Cell Free Synthesis of *o*-Succinylbenzoic Acid in Protein Extracts from Anthraquinone and Phylloquinone (Vitamin K_1) Producing Plant Cell Suspension Cultures. Occurrence of Intermediates between Isochorismic and *o*-Succinylbenzoic Acid

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Cell free protein extracts from heterotrophic anthraquinone ("Rubiatype") producing cell suspension cultures of three different *Galium* species and from a photoautotrophic phylloquinone (vitamin K_1) producing cell suspension culture of *Morinda lucida Benth.* (Rubiaceae) catalyzed the synthesis of *o*-succinylbenzoic acid (OSB) from isochorismic acid and α-oxoglutaric acid in the presence of thiamine diphosphate and Mn^{2+}.

At least two intermediates in the conversion of isochorismic to *o*-succinylbenzoic acid were detectable. One of these intermediates is likely to be identical to 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC), a metabolite known to occur in bacterial mutants *(menD⁺)* blocked in *o*-succinylbenzoic acid synthesis. The structure of the second intermediate is as yet unknown. A third intermediate may also occur.

Protein extracts from menaquinone (vitamin K_2) producing bacteria are known to catalyze the formation of *o*-succinylbenzoic acid (OSB) [1–9]. One of the substrates for the enzyme system (OSB-synthase system) was originally thought to be chorismic acid [1] but later shown to be isochorismic acid [4–9]. In addition α-oxoglutaric acid is a substrate of the OSB-synthase system. It is decarboxylated in the presence of thiamine diphosphate. The anion formed probably attacks isochorismic acid in a Michael type reaction [2, 7] (Fig. 1). The aromatization leading to *o*-succinylbenzoic acid is likely to be a multistep reaction sequence. An intermediate in this sequence has been isolated and a structure proposed [3].

The enzyme catalyzing the synthesis of this intermediate was called 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) synthase. The gene *(men D)* believed to encode this enzyme was sequenced [10]. The *men D* gene is linked to the *men C* gene on the *E. coli* chromosome. The latter gene encodes the enzyme that converts SHCHC to OSB. *o*-Succinylbenzoic acid (OSB) is the first aromatic intermediate not only in menaquinone biosynthesis in bacteria but also in phylloquinone and anthraquinone biosynthesis in higher plants [11–14].

Isolation of the enzymes from higher plants catalyzing the synthesis of OSB is, therefore, desirable but seems to pose great problems because anthraquinones occur predominantly in lignified tissue of the root bark [15] whereas phylloquinone producing young green leafs contain only insignificant amounts of protein. Cells cultivated in suspensions represent, however, a more suitable enzyme source. Hence we decided to use cells of a phylloquinone producing photoautotrophic suspension culture raised from *Morinda lucida* Benth. plants [16]) and cells of anthraquinone producing heterotrophic suspension cultures raised from *Galium mollugo, Galium verum* and *Galium uliginosum* plants [17, 18] as sources of the enzyme. Active preparations could be obtained, when protein was extracted following previously published methods [19, 20].

A preliminary account of part of this work has been published [21].

Abbreviations: CoASH, coenzyme A; DMOSB, OSB-dimethylester; GC, gas chromatograph; GC-MS gas chromatography-mass spectroscopy; OSB, *o*-succinylbenzoic acid [4-(2′-carboxyphenyl)-4-oxobutyrate]; SB, succinylbenzene; SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; ThPP, thiamine diphosphate.

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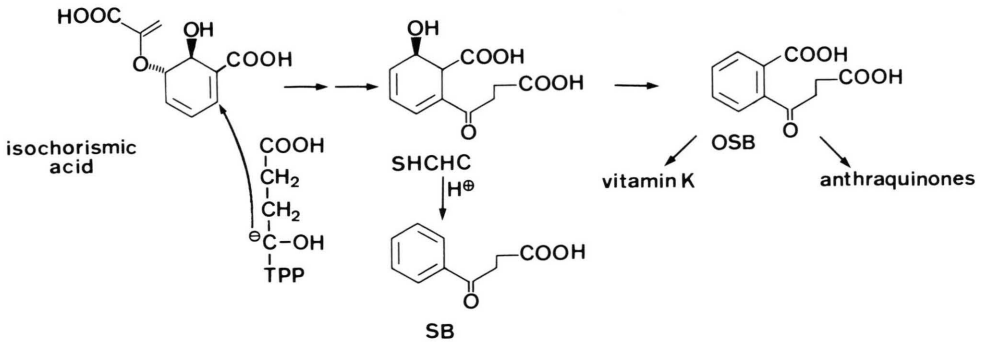

Fig. 1. Biosynthesis of OSB and chemical conversion of SHCHC to SB.

Materials and Methods

Cell cultures

Heterotrophic cell suspension cultures of the three *Galium* species, Rubiaceae, were cultured in 50 ml of medium A in 300 ml Erlenmeyer flasks kept under continuous white light (500 lux) [17], and photoautotrophic cell suspension cultures of *Morinda lucida* Benth., Rubiaceae, were cultured in 50 ml of sucrose free medium A [16, 17] in a two-tier culture vessel [22] kept under continuous white light (6000 lux). These cell cultures were agitated at 100 rpm on a rotary shaker. Cells were harvested 3 days after inoculation.

Bacteria

Enterobacter aerogenes 62-1 (*Klebsiella pneumoniae* ATCC 25306) has been described by Gibson and Gibson [23] and was kindly provided by Dr. N. Amrhein, ETH Zürich, Switzerland. *Escherichia coli* K 12 was obtained from the Institut für Mikrobiologie der Westfälischen Wilhelms-Universität Münster [24]. *E. coli* [24] and *E. aerogenes* 62-1 [25] were grown as described.

Chemicals

Barium chorismate and thiamine diphosphate were purchased from SIGMA (München, F.R.G.), α-oxo[U-^{14}C]glutarate (10.8 MBq/μmol), and α-oxo[1-^{14}C]glutarate (2.2 MBq/μmol) were purchased from NEN, Dreieich, F.R.G. Bovine serum albumin (BSA) and Tris(hydroxymethyl)-aminomethane-HCl were purchased from SERVA (Heidelberg, F.R.G.). OSB was synthesized according to the method of Roser [26] and OSB dimethylester was prepared as described previously [27]. Other chemicals were purchased from MERCK (Darmstadt, F.R.G.).

Purification of Ba-chorismate

Purified chorismic acid was prepared and detected by HPLC as described [5, 6].

HPLC analysis of OSB

For HPLC analysis (LKB HPLC system) samples containing OSB were injected onto a Knauer RP-8 (7 μm) Lichrosorb column (250 mm × 4 mm) connected to a precolumn (30 mm × 4 mm) packed with Perisorb RP-8 (30–40 μm). The columns were eluted (8 min) with water containing formic acid (0.13 M) followed by a gradient of methanol increasing from 0 to 30% within 30 min. Alternatively elution was carried out isocratically (20% methanol). The flow rate was 1 ml/min and the UV detector was set to 245 nm.

Enzyme preparation

Anthraquinone producing cells (20 g fresh weight) were homogenized (Branson Sonifier) after suspension in KPi buffer (40 ml, 0.1 M, pH 7.0) containing dithiothreitol (0.2 mM), EDTA (10 mM) and bovine serum albumin (10 g/l). The homogenate was cooled in ice. The temperature of the homogenate did not exceed 7 °C. After centrifugation (10 min, 48,000 × *g*, 4 °C), low-molecular weight compounds where removed from the supernatant by filtration through a PD 10 column (Sephadex G 25).

Phylloquinone producing *Morinda* cells (20 g) were suspended, homogenized, and centrifuged as described above. After centrifugation the supernatant was subjected to ammonium sulfate precipitation. The pellet of the 10–30% fraction was dissolved in 0.1 M KPi buffer (pH 7.0) containing 0.2 M dithiothreitol. This active crude enzyme extract was freed of low molecular weight compounds (Sephadex G25) and concentrated 2-fold by ultrafiltration (Amicon, centricon 10).

Assay for enzyme activity

In a final volume of 395 µl, the incubation mixture contained isochorismate (10 nmol) (or purified chorismate (10–200 nmol)), α-oxo[U-^{14}C]glutarate (1 nmol), 10.8 KBq), thiamine diphosphate (120 nmol), Tris-HCl (50 µmol, pH 8.1), $MnCl_2$ (2 µmol) and protein (3 mg). After incubation for 180 min, pH 7.80 at 30 °C, the reaction was terminated by addition of 50 µl HCl (12 N) and the protein centrifuged off.

Enzymatic synthesis of isochorismic acid

Isochorismate synthase was isolated from *E. aerogenes* 62-1 as described [28]. Isochorismic acid was prepared and isolated from the supernatant of the incubation mixture as described [6] except that after incubation the mixture was concentrated and centrifuged after addition of HCl (25 µl, 10 N).

Enzymatic synthesis of the coenzyme A ester of OSB

Coenzyme A ligase of OSB was isolated from *E. coli* K 12 [29] and incubated with enzymatically formed radioactively labelled OSB (2.5 KBq) in a 155 µl mixture containing ATP (1 µmol), CoASH (0.15 µmol), $MgCl_2$ (4.2 µmol) and crude enzyme (0.2 mg protein) in KPi buffer (0.1 M) for 30 min at 30 °C and pH 7.1. The reaction was terminated (10 µl HCl, 12 N) and the protein centrifuged off. The OSB coenzyme A ester formed was detected by HPLC as described [29, 30].

Identification of enzymatically formed OSB

In a final volume of 8.3 ml, isochorismic acid (400 nmol), α-oxoglutaric acid (30 µmol), ThPP (1 µmol), Tris-HCl pH 8.2 (847 µmol), $MnCl_2$ (20 µmol) and crude enzyme extract in 0.1 M KPi buffer, pH 7.0 (75 mg protein) were incubated for 30 h at 30 °C and pH 7.8. After incubation the mixture was evaporated at 30 °C. The residue (300 µl) was acidified (100 µl HCl, 12 N) and centrifuged (3 min, 15000 rpm, Eppendorf centrifuge type 5414 S). The OSB formed was detected by HPLC as described above (isocratic mode of operation). Formation of OSB was not observed in heat-denatured protein solutions. Treatment of the enzymatically formed unlabelled OSB with diazomethane [27] provided the methylated derivative which was injected in ethereal solution into a GC equipped with a DB-5 capillary column 30 m × 0.32 mm, 0.25 µm film; temperature programme: 150 °C for one min, increasing to 230 °C at a rate of 15 °C per min. Retention time of DMOSB 5.81 min. GC-MS: GC as above; MS: Finnigan/Mat 1020 B, 70 eV. *m/z* (relative intensity) = 250 $[M]^+$ (0.5%), 219 $[M-OCH_3]^+$ (2%), 163 $[M-CH_2CH_2COOCH_3]^+$ (100%).

The ^{14}C labelled OSB was also converted to its spirodilactone: The fraction containing OSB was collected from the HPLC apparatus and evaporated at 50 °C in a rotary evaporator at pH 2.5. The residue was dissolved in H_2O/MeOH (300 µl; 3:2 v/v) and analyzed by HPLC (retention time of OSB 11.2 min, of its spirodilactone 21.1 min). Yield 50–75% of OSB employed. The spirodilactone was collected from the HPLC apparatus, evaporated to dryness and dissolved in NaOH (300 µl, 30 min at 30 °C). The solution was acidified (HCOOH, 12 µl) and again analyzed for OSB by HPLC. The product of hydrolysis co-chromatographed with an authentic sample of OSB. The specific activity of the spirodilactone (7.80 KBq/µmol) was essentially identical to that of the starting material (OSB, 7.84 KBq/µmol).

Identification of unknown compound (t_R = 10.2 min, compare Fig. 2) as SHCHC

SHCHC (Fig. 2) was collected from the HPLC apparatus. Acid treatment (pH 0; 1 N HCl; 70 min; 30 °C) converted this compound to ^{14}C labelled OSB (yield 13%) and ^{14}C labelled SB (yield 24%) or to ^{14}C labelled SB alone at pH 2.6 and room temperature. Alkali treatment (pH 8.5, 30 °C, 180 min) gave ^{14}C labelled OSB (yield 20%). The ^{14}C labelled SB formed from ^{14}C labelled SHCHC was identified by co-chromatography with authen-

tic material. The specific activity remained constant when SB was taken through three HPLC purification steps (Solvents: 25% MeOH, 75% H_2O, HCOOH 0.097 M; 30% MeOH, 70% H_2O, HCOOH 0.091 M; 40% MeOH, 60% H_2O, HCOOH 0.078 M, spec. activity: 4.588; 4.570; 4.547 MBq/mmol).

Enzymic conversion of intermediates to OSB

Radiolabelled SHCHC (3.2 KBq) was incubated (60 min, 30 °C) in Tris HCl buffer (50 μM, pH 7.0), $MnCl_2$ (2 μM) and protein (2 mg) after ammonium sulfate precipitation (30–70% saturation). The incubation was terminated (1 N HCl) and centrifuged (2 min). Unlabelled OSB (100 nmol) was added and the mixture separated by HPLC.

The radiolabelled unknown compound (t_R = 23.4 min, compare Fig. 2) was incubated (30 °C, 180 min) in Tris HCl buffer (50 μM, pH 7.8) in the presence of $MnCl_2$ (2 μmol) and protein extract (28 mg) after ammonium sulfate precipitation (30–70% saturation) and subsequent gel filtration. The mixture was acidified (1 N HCl) and centrifuged (2 min). The OSB formed was isolated and identified as described. Formation of radiolabelled OSB was not observed in heat-denatured protein solutions.

Radioactivity-measurement

Berthold HPLC-monitor LB 506 A (compare Fig. 2) or scintillation counting with a BETA-SZINT BF 5001 A.

Protein determination

Protein was determined according to Bradford [31].

Results and Discussion

The radioactively labelled OSB obtained by enzymatic synthesis from α-oxo[U-^{14}C]glutaric acid and isochorismic acid in the presence of thiamine diphosphate and Mn^{2+} showed the same retention time as authentic unlabelled *o*-succinylbenzoic acid, which was added to the incubation mixture after termination of the reaction.

The OSB fraction (Fig. 2) was converted to its spirodilactone chemically. The spirodilactone was isolated and again converted to OSB by alkaline hydrolysis [26, 30]. The specific activity of the spirodilactone was essentially identical to that of the starting material.

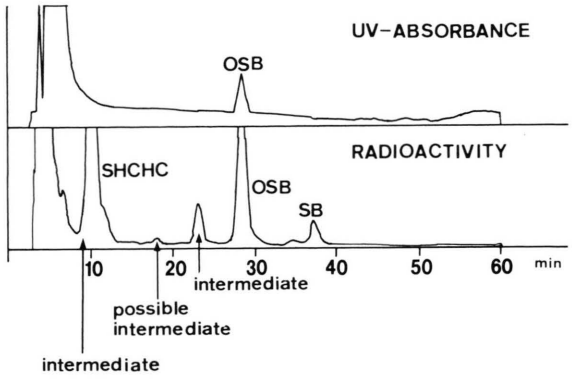

Fig. 2. HPLC separation of an incubation mixture after addition of authentic unlabelled OSB. Detection: UV absorbance at 245 nm (upper track, 0–1000 mV) and flow scintillation counting (lower track, 0–250 dpm).

The enzymatically formed radioactively labelled OSB was also converted to its coenzyme A ester by incubation with coenzyme A ligase from *E. coli* K_{12}. The OSB coenzyme A ester and the spirodilactone formed were detected by HPLC. OSB spirodilactone is a product of the chemical decomposition of OSB coenzyme A ester [32, 33]. The alkaline hydrolysis of these reaction products again resulted in the formation of OSB, which was detected by HPLC.

Esterification (diazomethane) of the enzymatically formed unlabelled OSB (yield 80% with respect to isochorismic acid) gave a compound which upon GC-MS analysis showed the same retention time and fragmentation pattern as an authentic sample of the dimethyl ester of OSB.

Formation of OSB was not observed in heat-denatured protein solutions. When α-oxo[U-^{14}C]glutaric acid was replaced by α-oxo[1-^{14}C]glutaric acid the OSB formed was inactive (Table I). This is in agreement with expectations because C-1 of α-oxoglutaric acid is removed during biosynthesis of OSB [5, 34].

When isochorismic acid was replaced by purified chorismic acid no formation of OSB was observed. This indicates that isochorismic acid is the immediate precursor of OSB in plant cells which produce anthraquinones or phylloquinone and that an active isochorismate synthase was not de-

Table I. Formation of OSB in protein extracts from anthraquinone and phylloquinone producing plant cell suspension cultures. The complete incubation mixture contained isochorismate, α-oxo[U-^{14}]glutarate, thiamine diphosphate and Mn^{2+} as described under Materials and Methods.

	OSB formed in extracts of	
	G. mollugo	*M. lucida*
Radiochemical yield in a complete incubation mixture (100% = α-oxo[U-^{14}C]glutaric acid)	2.37 KBq (22%)	0.54 KBq (5%)
α-oxo[1-^{14}C]glutaric acid instead of α-oxo[U-^{14}C]glutaric acid	no radioactivity	
Minus isochorismic acid	no radioactivity	
Chorismic acid instead of isochorismic acid	no radioactivity	
Minus thiamine diphosphate	1.18 KBq (11%)	0.27 KBq (2.5%)
Minus Mn^{2+}	0.23 KBq (2.1%)	0.05 KBq (0.4%)
Heat inactivated extract	no radioactivity	

tectable in these extracts. When thiamine diphosphate or Mn^{2+} was omitted from the incubation mixture formation of OSB was greatly reduced. This shows that limiting amounts of ThPP and Mn^{2+} are present in the crude enzyme extracts even after filtration through Sephadex G 25.

Formation of OSB was linear with time up to 3 h when 3 mg protein were employed. The pH optimum was 7.8 and the temperature optimum 30 °C. The yield of OSB in protein extracts from plant cell suspension cultures of three different *Galium* species which produce anthraquinones is shown in Table II. The most active preparation was obtained from *Galium mollugo* cells.

A time course study of OSB synthesis showed that maximum enzyme activity was followed by maximum anthraquinone accumulation in the cultured cells (Fig. 3).

Enzyme extracts from *Morinda lucida* and *Galium mollugo* cells were incubated with a complete mixture including α-oxo[U-^{14}C]glutaric acid. After addition of authentic unlabelled OSB the mixture was separated by HPLC and the effluent of the column passed through a scintillation flow counter (Fig. 2). Different radioactive peaks appeared at 10.2, 18.0, 23.4, 28.0 and 35.0 min after injection (Fig. 2).

Table II. Formation of OSB in crude protein extracts from anthraquinone producing plant cell suspension cultures. As opposed to the experiments listed in Table I protein solutions were not concentrated (compare Materials and Methods). (100% = amount of α-oxoglutaric acid employed.)

Plant tissue	%	Yield of *o*-succinylbenzoic acid KBq	nmol OSB/min × mg protein
G. mollugo	4.0	0.43	9.07×10^{-5}
G. uliginosum	3.2	0.34	7.22×10^{-5}
G. verum	2.7	0.29	6.11×10^{-5}

The formation of these peaks was isochorismic acid, α-oxoglutaric acid and ThPP dependent. The compound with a retention time of 10.2 min was collected from the HPLC apparatus. Alkali treatment of this peak gave OSB. Acid treatment, however, gave two products which cochromatographed with succinylbenzene (SB) and OSB. Such a reaction had previously been observed [3] when SHCHC was treated with acid. Indeed enzymic incubation of this compound (SHCHC) with the OSB synthase system gave OSB. It is therefore very likely identical to SHCHC (Fig. 1). The compound (SHCHC) accumulates in bacteria blocked in OSB synthesis [3]. The succinylbenzene (SB) formed by acid treatment of SHCHC was identified by comparison (HPLC) with an authentic sample of SB in these solvent systems. The specific activity of the SB remained constant after chromatography in three different solvent systems.

The compound eluting at 23.4 min is another intermediate in OSB biosynthesis because enzymic incubation with this compound also gave OSB, which was again identified as described. Enzymic conversion of these intermediates to OSB took place in the absence of isochorismic, 2-oxoglutaric

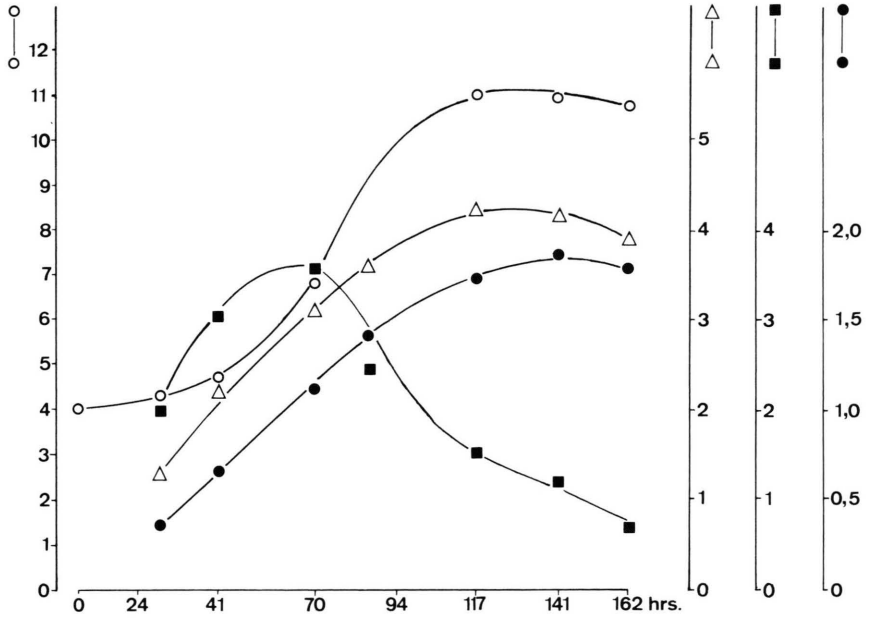

Fig. 3. Correlation between growth of culture, protein synthesis, anthraquinone formation and activity of OSB synthase in a *Galium mollugo* cell suspension culture. ○——○, Fresh weight (g) in 50 ml medium; △——△, protein (mg/g fresh weight); ■——■, yield of OSB (% of α-oxoglutaric acid); ●——●, anthraquinone (mg/g fresh weight).

acid and ThPP indicating that these intermediates contain the complete carbon skeleton of OSB. The amount of radioactivity present in the fraction eluting at 18.0 min was too low to allow a conversion to OSB. Formation of this peak depends also on isochorismic and α-oxoglutaric acid.

A reaction mechanism for the conversion of isochorismic acid to OSB has been proposed [3].

The experiments described herein show that isochorismic acid is a metabolite which plays an important role not only in procaryotic [4–9, 25, 35–38] but also in eucaryotic organisms [21, 39]. The *Morinda lucida* cell cultures employed in this study produce phylloquinone [16] a compound which is very likely involved in photosynthesis [40, 41]. If so the OSB synthase system is distributed in nature as widely as is photosynthesis and bacterial menaquinone formation.

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