDCA	7,500 ± 460	39
+500 nmol UDPG +500 nmol SAM	9,480 ± 920	50
+500 nmol UDPG	10,550 ± 1,210	55

Table II. *Formation of p-[^{14}C]hydroxybenzoate from p-[U-^{14}C]coumaroyl-CoA and dependency on cofactors*

In the control assay, *p*-[U-^{14}C]coumaroyl-CoA (20,000 dpm) was incubated for 20 min at 30°C with ascorbate, NAD, and cell-free extract (see "Materials and Methods"). Concentrations: ATP, 5 mM; Mg^{2+}, 5 mM; CoA, 0.5 mM; NADP, 1 mM. Data are mean values ± SE of three replicates.

Assay	Formation of *p*-[^{14}C]Hydroxybenzoate	
	dpm	%
Control	5510 ± 940	100
+ATP/+CoA/$+Mg^{2+}$	6270 ± 630	114
+CoA	5780 ± 370	105
−NAD	<300	<5.4
−NAD/+NADP	550 ± 60	10

observed within 5 min, whereas p-coumaroyl-CoA was stable in a heat-denatured extract (data not shown).

Since p-coumaroyl-CoA was hydrolized so quickly to free p-coumarate, presumably by thioesterases, the previous results did not completely exclude the possibility that free p-coumarate rather than p-coumaroyl-CoA is converted to p-hydroxybenzoate.

To clarify this point, we added unlabeled p-coumarate to an enzyme assay containing p-[U-^{14}C]coumaroyl-CoA of known specific radioactivity (8000 dpm/nmol), as well as ATP, Mg^{2+}, CoA, and NAD. During subsequent incubation, p-[^{14}C]hydroxybenzoate and, by thioesterase activity, p-[^{14}C]-coumarate were formed. The specific radioactivity of the labeled products was measured by HPLC. After 20 min of incubation, the specific radioactivity of the enzymatically formed p-[^{14}C]hydroxybenzoate was up to 10 times higher than that of the p-coumarate, which had been diluted by the added unlabeled p-coumarate (Table III). Since specific radioactivity cannot increase in such a bioconversion, this proves that the major part of the radioactivity in p-[^{14}C]hydroxybenzoate is directly derived from p-[U-^{14}C]coumaroyl-CoA and not via a free pool of p-[U-^{14}C]coumarate. In the control assay, the specific radioactivity of the p-[^{14}C]hydroxybenzoate (5730 dpm/nmol) is about seven-ninths of that of free p-[U-^{14}C]coumarate (7760 dpm/nmol), because two-ninths of the radioactivity per mol are lost by removal of two carbons. The specific radioactivity of the p-[^{14}C]hydroxybenzoate in the assays with addition of unlabeled p-coumarate is slightly lower than that of the control assay. This can be explained by the formation of p-hydroxybenzoate from unlabeled p-coumarate via the 4CL reaction (see below).

To provide further proof that p-hydroxybenzaldehyde is not an intermediate in p-hydroxybenzoate formation, we performed a similar experiment adding unlabeled p-hydroxybenzaldehyde. After 20 min of incubation, no detectable amount of radioactivity could be found in the p-hydroxybenzaldehyde (Table III), showing that a conversion of p-[U-^{14}C]-coumaroyl-CoA to labeled p-hydroxybenzaldehyde has not taken place. However, radioactivity was clearly detectable in p-hydroxybenzoate. This indicates that the radioactivity in p-[^{14}C]hydroxybenzoate is not derived from a free pool of p-hydroxybenzaldehyde. The specific radioactivity of p-[^{14}C]-hydroxybenzoate in assays containing p-hydroxybenzaldehyde is lower than that of the control assay, because unlabeled p-hydroxybenzaldehyde is enzymatically oxidized to p-hydroxybenzoate in a NAD-dependent reaction (Yazaki et al., 1991).

These results clearly demonstrate that the main pathway for the formation of p-hydroxybenzoate from p-coumarate proceeds via p-coumaroyl-CoA as activated intermediate. p-Hydroxybenzaldehyde is not involved in this biosynthesis under our experimental conditions.

Formation of p-Coumaroyl-CoA from p-Coumarate (4CL-Reaction)

If p-coumaroyl-CoA is the activated intermediate in p-hydroxybenzoate biosynthesis from p-coumarate, 4CL activity must be present. Indeed, the ATP-, Mg^{2+}-, and CoA-dependent formation of *trans*-p-coumaroyl-CoA from *trans*-p-coumarate could be detected by HPLC (data not shown). The reaction is rapid, but due to the presence of p-coumaroyl-CoA-hydrolyzing activity (presumably thioesterases), an equilibrium between synthesis and hydrolysis is reached within 10 min. The reaction is linear with time for approximately 5 min. Still, sufficient p-coumaroyl-CoA is formed by 4CL to explain the biosynthesis of p-hydroxybenzoate through this intermediate in the complete assay using free p-coumarate as substrate and ATP, Mg^{2+}, CoA, and NAD as cofactors.

Identification of Intermediates between p-Coumaroyl-CoA and p-Hydroxybenzoate

The results mentioned above prove that p-coumaroyl-CoA is the activated intermediate in the synthesis of p-hydroxybenzoate from p-coumarate. The removal of a two-carbon unit from p-coumaroyl-CoA, after oxidation, might be hydrolytic or thioclastic, i.e. either p-hydroxybenzoate or p-hydroxybenzoyl-CoA may result as the immediate product of the cleaving reaction. Furthermore, it remained to be shown that acetyl-CoA rather than free acetate is the C_2 compound that is removed. In the assays mentioned above, we found free p-hydroxybenzoate and free acetate as products in our HPLC analysis, but not the corresponding CoA esters. However,

Table III. *Formation of p-hydroxybenzoate, p-hydroxybenzaldehyde, and trans-p-coumarate from p-[U-^{14}C]coumaroyl-CoA in the presence of unlabeled p-coumarate and p-hydroxybenzaldehyde*

p-[U-^{14}C]Coumaroyl-CoA (40,000 dpm; 5 nmol) was incubated for 20 min at 30°C with ascorbate, ATP, Mg^{2+}, CoA, NAD, and cell-free extract (see "Materials and Methods"). Different amounts of unlabeled p-coumarate (PCA) or p-hydroxybenzaldehyde (PHAL) were added to the assay prior to incubation. Specific radioactivity of the products was analyzed by HPLC. Data are mean values of three replicates. Average SE was 9.5% of the mean value for radioactive data and 2.8% for nonradioactive data.

Assay	p-Hydroxybenzoate			*trans*-p-Coumarate			p-Hydroxybenzaldehyde		
	dpm	*nmol*	*dpm/nmol*	*dpm*	*nmol*	*dpm/nmol*	*dpm*	*nmol*	*dpm/nmol*
Control	5150	0.9	5730	17,840	2.3	7760	<300	<0.01	
+10 nmol PCA	4710	0.9	5230	18,870	8.0	2360	<300	<0.01	
+40 nmol PCA	4450	0.9	4940	19,760	42.3	470	<300	<0.01	
+5 nmol PHAL	4980	1.5	3320	19,910	2.5	7970	<300	1.3	<231
+20 nmol PHAL	4220	2.0	2110	18,280	2.3	7950	<300	18.6	<16

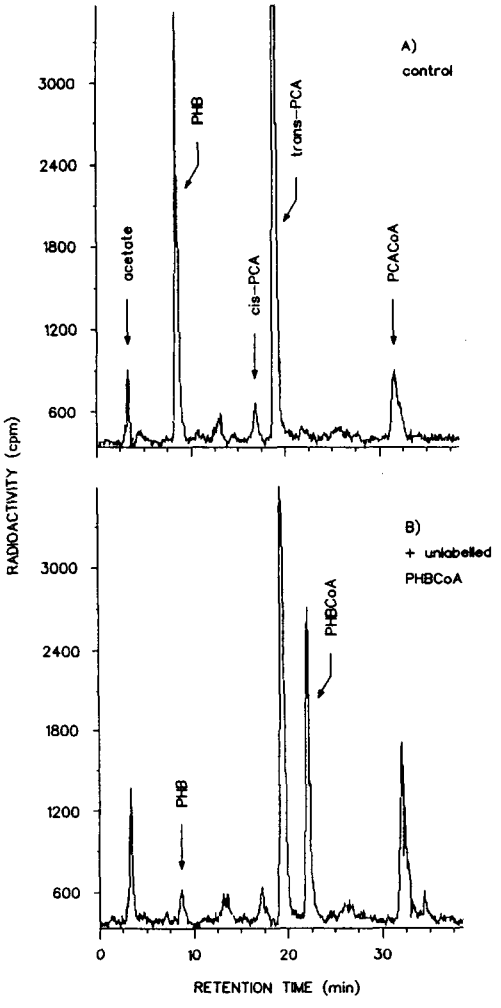

Figure 3. HPLC analysis of assays for *p*-[^{14}C]hydroxybenzoate formation from *p*-[^{14}C]coumaroyl-CoA. A, Native cell extract was incubated with *p*-[U-^{14}C]coumaroyl-CoA, ascorbate, and NAD for 5 min at 30°C. B, The same incubation as in A, but unlabeled *p*-hydroxybenzoyl-CoA (55 μM) was added. PHB, *p*-Hydroxybenzoate; PCA, *p*-coumarate; PCACoA, *p*-coumaroyl-CoA; PHBCoA, *p*-hydroxybenzoyl-CoA.

this fact could be due to high thioesterase activities hydrolizing the CoA esters before they could be detected. Indeed, fast enzymatic hydrolysis of externally added *p*-hydroxybenzoyl-CoA and acetyl-CoA in the extracts could be demonstrated (data not shown).

We synthesized *p*-hydroxybenzoyl-CoA (see "Materials and Methods") and added it to the assay for *p*-[^{14}C]hydroxybenzoate formation (from *p*-[U-^{14}C]coumaroyl-CoA) in an amount (55 μM) large enough to prevent complete hydrolysis during a short incubation time (5 min). Under these conditions, we were able to detect radioactive *p*-hydroxybenzoyl-CoA as product (Fig. 3), and the formation of free *p*-[^{14}C]-hydroxybenzoate was reduced by a corresponding amount.

The identity of *p*-[^{14}C]hydroxybenzoyl-CoA could be confirmed by alkaline hydrolysis of the isolated compound and identification of the resulting free *p*-[^{14}C]hydroxybenzoate by HPLC.

In an analogous experiment, we added an excess (5 mM) of unlabeled acetyl-CoA to an assay for the formation of *p*-[^{14}C]hydroxybenzoate from *p*-[U-^{14}C]coumaroyl-CoA. Under these conditions we were able to detect labeled acetyl-CoA, and the peak of free acetate was reduced by a corresponding amount (Fig. 4).

The enzymatically formed [^{14}C]acetyl-CoA was further identified after isolation by HPLC and enzymatic conversion to [^{14}C]citrate using the CS reaction. The latter compound was identified by ion pair HPLC (see "Materials and Methods").

To exclude that the observed products, *p*-[^{14}C]hydroxybenzoyl-CoA or [^{14}C]acetyl-CoA, were formed from the free acids

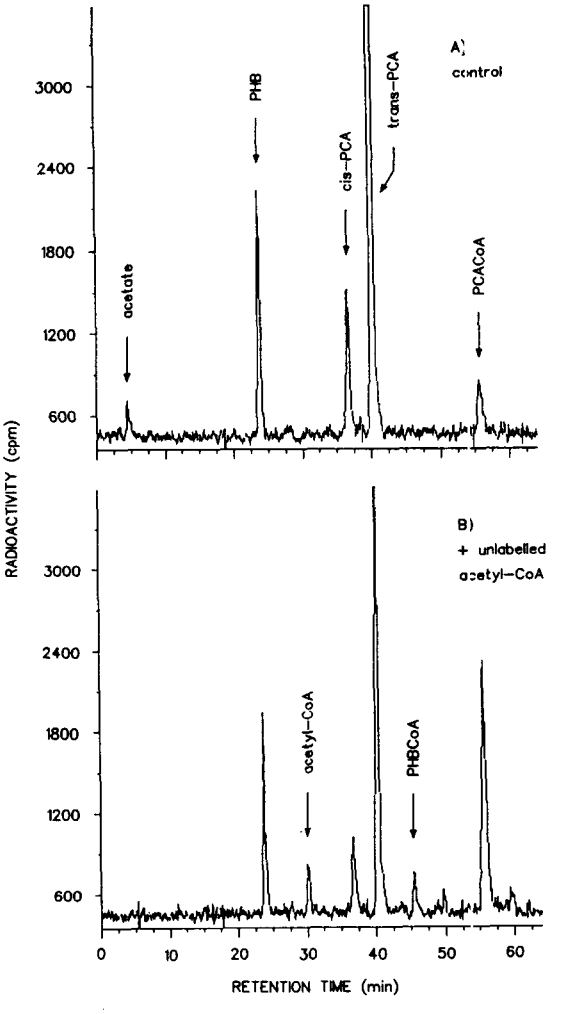

Figure 4. HPLC analysis of assays for *p*-[^{14}C]hydroxybenzoate formation from *p*-[^{14}C]coumaroyl-CoA. A, Native cell extract was incubated with *p*-[U-^{14}C]coumaroyl-CoA, ascorbate, and NAD for 5 min at 30°C. B, The same incubation as in A, but unlabeled acetyl-CoA (5 mM) was added. PHB, *p*-Hydroxybenzoate; PCA, *p*-coumarate; PCACoA, *p*-coumaroyl-CoA; PHBCoA, *p*-hydroxybenzoyl-CoA.

by transesterification or CoA ligase reactions, we incubated cell-free extracts with p-[carboxyl-^{14}C]hydroxybenzoate and [2-^{14}C]acetate in the presence of either free CoA or possible CoA donors such as p-hydroxybenzoyl-CoA, p-coumaroyl-CoA, and acetyl-CoA. The formation of p-[^{14}C]hydroxybenzoyl-CoA and [^{14}C]acetyl-CoA could not be detected in any of these incubations, so the radioactive CoA esters observed in the experiments described above are direct products of p-[U-^{14}C]coumaroyl-CoA.

The results show that p-coumaroyl-CoA is cleaved, presumably after oxidation, in a thioclastic reaction to give p-hydroxybenzoyl-CoA and acetyl-CoA. The CoA esters cannot be detected without trapping by the addition of an excess of the unlabeled substances. This is due to a rapid cleavage of these compounds by thioesterases.

100,000*g* Centrifugation of Enzyme Extracts

We performed a centrifugation step (100,000*g*) to test wether the enzymes involved in p-hydroxybenzoate biosynthesis are membrane associated or soluble in the cytosol. The enzyme activity forming p-hydroxybenzoate from p-coumarate or p-coumaroyl-CoA was found entirely in the 100,000*g* supernatant (data not shown).

DISCUSSION

The exact mechanism of the biosynthesis of benzoic acids in plants has been enigmatic over the last 25 years. Since feeding experiments established that hydroxybenzoic acids are derived from phenylpropanoid precursors by removal of acetate or acetyl-CoA from the side chain (El-Basyouni et al., 1964; Vollmer et al., 1965; Zenk, 1965; Heide et al., 1989), the readily available hypothesis was that the reaction proceeds analogous to the β-oxidation of fatty acids, i.e. via the CoA esters. However, in vitro studies in a number of plant systems (Alibert and Ranjeva, 1971; Kindl and Ruis, 1971; Alibert et al., 1972; Hagel and Kindl, 1975; French et al., 1976; Löffelhardt and Kindl, 1976; Yazaki et al., 1991; Schnitzler et al., 1992) could supply no biochemical evidence for this hypothesis. No CoA esters were identified as intermediates, and most studies reported that the reaction is not dependent on the addition of ATP and CoA. Therefore, alternative hypotheses for the biosynthesis of benzoic acids have been suggested (French et al., 1976; Funk and Brodelius, 1990a, 1990b; Yazaki et al., 1991; Funk and Brodelius, 1992; Schnitzler et al., 1992).

Cell cultures of *L. erythrorhizon*, which form large amounts of secondary metabolites derived from p-hydroxybenzoate (Tabata et al., 1974), present an exceptionally well-suited system for biosynthetic studies of this compound. In a previous investigation using this system (Yazaki et al., 1991), the enzymatic formation of p-hydroxybenzoate from p-coumarate was demonstrated, and p-hydroxybenzoate was identified by chemical derivatization and GC-MS analysis. The reaction was specific for p-coumarate, whereas o- and m-coumarate as well as cinnamate were not accepted as substrates. This, together with the very efficient enzymatic conversion observed in the present study (25% conversion of p-coumaroyl-CoA in 5 min, see Figs. 3 and 4), indicates that *L. erythrorhizon* possesses specific enzymes for the biosynthesis of p-hydroxybenzoate.

For the present study we devised an improved assay system to avoid the decomposition of the substrate that was observed previously (Yazaki et al., 1991). In addition, we synthesized p-[^{14}C]coumarate and p-[^{14}C]coumaroyl-CoA as substrates and developed HPLC systems for the detection of the CoA esters.

Using these methods, the enzymatic formation of p-coumaroyl-CoA by a 4CL reaction could be readily demonstrated, as could the conversion of p-coumaroyl-CoA to p-hydroxybenzoate. Although p-coumaroyl-CoA is enzymatically hydrolized very quickly in our extracts, dilution experiments with radioactive substrate (Table III) unequivocally proved that p-coumaroyl-CoA is an intermediate in the conversion of p-coumarate to p-hydroxybenzoate. The products p-[^{14}C]hydroxybenzoyl-CoA and [^{14}C]acetyl-CoA first escaped detection due to quick enzymatic hydrolysis. However, by addition of unlabeled CoA esters, in amounts sufficient to prevent complete enzymatic hydrolysis within 5 min, both p-[^{14}C]hydroxybenzoyl-CoA and [^{14}C]acetyl-CoA could be identified as products (Figs. 3 and 4). The possibility that these CoA esters were formed from free p-[^{14}C]hydroxybenzoate or [^{14}C]acetate could be excluded by appropriate controls.

Thus, our study provides the first clear-cut biochemical evidence that the biosynthesis of p-hydroxybenzoate proceeds via p-coumaroyl-CoA and a thioclastic cleavage to p-hydroxybenzoyl-CoA and acetyl-CoA. This thioclastic reaction requires CoA as cofactor. In contrast, the conversion of p-coumaroyl-CoA to p-hydroxybenzoate was only slightly stimulated by addition of CoA in our experiments (Table II). This can be explained by the very fast enzymatic hydrolysis of p-coumaroyl-CoA, which immediately provides free CoA once the incubation is started. The enhancement of p-hydroxybenzoate formation when ATP, CoA and Mg^{2+} were added (Table II) might be due to a replenishment of the p-coumaroyl-CoA pool by the 4CL reaction.

All enzymes involved in the conversion of p-coumarate to p-hydroxybenzoate remained in the supernatant of a 100,000*g* centrifugation. Since the general phenylpropanoid metabolism is localized in the cytosol (Hrazdina and Jensen, 1992), and shikonin biosynthesis in *Lithospermum* takes place in vesicles formed from the ER (Tsukada and Tabata, 1984; Yamaga et al., 1993), it appears plausible that the enzymes of p-hydroxybenzoate biosynthesis are localized in the cytosol of *Lithospermum* cells. This is in contrast with the results in other plant systems in which the enzymes of p-hydroxybenzoate biosynthesis are reported to be associated with glyoxysomes in castor beans (Kindl and Ruis, 1971), with mitochondrial membranes in *Cucumis sativus* (Hagel and Kindl, 1975); and with thylakoid membranes in a blue-green alga (Löffelhardt and Kindl, 1976).

Experiments with cell-free extracts of potato tubers (French et al., 1976) and carrot cell cultures (Schnitzler et al., 1992) provided evidence that p-hydroxybenzaldehyde may be an intermediate in p-hydroxybenzoate biosynthesis (Fig. 1). Similar results were obtained in the previous study using *L. erythrorhizon* cell cultures (Yazaki et al., 1991), in which unlabeled p-coumarate was incubated with enzyme extract

for 3 or 4 h. Under these conditions, we could reproduce the formation of *p*-hydroxybenzaldehyde as well as its enzymatic oxidation to *p*-hydroxybenzoate (data not shown). However, no *p*-hydroxybenzaldehyde formation was observed under the improved assay conditions (using radioactive substrates, shorter incubation times, and inclusion of ascorbate), and the rate of *p*-hydroxybenzoate formation from *p*-coumaroyl-CoA greatly exceeded the ATP- and CoA-independent formation of *p*-hydroxybenzoate from *p*-coumarate reported in the previous study (Yazaki et al., 1991). Dilution experiments with radioactive *p*-coumaroyl-CoA refuted free *p*-hydroxybenzaldehyde as an intermediate in the conversion of *p*-coumaroyl-CoA to *p*-hydroxybenzoate (Table III). Clearly, the reaction via the CoA esters is the major biosynthetic route to *p*-hydroxybenzoate. It cannot be decided at present whether the route via *p*-hydroxybenzaldehyde is an alternative, minor pathway or an artifact obtained only in vitro.

It remains to be seen whether the pathway via the CoA esters can be demonstrated in other plant systems, especially in those for which a different mechanism for the biosynthesis of benzoic acids has been proposed (French et al., 1976; Funk and Brodelius, 1990b; Schnitzler et al., 1992) or in which a different compartmentalization of the enzymes was observed (Kindl and Ruis, 1971; Hagel and Kindl, 1975; Löffelhardt and Kindl, 1976). Of special interest is the question of whether the biosynthesis of salicylic acid proceeds in a similar way. Salicylic acid has recently been identified as a potential signal substance in plant defense mechanisms and is supposed to be formed from cinnamic acid via benzoic acid (Yalpani et al., 1993), although the enzymes involved have not yet been identified.

If the conversion of *p*-coumaroyl-CoA to *p*-hydroxybenzoyl-CoA in *Lithospermum* extracts proceeds in a manner analogous to the β-oxidation of fatty acids, it would involve three different enzymatic reactions: addition of water to the double bond, oxidation to the keto function, and thioclastic cleavage. So far we have not detected intermediates between *p*-coumaroyl-CoA and *p*-hydroxybenzoyl-CoA. Purification of the enzymes involved should provide further evidence for the exact reaction mechanism and the number of enzymes involved.

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