

Conversion of Flavanone to Flavone, Dihydroflavonol and Flavonol with an Enzyme System from Cell Cultures of Parsley

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Soluble enzyme preparations from irradiated cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.) catalyse the conversion of flavanone to flavone, dihydroflavonol and flavonol. These reactions require 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors. In the presence of these cofactors conversion of dihydroflavonol to flavonol was also observed. With this system *in vitro* biosynthesis of radioactive flavone, dihydroflavonol and flavonol from $[2-^{14}\text{C}]$ malonyl-CoA and 4-coumaroyl-CoA in good yield and with high specific activity is possible.

We postulate that synthesis of flavone and flavonol from flavanone proceeds via 2-hydroxy- and 2,3-dihydroxyflavanone, respectively, with subsequent dehydration.

The microsomal fraction of the parsley cells contains an NADPH-dependent flavanone 3'-hydroxylase.

Introduction

Investigations on the biosynthesis of the flavones apigenin (**5**, Fig. 1) and chrysoeriol (3'-methylether of **6**) in parsley with radioactive precursors had shown that naringenin (**1**) but not dihydrokaempferol (**3**) is readily incorporated into these flavones [1]. It was therefore concluded that flavones originate from flavanones by dehydrogenation and not by dehydration of dihydroflavonols. Oxidation of flavanone to flavone was later shown with a cell-free extract from very young primary leaves of parsley, and furthermore it was proved that a flavanone and not the isomeric chalcone is the substrate of this reaction [2]. In addition to molecular oxygen and Fe^{2+} ions, the soluble enzyme preparation required another nonproteinaceous cofactor which was not identified.

Recently we had shown that flowers of *Matthiola incana* contain a soluble flavanone 3-hydroxylase which requires 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors [3]. We now report on the occurrence of the flavanone 3-hydroxylase reaction in illuminated cell suspension cultures of parsley. Much to our surprise this enzyme preparation also catalyzed a

conversion of flavanone to flavone and of dihydroflavonol to flavonol dependent on 2-oxoglutarate, ascorbate and Fe^{2+} . In addition the microsomal fraction contained an NADPH-dependent flavanone 3'-hydroxylase.

Materials and Methods

Parsley cell cultures

Cell suspension cultures of parsley (*Petroselinum hortense* Hoffm.) were propagated in the dark and irradiated for 24 h for enzyme induction as described [4].

Buffers

Buffer A, 0.1 mol/l Tris-HCl, pH 7.5, 14 mmol/l 2-mercaptoethanol;
Buffer B, 0.1 mol/l Tris-HCl, pH 7.5, 2.8 mmol/l 2-mercaptoethanol;
Buffer C, buffer A, containing 10% glycerol;
Buffer D, 0.01 mol/l Tris-HCl, pH 7.5, 2.8 mmol/l 2-mercaptoethanol, 10% glycerol.

Chemicals and synthesis of substrates

$[2-^{14}\text{C}]$ Malonyl-CoA (2.18 GBq/mmol) obtained from Amersham Buchler was diluted to 0.78 GBq/mmol with unlabelled material from Sigma. $[4\text{a},6,8-^{14}\text{C}]$ Naringenin and $[4\text{a},6,8-^{14}\text{C}]$ eriodictyol (both 2.36 GBq/mmol) were prepared enzymatically with crude chalcone synthase (45–80% saturation am-

Abbreviations: API, apigenin; DHK, dihydrodaempferol; NAR, naringenin; KAE, kaempferol; BNY, bis-noryangonin; MAA, malonic acid; DEDTC, diethyldithiocarbamate; DEPC, diethylpyrocarbonate; *p*CMB, *p*-chloromercuribenzoate.

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monium sulfate precipitate desalted over Sephadex G-50 using buffer A) as described which includes chalcone isomerase [3]. [$4a,6,8^{-14}C$]Dihydrokaempferol was prepared by addition of 250 $\mu\text{mol/l}$ 2-oxoglutarate and 50 $\mu\text{mol/l}$ ferrous sulfate to the incubation mixture for synthesis of radioactive naringenin or from isolated [^{14}C]naringenin as described below. The substrates were purified by thin-layer chromatography on cellulose plates using solvent system 1 and were extracted from the adsorbent with methanol. Aliquots containing 10000 dpm (73 pmol) were evaporated directly before use. Authentic flavonoids were from our collection.

Preparation of crude extract and of microsomal fraction

20 g freshly harvested parsley cells were homogenized in a chilled mortar together with 10 g quartz sand, 2 g equilibrated Dowex 1 \times 2 and 10 ml buffer A. Centrifugation at 9000 $\times g$ for 10 min gave supernatant S_9 . The membrane fraction was prepared from S_9 by Mg^{2+} precipitation [3]. Addition of 30 mmol/l magnesium chloride and centrifugation at 40000 $\times g$ for 20 min gave supernatant S_{40} and a pellet. After carefully washing the pellet with buffer B, it was dissolved in 2 ml buffer B and homogenized in a glass homogenizer.

For enzyme preparations from frozen cells stored at -20°C the 40000 $\times g$ supernatant (SS_{40}) was prepared without Mg^{2+} precipitation. The ammonium sulfate fraction (45–80% saturation) was obtained from extracts prepared as described in [5]. The protein was stored in buffer C at -20°C after freezing with liquid nitrogen.

For removal of cofactors or for desalting protein preparations were subjected to gel filtration on Sephadex G-50 with buffer B.

Analytical methods

Protein was determined as described in [3]. Column chromatography was performed on Sephadex LH-20 (0.9 \times 26 cm) with methanol (7 ml/h) [6]. The elution profiles of products were determined either by measuring aliquots of fractions for radioactivity in a scintillation spectrometer using 0.5% PPO in toluene or by determining optical density in a Perkin-Elmer UV/VIS spectrophotometer at the appropriate wavelengths [7]. For thin-layer chromatography on cellulose plates (E. Merck) solvent system

(1) 15% acetic acid and (2) chloroform/acetic acid/water (10:9:1, v/v/v) were used. On silica gel plates (Nano plates SIL-20 UV₂₅₄, Macherey-Nagel and Co.) solvent systems (3) chloroform/methanol/formic acid (93:6:1, v/v/v) and (4) benzene/acetic acid/water (125:72:3, v/v/v) were used. Flavonoids were detected under UV light or after spraying with 0.1% aqueous fast blue B salt followed by exposure to ammonia vapors. Radioactivity was localized either by scanning the plates or by autoradiography (Kodak X-Omat XR-5). The radioactive zones were scraped off and counted as described for radioactive column fractions.

Identification of reaction products

A mixture of approximately 22000 dpm of each product was chromatographed together with 100 nmol authentic carrier on Sephadex LH-20 and localized as described under analytical methods. The products were further identified on cellulose plates (2000 dpm) with solvent systems 1 and 2 and on high-performance thin-layer plates (400 dpm) by a two-dimensional technique with solvent system 3 (twice) and 4. Autoradiography (5 d at -20°C) and visualization of carrier was performed as described under analytical methods.

Assay for flavanone 3-hydroxylase

The assay system contained in 100 μl total volume 5 μmol Tris-HCl (pH 7.5), 0.28–0.7 μmol 2-mercaptoethanol, 73 pmol radioactive substrate, 25 nmol 2-oxoglutaric acid, 5 nmol ferrous sulfate and enzyme. After incubation for 5–60 min at 30 $^\circ\text{C}$ the reaction was stopped by addition of approx. 10 mg EDTA. After sonication for 2 min (Bandelin sonifier bath) 5 μl ethanolic carrier solution containing 4 μg naringenin, 2 μg apigenin, 2 μg kaempferol, 4 μg dihydroquercetin, 2 μg luteolin and 2 μg quercetin was added. The phenolics were extracted with ethyl acetate and chromatographed on a cellulose plate with solvent system 1. Radioactivity was measured as described under analytical methods.

Assay for flavone formation

The assay was carried out as described for flavanone 3-hydroxylase except that the incubation mixture also contained 100 nmol sodium ascorbate and S_9 or SS_{40} after equilibration with buffer A on

Sephadex G-50. The extracted phenolics were chromatographed with solvent system 2 or with solvent system 1 followed by solvent system 2 up to the previously marked spot of dihydroquercetin or dihydrokaempferol as described under identification of reaction products.

Assay for flavonol formation

The assay was carried out as described for apigenin formation using (73 pmol) dihydrokaempferol as substrate.

Dependence of reaction on pH

S_9 or SS_{40} was equilibrated with buffer D on Sephadex G-50. Enzyme assays were carried out in 0.15 mol/l K-phosphate buffer.

Results

Enzyme preparation

Seven-day-old dark grown parsley cell cultures which had been illuminated for 24 h [4] were used for enzyme preparations as described in Methods. The enzyme preparations used are listed in Table I.

Enzymatic products from $[^{14}C]naringenin$ and $[^{14}C]dihydrokaempferol$

Incubation of $[^{14}C]naringenin$ (1, Fig. 1) with a cell-free extract of parsley cells from which low

Table I. Enzyme preparations used in the experiments. For details see Methods.

Enzyme source	Type of preparation	Abbreviation
Fresh parsley cells	9000 $\times g$ supernatant	S_9
	40000 $\times g$ supernatant after precipitation of microsomes with Mg^{2+}	S_{40}
	microsomal pellet	P_{40}
Frozen parsley cells	40000 $\times g$ supernatant	SS_{40}
	45–80% ammonium sulfate fraction	—

Enzyme preparations were used as such or after removal of low molecular weight substances by gel filtration on Sephadex G-50.

molecular weight substances had been removed by gel filtration on Sephadex G-50 gave no reaction products (Fig. 2a). When however 2-oxoglutarate, Fe^{2+} and ascorbate were added to such an incubation mixture, two radioactive reaction products were detected by thin-layer chromatography (Fig. 2b). These two products were identified as apigenin (5) and dihydrokaempferol (3) as described in the next section. By rechromatography of the zone near the origin in solvent system 2 it was shown that in addition a small amount of kaempferol (7) was formed which ran together with apigenin in solvent system 1.

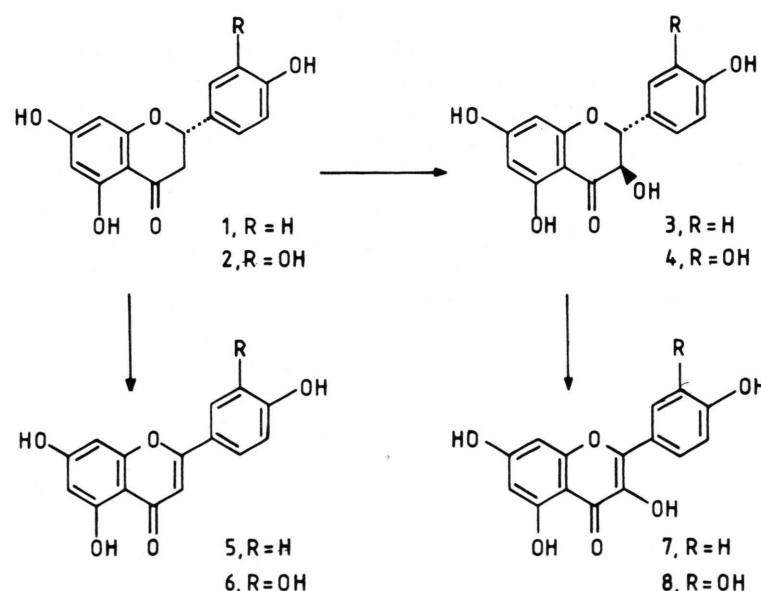


Fig. 1. Reaction products of naringenin (1) or eriodictyol (2) with enzyme preparations of parsley cell cultures in presence of 2-oxoglutarate, Fe^{2+} , and ascorbate. 3, Dihydrokaempferol; 4, dihydroquercetin; 5, apigenin; 6, luteolin; 7, kaempferol; 8, quercetin.

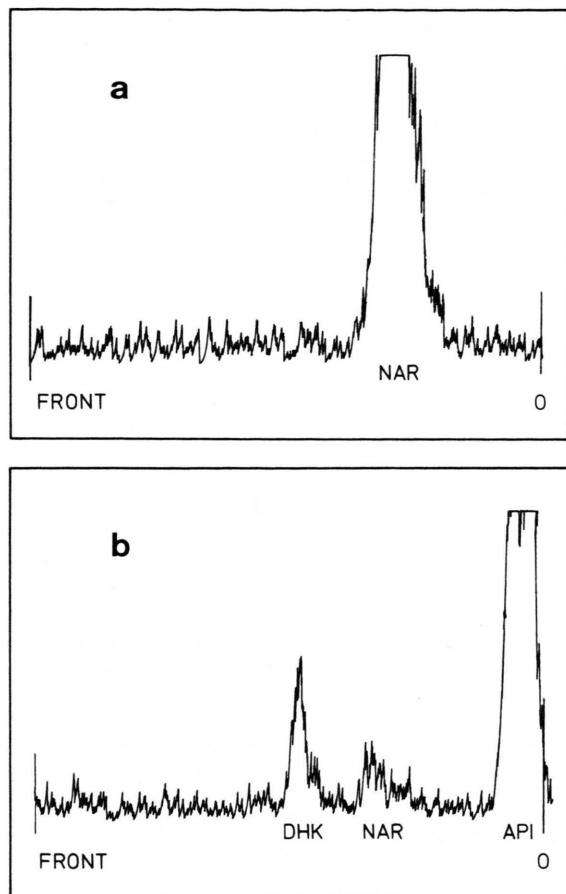


Fig. 2. a) Radioscan of TLC on cellulose with solvent system 1 from an incubation of [¹⁴C]naringenin with S₉ after gel filtration without additions. b) Same as (a) but with addition of 2-oxoglutarate, Fe²⁺, and ascorbate. A small amount of kaempferol runs together with apigenin.

Apigenin, dihydrokaempferol and kaempferol were also formed, albeit in varying yields, when cell-free extracts obtained from fresh cells or cells frozen for a short time were used which had not been subjected to gel filtration.

Incubation of [¹⁴C]dihydrokaempferol with this enzyme preparation and the three cofactors leads to formation of kaempferol (Fig. 3b). Again it was shown that all three cofactors are needed for this reaction (Fig. 3a).

It was clear from these results that the crude extract contains several enzyme activities. To obtain more information on this complex system the different enzyme preparations listed in Table I were incubated under various conditions and the prod-

ucts analysed. The results summarized in Table II allow the following conclusions. With naringenin as substrate apigenin is produced as the major product with concomitant formation of dihydrokaempferol. In some experiments in which the yield of apigenin is high the yield of dihydrokaempferol seems to decrease. This is however due to competition for the common substrate, which becomes yield limiting. In all cases the highest yields are obtained when all 3 cofactors are added to the incubation. Whereas kaempferol formation from naringenin is low, a good yield of kaempferol is obtained with dihydrokaempferol as substrate. No conversion of dihydrokaempferol to apigenin could be detected.

In addition to these soluble enzyme activities the microsomal pellet contains an NADPH-dependent

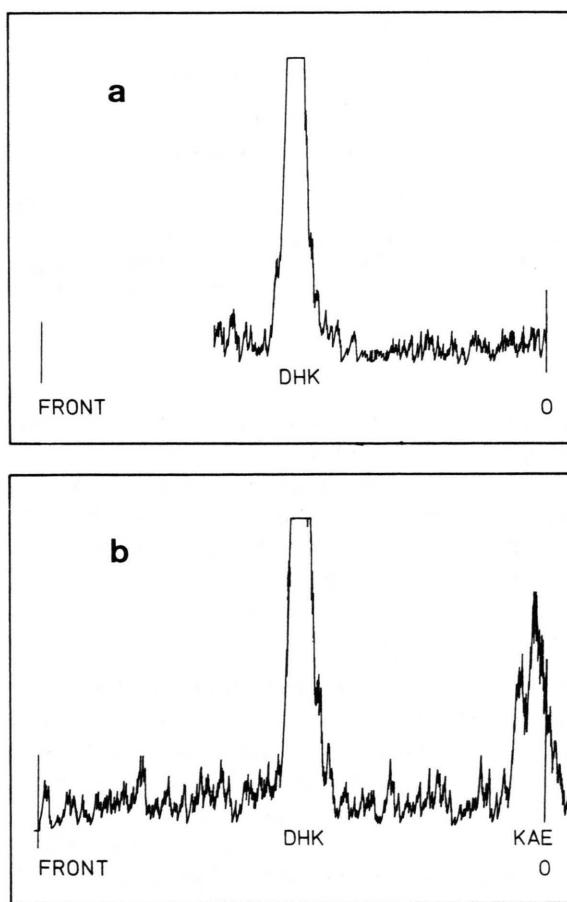


Fig. 3. a) Radioscan of TLC on cellulose with solvent system 1 from an incubation of [¹⁴C]dihydrokaempferol with S₉ after gel filtration without additions. b) Same as (a) but with addition of 2-oxoglutarate, Fe²⁺, and ascorbate.

Table II. Cofactor requirement and subcellular localization of hydroxylating activities in cell suspension cultures of parsley.

Enzyme source (μ g protein per incubation)	Cofactor added	cpm in:			
		Apigenin ^a	Dihydrokaempferol ^a (Dihydroquercetin) ^b	Kaempferol	Eriodictyol ^a
S ₉ (23)	none	1750	230	0	0
	NADPH	2460	300	0	0 ^c
	ascorbate + 2-oxoglutarate + Fe ²⁺	6130 ^d	370	—	0
S ₄₀ (22)	none	3190	500	0	0
	ascorbate + 2-oxoglutarate + Fe ²⁺	5560 ^d	380	—	0
S ₄₀ after gel filtration (15)	2-oxoglutarate + Fe ²⁺	2170	1230	0	0
	ascorbate + 2-oxoglutarate + Fe ²⁺	5040 ^d	830	(1200) ^e	0
P ₄₀ (29)	NADPH	180	(840) ^b	0	3900
	NADH	170	(200) ^b	0	1470
	ascorbate + 2-oxoglutarate + Fe ²⁺	690	270	0	0 ^c
SS ₄₀ after gel filtration	none	0 ^f	0 ^f	0 ^g	0
	NADPH	0	0	0	0
	ascorbate	0	0	0	0
	2-oxoglutarate	130	0	0	0
	Fe ²⁺	410	110	0	0
	ascorbate + 2-oxoglutarate	730	190	0	0
	ascorbate + Fe ²⁺	670	180	0	0
	2-oxoglutarate + Fe ²⁺	1010	840	0	0
	ascorbate + 2-oxoglutarate + Fe ²⁺	2720 ^d	920	(980) ^e	0

^a Product formed from 10000 dpm [¹⁴C]naringenin. Total radioactivity recovered from the plates was 70 per cent.

^b Mostly dihydroquercetin in incubations with P₄₀ and NADPH. ^c Eventual traces may be hidden under tailing naringenin. ^d Eventual traces of kaempferol are included in apigenin. ^e Product formed from 10000 dpm [¹⁴C]dihydrokaempferol. Total radioactivity recovered from the plates was 50 per cent. ^f Low enzyme activities may be observed due to the presence of residual traces of endogenous cofactors. ^g Not detectable in radioscans.

flavanone 3'-hydroxylase which catalyzes the formation of eriodictyol (**2**) from naringenin.

Eriodictyol is also substrate for the soluble enzyme system mentioned above; in this case luteolin (**6**) dihydroquercetin (**4**) and quercetin (**8**) are formed.

Product identification

All enzymatic products were unequivocally identified by chromatography with authentic carrier substances on cellulose plates with solvent systems 1 and 2 and by two-dimensional high performance thin-layer chromatography and autoradiography as described under Methods. Furthermore the elution volumes and radioactivity profiles were determined on a Sephadex LH-20 column (Table III). Fig. 4 shows a representative example in which the presence of radioactive dihydrokaempferol and apigenin is demonstrated.

Further properties of the soluble enzyme system

Enzyme activity for conversion of naringenin or dihydrokaempferol is only present in extracts from illuminated parsley cell cultures. Dark grown cultures are devoid of this activity.

A desalted ammonium sulfate fraction (45–80% saturation) from an extract of frozen cells (SS₄₀)

Table III. Ratios of elution volume to void volume of flavonoids on Sephadex LH-20 with methanol (cf. ref. [6]).

Substance	Ve/Vo
Naringenin	4.8
Eriodictyol	5.4
Dihydrokaempferol	4.3
Dihydroquercetin	4.7
Kaempferol	6.7
Quercetin	7.6
Apigenin	5.2
Luteolin	5.9

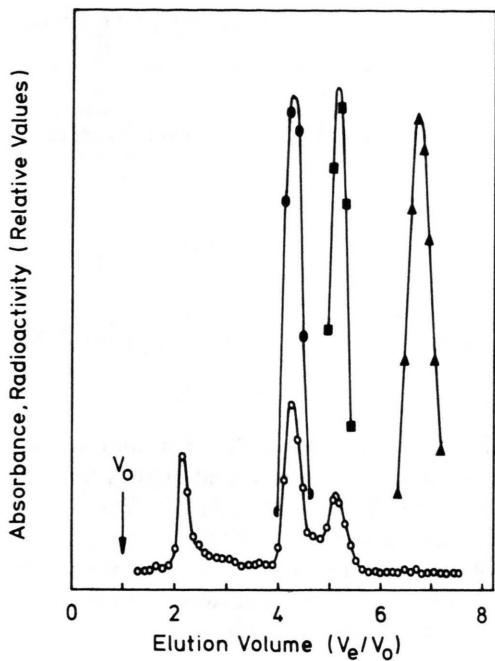


Fig. 4. Sephadex LH-20 chromatogram of ethyl acetate extracts from incubations of S_8 after gel filtration and addition of 2-oxoglutarate and Fe^{2+} . (●) Dihydrokaempferol, (■) apigenin, (▲) kaempferol, (○) radioactivity. The peak at V_e/V_0 2.1 is from an unknown product. A small amount of naringenin is located at V_e/V_0 4.8.

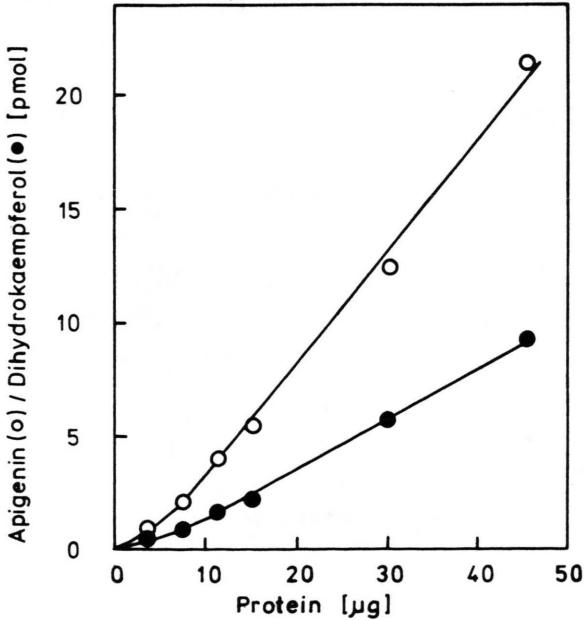


Fig. 5. Dependence of apigenin and dihydrokaempferol synthesis on protein concentration. The assay contained 73 pmol naringenin.

gave upon incubation with [^{14}C]naringenin, 2-oxoglutarate, Fe^{2+} and ascorbate about equal amounts of apigenin, dihydrokaempferol and kaempferol. With the same incubation system but without addition of ascorbate the relative yield of dihydrokaempferol increased and that of kaempferol decreased.

In the complete assay system synthesis of apigenin and dihydrokaempferol was linear with protein concentration up to about 50 μ g protein (Fig. 5) and with time for about 30 min.

The pH optimum for apigenin formation is about 7. For conversion of naringenin to dihydrokaempferol and of dihydrokaempferol to kaempferol a pH optimum around 6.5 was observed.

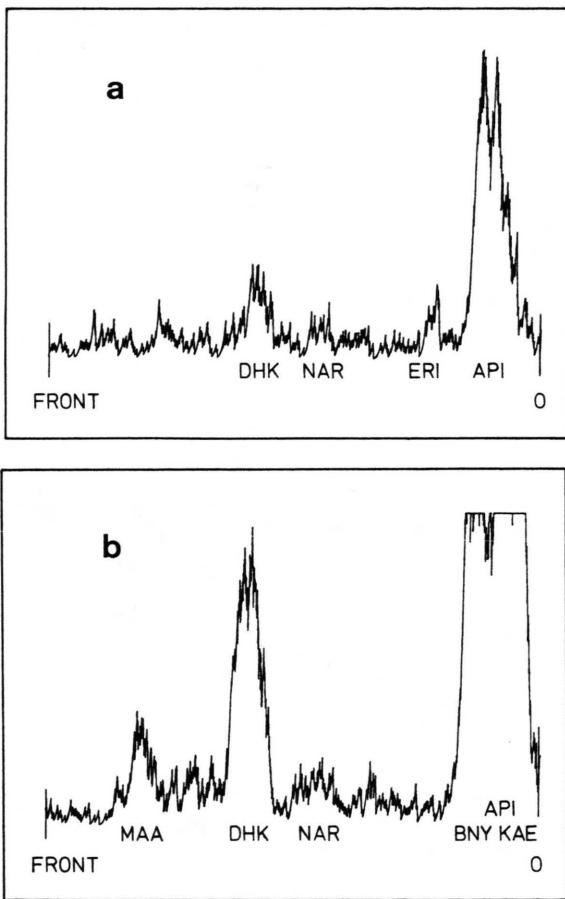


Fig. 6. a) Radioscan of TLC on cellulose with solvent system 1 from an incubation of [$2-^{14}C$]malonyl-CoA and 4-coumaroyl-CoA with S_8 in presence of 2-oxoglutarate, Fe^{2+} , and ascorbate. b) Same as (a) but with ammonium sulfate fraction (Table I).

Table IV. Effect of various inhibitors on the formation of apigenin and dihydrokaempferol from naringenin. Enzyme preparation S₉ with 2-oxoglutarate, Fe²⁺ and ascorbate was used.

Inhibitor	Concentration [mmol/l]	Apigenin		Relative activity [%] ^b	
		[cpm] ^a	[cpm] ^a	Apigenin	Dihydrokaempferol
none		3190	700	100	100
EDTA	1	500	260	16	34
KCN	5	380	n.d. ^c	12	—
DEDTC	2	2200	770	68	100
DEPC	0.2	2820	700	88	90
pCMB	1	3235	790	100	100

^a The assay contained 10000 dpm [¹⁴C]naringenin. ^b Relative activities are corrected for recovered radioactivity. ^c Not detectable.

Table V. Effect of various inhibitors on the formation of kaempferol from dihydrokaempferol. Enzyme assay as in Table III.

Inhibitor	Concentration [mmol/l]	Kaempferol [cpm] ^a	Relative Activity [%]
none		820	100
EDTA	1	44	5
KCN	5	100	11
DEDTC	2	80	9
DEPC	0.5	830	100
pCMB	1	390	43

^a The assay contained 10000 dpm [¹⁴C]dihydrokaempferol.

In inhibition experiments with naringenin as substrate strong inhibition was found with 1 mmol/l EDTA and 5 mmol/l KCN (Table IV). These substances also had a strong inhibitory effect on conversion of dihydrokaempferol to kaempferol. In addition diethyldithiocarbamate was a strong inhibitor for the latter reaction (Table V).

In vitro biosynthesis of flavonoids from 4-coumaroyl-CoA and malonyl-CoA

Since chalcone synthase and chalcone isomerase are present in soluble enzyme preparations from illuminated parsley cells [16], incubation of 4-coumaroyl-CoA and [2-¹⁴C]malonyl-CoA with S₉ (Table

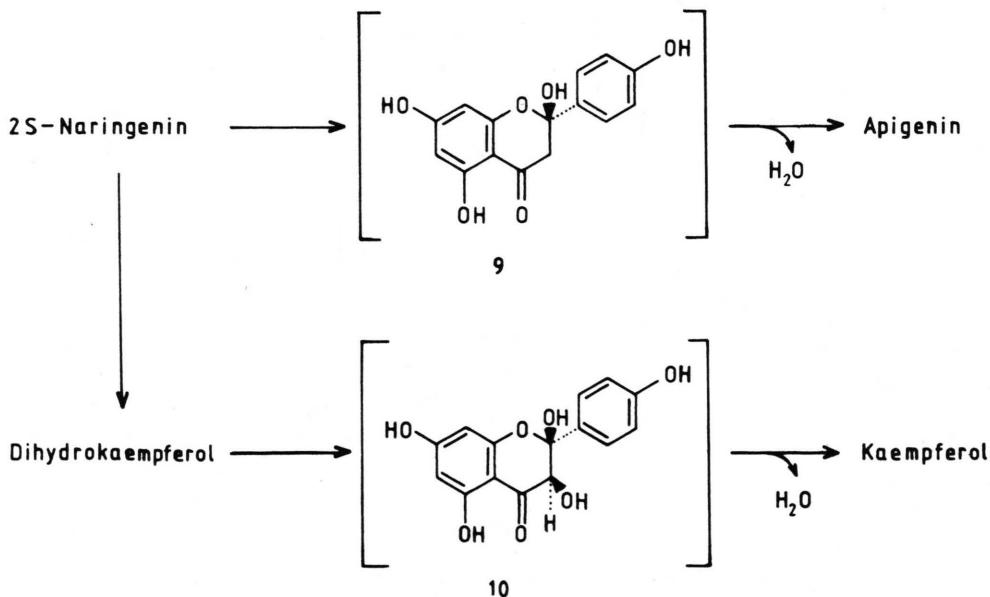


Fig. 7. Hypothetical reaction sequence from naringenin to apigenin and kaempferol *via* 2-hydroxyflavanones.

I) in presence of 2-oxoglutarate, Fe^{2+} and ascorbate leads directly to formation of apigenin as major product together with a small amount of dihydro-kaempferol and eventually kaempferol (Fig. 6a). When the ammonium sulfate fraction (Table I) is used as enzyme source the yield of dihydro-kaempferol and kaempferol increases (Fig. 6b). In addition the formation of apigenin, bis-noryangonin [17] and free malonic acid is observed. When ascorbate is omitted from such an incubation dihydro-kaempferol becomes the major reaction product.

Discussion

The missing nonproteinaceous cofactor for the biosynthesis of flavones from flavanones [2] has now been identified as 2-oxoglutarate. The *in vivo* incorporation of ^{14}C -labelled dihydroflavonols into flavonols had been shown previously in *Pisum sativum* [8] and *Datisca cannabina* [9]. The present results prove the *in vitro* conversion of dihydroflavonols to flavonols, which also requires 2-oxoglutarate, Fe^{2+} ions and ascorbate. The fact that both flavanone 3-hydroxylation and formation of flavones and flavonols require the typical cofactors for 2-oxoglutarate-dependent dioxygenases [10] is best rationalized by the hypothesis that flavone and flavonol synthesis proceeds *via* 2-hydroxyflavanone and 2,3-dihydroxyflavanone, respectively. Such a reaction sequence would be expected to involve two enzymes: a 2-hydroxylase and a dehydratase. This hypothetical reaction sequence is shown in Fig. 7.

Indirect evidence for a role of 2-hydroxyflavanones in flavone biosynthesis is available from the literature.

Because 2,4,6-trihydroxydibenzoylmethane 4-glucoside occurs together with the corresponding flavone (chrysanthemum 7-glucoside) in leaves of a *Malus* species, it was assumed that *o*-hydroxydibenzoylmethanes could be intermediates in flavone bio-

synthesis [11]. Chopin *et al.* later showed that all 2,6-dihydroxydibenzoylmethanes exist in cyclic hemiketalic form as 2,5-dihydroxyflavanones [12, 13]. Such compounds are easily converted to flavones by loss of water. It was further shown that 2,3,5,7,4'-pentahydroxyflavanone (**10**, Fig. 7) and 2,3,4,7,3'4'-hexahydroxyflavanone dehydrate under mild conditions to kaempferol and quercetin, respectively [14]. In *Populus nigra* buds [18] and in stems of *Unona lawii* [19] substituted 2,5-dihydroxyflavanones occur together with the corresponding flavones.

Separation and purification of the individual enzyme is necessary before studies on their mechanism can be carried out. The *in vitro* synthesis of flavones, flavonols and dihydroflavonols from $[2-^{14}\text{C}]$ malonyl-CoA and 4-coumaroyl-CoA is a convenient method to obtain these compounds in labelled form in good yield and with high specific activity.

Parallel to our investigation Stotz and Forkmann have studied the oxidation of flavanones to flavones with extracts of snapdragon flowers (*Antirrhinum majus*) [15]. Surprisingly the enzymatic activity for this reaction in *A. majus* is localized in the microsomal fraction and requires NADPH as cofactor. This is one of the rare cases in which the same product is formed by two distinctly different enzymatic reactions. Perhaps this is of significance in the consideration of the evolution of flavonoid biosynthesis in plants.

Cell cultures of parsley again proved to be a treasure chest for new enzymes of flavonoid biosynthesis. Work to separate and purify the individual enzymes is in progress.

Acknowledgements

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