Phytochemistry, 1975. Vol. 14. pp. 2437 2442. Pergamon Press. Printed in England.

BIOSYNTHESIS OF CYANIDIN IN CELL CULTURES OF *HAPLOPAPPUS GRACILIS*

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(*Received* 21 *May* 1975)

Key Word Index—*Haplopappus gracilis*; Compositae; tissue culture; biosynthesis; flavonoids; cyanidin.

Abstract—Dihydroquercetin proved to be a good precursor for light-stimulated cyanidin biosynthesis in cell suspension cultures of *Haplopappus gracilis*. With [2-^{14}C-2-^{3}H]-naringenin as precursor all tritium was lost upon conversion to cyanidin. In the presence of dimethyl sulphoxide, naringenin was converted to eriodictyol and dihydrokaempferol by suspension cultures of *H. gracilis*. This conversion was also observed in the absence of DMSO but in much lower yield. The hydroxylation of naringenin to eriodictyol and of dihydrokaempferol to dihydroquercetin was found with a microsomal preparation from illuminated cell suspension cultures. This reaction requires NADPH and oxygen. The microsomal fraction also contained cinnamic acid 4-hydroxylase activity but did not catalyse the hydroxylation of *p*-coumaric to caffeic acid. On the basis of these results, it is suggested that biosynthesis of anthocyanins takes place partly or completely on membranes.

INTRODUCTION

Earlier investigations with buck wheat seedlings [1] and cell suspension cultures of *Haplopappus gracilis* [2] had shown that dihydrokaempferol (3,5,7,4′-tetrahydroxy-2,3-*trans*-flavanone) was a better precursor for cyanidin than L-phenylalanine, 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside or kaempferol. According to these results we have formulated a biosynthetic pathway to anthocyanins via naringenin (5,7,4′-trihydroxyflavanone) and dihydrokaempferol (or dihydroquercetin) [3].

In this study, we present further evidence for this pathway and report on the hydroxylation of naringenin to eriodictyol (5,7,3′,4′-tetrahydroxyflavanone) and dihydrokaempferol with permeabilized cells and with a microsomal preparation from *H. gracilis* suspension cultures.

RESULTS

Production of anthocyanin by H. gracilis *cell cultures*

When cell suspension cultures of *H. gracilis* which had been transferred to fresh medium were kept in the dark for 48–72 hr and then illuminated with blue fluorescent light, anthocyanin production started about 24 hr after onset of illumination. This light induced anthocyanin production remained linear for at least 50 hr (Fig. 1). When the cells were illuminated immediately after transfer to fresh medium, anthocyanin production also did not start until about 90 hr after the transfer (Fig. 1). All the following results were obtained with cells in the linear phase of anthocyanin production.

Incorporation of [G-^{3}H]-*dihydroquercetin and* [2-^{14}C-2-^{3}H]-*naringenin into cyanidin*

The incorporation into cyanidin of [G-^{3}H]-dihydroquercetin was compared with that of [2-^{14}C]-naringenin. The labeled compounds were added to cells of different age. After 6 hr incubation cyanidin was isolated as described previously [2] and purified to radiopurity by PC and chromatography on Sephadex-LH-20. In comparing the incorporation rates and dilution values for

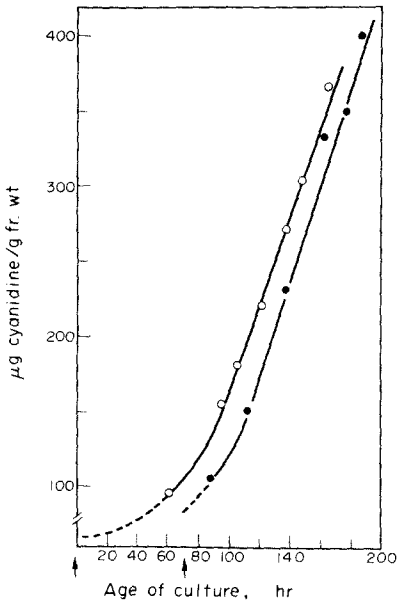

Fig. 1. Anthocyanin production of illuminated *H. gracilis* suspension cultures. Arrows indicate start of illumination: O——O, cells illuminated immediately after transfer to fresh medium; ●——●, cells illuminated after 72 hr in the dark.

cyanidin shown in Table 1, it must be taken into consideration that tritium at C-2 and probably also at C-3 of [G-^{3}H]-dihydroquercetin is lost upon incorporation into cyanidin (see below). The percentage of tritium at these positions in [G-^{3}H]-dihydroquercetin was not known but it had been determined to be about 40% in the case of dihydrokaempferol. The latter compound was also obtained by Wilzbach labeling [4]. It can therefore be assumed that the conversion of dihydroquercetin to cyanidin is at least 1·5 times higher than that actually determined.

Table 2. Incorporation of [2-^{3}H-2-^{14}C]-naringenin into cyanidin in *H. gracilis* cell cultures

Precursor	Uptake of precursor (%)	Specific incorporation‡ into cyanidin (%)	Specific activity of cyanidin (μCi/μmol)	Dilution
[2-^{3}H]-Naringenin*	89	0	–	–
[2-^{14}C]-Naringenin*	79	5·7	0·24	10
[2-^{3}H-2-^{14}C]-Naringenin†	^{3}H:86 ^{14}C:83	^{3}H:0 ^{14}C:5	^{14}C:0·14	^{14}C:22

* Parallel experiments in two flasks. † A mixture of the ^{3}H- and ^{14}C-labeled compound in one flask was used. ‡ Corrected for uptake of precursor.

Mechanisms formulated for the conversion of dihydroflavonols into anthocyanidins could involve a 2·4 or 3·4 hydride shift [5,6]. To test the hypothesis involving a 2·4 hydride shift, we used [2-^{3}H]-naringenin as precursor because this compound can be converted to dihydrokaempferol and dihydroquercetin by the cells (see below). [2-^{14}C]-Naringenin served as internal standard in these experiments. The results shown in Table 2 prove that tritium in the 2-position is lost upon conversion of naringenin to cyanidin.

Experiments with permeabilized cells

Permeabilization of intact plant cells with dimethyl sulphoxide (DMSO) has been used for the assay of enzymes in tobacco cells [7,8]. For the permeabilization experiments with *H. gracilis* suspension cultures grown in Steward flasks [9] were used. These cultures consisted of smaller aggregates than cells grown in Erlenmeyer flasks. Cells preincubated for 20–30 min in buffer containing 10% DMSO were incubated with [2-^{14}C]-naringenin for 1·5 hr. The ethyl acetate extract of

Table 1. Incorporation of [G-^{3}H]-dihydroquercetin and [2-^{14}C]-naringenin into cyanidin in *H. gracilis* cell cultures

Precursor	Age of cells at time of precursor addition (hr in light)	Uptake of precursor (%)	Specific incorporation into cyanidin* (%)	Specific activity of cyanidin (μCi/μmol)	Dilution
DQ	98	38	0·1	0·04	125
DQ	104	32	2·7	0·37	13
DQ	110	34	1·3	0·49	10
DQ	116	33	2·0	0·68	7
Nar	114	80	5·2	0·22	11
Nar	114	77	6·2	0·26	10

DQ = [G-^{3}H]-Dihydroquercetin; Nar = [2-^{14}C]-naringenin. * Corrected for uptake of precursor. The specific incorporation and dilution with dihydroquercetin as precursor is not corrected for loss of ^{3}H from C-2 and probably also from C-3 upon conversion to cyanidin.

Table 3. Subcellular distribution of hydroxylating activity in cell-free extracts from *H. gracilis* suspension cultures

	Eriodictyol + dihydrokaempferol (dpm)	Protein (mg/ml)	dpm in hydroxylation products/mg protein
Crude extract	(90)	0·6	(1510)
90000 *g* supernatant	0	0·5	0
Microsomal pellet	13600	4·5	31000

the incubation mixtures showed two reaction products on radiochromatograms (solvent 3). The major product corresponded in its chromatographic mobility (R_f 0·55) to eriodictyol and the minor product (R_f 0·33) to dihydroquercetin. Weak radioactivity was also present in cyanidin isolated from the aqueous residue after the ethyl acetate extraction. The product with the lower R_f corresponding to dihydroquercetin was quite labile and could not be identified unequivocally. The major product upon PC with 15% HOAc separated into two products, identified as eriodictyol and dihydrokaempferol by co-chromatography on paper (solvents 1–4), cellulose (solvent 5) and polyamide TLC (solvents 6 and 7). Dihydrokaempferol was further identified by oxidation with magnesium bisulfite to kaempferol [10]. Eriodictyol was also identified by oxidation to luteolin with a cell free extract from young primary parsley leaves [11].

In a typical experiment the conversion of naringenin to eriodictyol, dihydrokaempferol and cyanidin was about 17, 3 and 1·4%, respectively. The same products were observed when DMSO was omitted from the preincubation buffer. However, without DMSO the uptake of naringenin and the yield of hydroxylation products was lower by about 50%. In a nitrogen atmosphere the yield of eriodictyol + dihydrokaempferol was approximately 80% lower than in air.

Table 4. Cofactor requirement for hydroxylation of naringenin with microsomal preparation from *H. gracilis* suspension cultures

Experiment		Eriodictyol + dihydrokaempferol (dpm)
	Control (5 min at 100°)	0
I	+NADPH	11300
	−NADPH	0
	+NADPH-regenerating system	10900
	+NADH	4200
	Control	7300
II	$-O_2$	2360

Table 5. Naringenin hydroxylation with microsomal preparation from light- and dark-grown suspension cultures

	Eriodictyol + dihydrokaempferol (dpm)	μg Cyanidin/ g fr. wt
Light	39600	85
Dark	7600	25

After transfer to fresh medium cells were kept for 2·5 days in light or dark.

Experiments with cell-free extracts and microsomal preparations

Attempts to convert [G-^{3}H]-dihydroquercetin to cyanidin with cell-free extracts of *H. gracilis* suspension cultures have so far been unsuccessful. Various possible cofactors were used in these experiments.

The hydroxylation of naringenin to eriodictyol and dihydrokaempferol observed with the permeabilized cells could not be detected in the cell-free extract. However, when the 90000 *g* microsomal pellet was incubated with [2-^{14}C]-naringenin, mercaptoethanol and NADPH in the presence of air a conversion to eriodictyol and dihydrokaempferol took place. Subcellular localization of the hydroxylating activity is shown in Table 3. Addition of polyvinylpyrrolidone during cell disintegration gave a more active microsomal preparation.

The cofactor requirement for the formation of eriodictyol and dihydrokaempferol is shown in Table 4. The reaction was dependent on NADPH; use of NADH as a cofactor gave a much lower yield. Incubation in an argon atmosphere decreased the yield of hydroxylation products by about 70%. Microsomal preparations obtained from dark-grown cultures were much less active than from illuminated cultures (Table 5).

An exact pH optimum of the reaction was not determined. Approximately the same product yield was obtained in 0·1 M K-Pi buffer at pH 7·5 and 8·2. The same reaction at pH 6·5 reduced only 20% of the yield at pH 7·5. The reaction was linear with protein concentration up to about 0·25 mg protein/130 μl and with time to 15 min.

The substrate specificity of the microsomal system was investigated. The results shown in Table 6 demonstrate that the microsomal system also has cinnamic acid 4-hydroxylase activity but that

Table 6. Substrate specificity of the microsomal system from *H. gracilis* suspension cultures

Substrate	Activity in incubation	Product	dpm in product
[3-^{14}C]-Cinnamic acid	10^5 dpm/24 nmol	*p*-Coumaric acid	31500
p-[3-^{14}C]-Coumaric acid	$1{\cdot}3 \times 10^5$ dpm/15 nmol	(caffeic acid)	0
[2-^{14}C]-Naringenin	7×10^4 dpm/13 nmol	Eriodictyol + dihydrokaempferol	11500
[G-^{3}H]-Dihydrokaempferol	$2{\cdot}22 \times 10^5$ dpm/10 nmol	Dihydroquercetin	10000

p-coumaric acid is not converted to caffeic acid. Hydroxylation of dihydrokaempferol to dihydroquercetin was also observed.

To find out whether a phenolase-type reaction is involved in the hydroxylation of naringenin to eriodictyol the incubation was carried out in 0·1 M Pi–citrate buffer, pH 5·4, with ascorbate as electron donor [12]. No conversion of naringenin to eriodictyol was found under these conditions. Furthermore, addition of the specific copper chelating agent diethyldithiocarbaminate did not inhibit microsomal hydroxylation of naringenin in the standard incubation mixture.

DISCUSSION

The high incorporation of dihydroquercetin into cyanidin and the low dilution value again demonstrate that dihydroflavonols are efficiently converted into anthocyanins [1,2]. Tritium at the 2-position of naringenin was not retained in cyanidin. Any mechanism which would imply retention of hydrogen (tritium) from C-2, e.g. allylic rearrangement of flav-3-en-3,4-diol to flav-2-en-3,4-diol involving a hydride shift from C-2 to C-4 [5], can therefore be excluded. A cyclic process in which a hydride ion from C-3 of dihydroflavonol shifts to C-4 from the anthocyanin pseudo-base [6] is feasible.

The results on the hydroxylation of naringenin with DMSO treated cells led to the discovery that the microsomal system of *H. gracilis* is capable of catalyzing the hydroxylation of naringenin in the presence of NADPH and O_2 to dihydrokaempferol and eriodictyol. A further labile product was tentatively identified as dihydroquercetin, which was also formed with dihydrokaempferol as substrate. The cofactor requirement for this reaction characterizes the system as a microsomal mixed-function oxidase (mono-oxygenase). This is the first time that hydroxylation of a flavanone by such an oxidase has been demonstrated. Hydroxylation of naringenin, dihydrokaempferol and kaempferol at the 3′-position had been shown previously with a phenolase from leaves of spinach beet (*Beta vulgaris* L.) [12,13]. However, as a typical phenolase this enzyme also catalyzed hydroxylation of *p*-coumaric acid to caffeic acid [14]. In contrast, no hydroxylation of *p*-coumaric acid was observed with the microsomal preparation.

It is not surprising that the microsomal system from *H. gracilis* also catalyzed the hydroxylation of cinnamic acid to *p*-coumaric acid. Microsomal cinnamic acid 4-hydroxylase has been found previously in other plants and plant cell cultures [15]. Whether hydroxylation of naringenin at different positions and hydroxylation of cinnamic acid to *p*-coumaric acid are catalyzed by one or by several mono-oxygenases is unknown.

Other enzymes of flavonoid biosynthesis which have been found in *H. gracilis* cell cultures are: *p*-coumarate: CoA ligase, flavanone synthase (Hrazdina, unpublished results) and a cyanidin-3-O-glucosyltransferase (Fritsch and Witkop, unpublished results). These are "soluble" enzymes. On the basis of the known enzymatic reactions and the solubility of the enzymes the following sequence of reactions for the biosynthesis of cyanidin can be proposed. L-Phenylalanine is deaminated in the cytoplasm to cinnamic acid, which is then hydroxylated to *p*-coumaric acid at a membrane. Activation to the CoA thiolester and condensation with 3 malonyl CoA to naringenin [20] occurs in the cytoplasm, whereas hydroxylation of naringenin to dihydroquercetin and possibly also its conversion to cyanidin again take

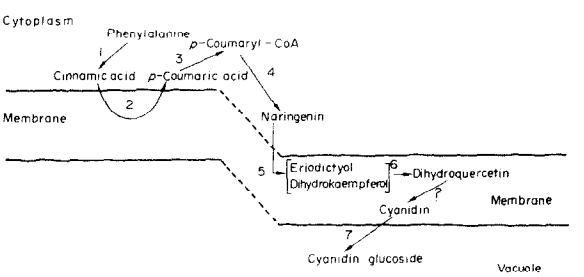

Fig. 2. Proposed scheme for compartmentalization of enzymes involved in anthocyanin biosynthesis: (1) Phenylalanine ammonia-lyase; (2) cinnamic acid 4-hydroxylase; (3) *p*-coumarate:CoA ligase; (4) flavanone synthase; (5 and 6) hydroxylases; (7) glucosyltransferase.

place at a membrane. Glucosylation of cyanidin then occurs by action of a glucosyltransferase which may be located in the vacuole or at the inner side of the tonoplast; the hydrophilic cyanin could then be excreted into the aqueous milieu of the vacuole (Fig. 2). Another possibility which must be considered is that under physiological conditions all enzymes involved in anthocyanin biosynthesis are associated with a membrane.

EXPERIMENTAL

Radioactive substances. Preparation of [G-^{3}H]-dihydrokaempferol [4] and [G-^{3}H]-dihydroquercetin [16] has been described previously. [3-^{14}C]-Cinnamic acid was purchased from Commisariat à l'Energie Atomique, France. *p*-[2-^{14}C]-Coumaric acid was obtained by reaction of *p*-hydroxybenzaldehyde with [2-^{14}C]-malonic acid [21]. [2-^{14}C]-Naringenin was obtained from 4,2',4',6'-tetrahydroxy-[β-^{14}C]-chalcone-2'-glucoside [17] as described below for [2-^{3}H]-naringenin.

Synthesis of [2-^{3}H]-*naringenin.* The morpholinoacetonitrile derivative of *p*-benzyloxybenzaldehyde [18] was synthesized in analogy to the procedure of Bennett *et al.* [19]. α-(4-Benzyloxyphenyl) α-morpholinocetonitrile crystallized from $CHCl_3$–petrol as yellow needles, mp 138–140° (Found: C, 74·0, H, 6·9; O, 9·5; N, 9·0. $C_{19}H_{20}O_2N_2$: requires C, 74·0; H, 6·5; O, 10·4; N, 9·1%. For exchange with tritiated H_2O 924 mg of the morpholinoactonitrile derivative was treated with 1·2 ml ^{3}HHO (3·4 Ci) as described by Bennett *et al.* [19]. For hydrolysis to *p*-hydroxybenzaldehyde the morpholino derivative (390 mg) was dissolved in a soln made up of 3 ml EtOH, 30 ml 10% HCl and 6 ml concn HCl, and the soln was heated under N_2 for 2·5 hr at 100°. The soln was then conc *in vacuo* and the aldehyde extracted with Et_2O. The aldehyde was purified by vacuum sublimation at 40–60°. 4,2',4',6'-Tetrahydroxy-[β-^{3}H]-chalcone-2'-glucoside was obtained from the tritiated aldehyde and tetraacetylglucosidophloracetophenone [17] and purified by PC with 30% HOAc. For preparation of [2-^{3}H]-naringenin the chalcone-glucoside was refluxed for 1 hr with 20 ml 2·5% H_2SO_4 in 50% MeOH. After concn of the solution and addition of H_2O naringenin was extracted with Et_2O and purified by PC with 30% HOAc followed by chromatography on Sephadex LH-20 in MeOH.

Chromatography. For descending PC on Whatman 3 MM the following solvent systems were used: (1) HOAc–H_2O–conc HCl (30:10:3); (2) HCO_2H–2 N HCl–H_2O (5:2:3); (3) $CHCl_3$–HOAc–H_2O (10:9:3); (4) C_6H_6–HOAc H_2O (115:72:3). For TLC on cellulose (cellulose plates Merck AG, Darmstadt): (5) C_6H_6–HOAc–H_2O (2:2:1, upper phase) and on polyamide (polyamide-PA 11 plates, Merck AG, Darmstadt): (6) 15% HOAc; and (7) 30% HOAc were used.

Cell cultures. H. gracilis cell cultures were grown in the dark at 25–27° as described previously [2]. For illumination of the cultures light blue fluorescent tubes (Philips K40 W/18) with a light intensity of 27000 lx were used.

Experiments with addition of DMSO. Cells (0·5 fr. wt) were agitated for 30 min at 30° with 1·5 ml 0·1 M Tris–HCl, pH 7·5, containing 28 mM mercaptoethanol and 10% DMSO. After addition of the radioactive substrate incubation was continued for the time shown in the tables and the reaction stopped by heating the incubation mixture for 5 min in a boiling H_2O bath. After centrifugation for 10 min at 20000 *g* the supernatent was acidified with 1 drop of conc HCl and then extracted 2× with 1·5 ml EtOAc. The EtOAc extract was applied to paper and the chromatogram developed with solvent 3 or 4. To the aq. anthocyanin-containing phase was then added 1 g Duolite C-25 (H^+ form, C. Roth, Karlsruhe). After decoloration of the soln the ion exchanger was transferred to a small column and washed with H_2O, MeOH and 1% HCl. Anthocyanins were then eluted with MeOH–conc HCl (94:6).

Oxidation of dihydrokaempferol to kaempferol with magnesium bisulfite. Magnesium bisulfite soln was obtained by passing SO_2 under stirring and cooling with ice for 8 hr through an aq. suspension of $Mg(OH)_2$ [20]. Radioactive dihydrokaempferol (40000 dpm) was diluted with 0·5 mg carrier substance and the soln heated for 15 min at 100° with 4 ml of the Mg-bisulfite soln. After acidification with 2 N HCl the soln was extracted with EtOAc and the extract applied to a polyamide plate.

Preparation of microsomal fraction and incubation with substrates. In all expts the buffer used was 0·1 M K-Pi buffer, pH 7·5, containing 28 mM mercaptoethanol. In a prechilled mortar 20 g (fr. wt) of cells were mixed with 4 g PVP and 10 g quartz sand. After addition of 40 ml buffer the cells were homogenized for 10 min at 4°, and the homogenate was then centrifuged for 10 min at 10000 *g*. The supernatant was filtered through nylon gauze and centrifuged for 90 min at 90000 *g*. The microsomal pellet was taken up in 0·5 ml buffer and homogenized by hand in a glass homogenizer with a Teflon pestle. The incubation mixture consisted of 10 μl DMSO, 10 μl of NADPH (20 mM), 10 μl labeled substrate dissolved in ethyleneglycol monomethyl ether and 100 μl microsomal fraction. After incubation for 1·5 hr at 30° the reaction was terminated by addition of 0·2 ml 2 N HCl.

Measurement of radioactivity. Measurements of radioactivity were made with a Beckman model LS-233 scintillation spectrometer. Counting yield on chromatograms was about 55% for ^{14}C and 5% for ^{3}H.

Acknowledgements—This work was supported by Deutsche Forschungsgemeinschaft (SFB 46) and Fonds der Chemischen Industrie. We should like to thank Mr. H. Müller for skilled technical assistance.

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