COMPARATIVE BIOSYNTHETIC PATHWAYS IN HIGHER PLANTS

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ABSTRACT

During the last ten years or so, variation in secondary plant products between different taxa has been increasingly used in systematic and evolutionary studies. However successful this approach has been, it now needs to be tempered by a consideration of the underlying variation in the biosynthetic pathways leading to the compounds in question. In some cases cognate compounds are formed by two or more pathways in different taxa; in others the same biosynthetic route is regulated in different ways. It is necessary, therefore, to examine the ontogeny and characteristics of the individual enzymes involved. Examples of such approaches are given for a large number of different classes of compound, including naphthoquinones, flavonoids, *meta*-carboxy aromatic amino acids, branched chain sugars and lignin.

INTRODUCTION

The contribution which the investigation of biosynthetic pathways can make to the study of evolution and systematics can be divided into three main areas: (*a*) the elucidation of general biosynthetic patterns by tracer techniques; (*b*) comparative biochemistry of individual biosynthetic steps and of the enzymes involved; and (*c*) comparative biochemistry of regulatory systems.

A large number of biosynthetic pathways in higher plants have now been outlined, mainly by tracer techniques, and it is not my intention to cover the whole field in this review. Rather I should like to concentrate my presentation on a few selected pathways. Unfortunately, the situation is completely different in the case of areas (*b*) and (*c*). The enzymology of biosynthetic pathways in higher plants is just in its infancy, and here we have to rely heavily on knowledge gained with enzymes from lower plants and microorganisms. The same applies to our knowledge of regulatory systems.

DIFFERENT BIOSYNTHETIC ROUTES LEADING TO IDENTICAL OR SIMILAR PRODUCTS

To begin with I should like to discuss briefly the importance of different biosynthetic routes which lead to identical or similar products. In chemical plant taxonomy, the analysis of secondary plant products is the predominant

method of investigation. The study of biosynthetic pathways has shown that the occurrence of the same secondary plant product in different taxa of the plant kingdom cannot be taken as evidence for a close relationship unless the identity of the biosynthetic pathway has been proved.

The most impressive example of the fact that compounds with the same carbon skeleton can be formed by different biosynthetic routes is that not less than four different pathways have been found which lead to the formation of the carbon skeleton of naphthoquinones in higher plants. These are: the acetate, shikimate, *p*-hydroxybenzoic acid, and toluhydroquinone pathways[1] (*Figure 1*).

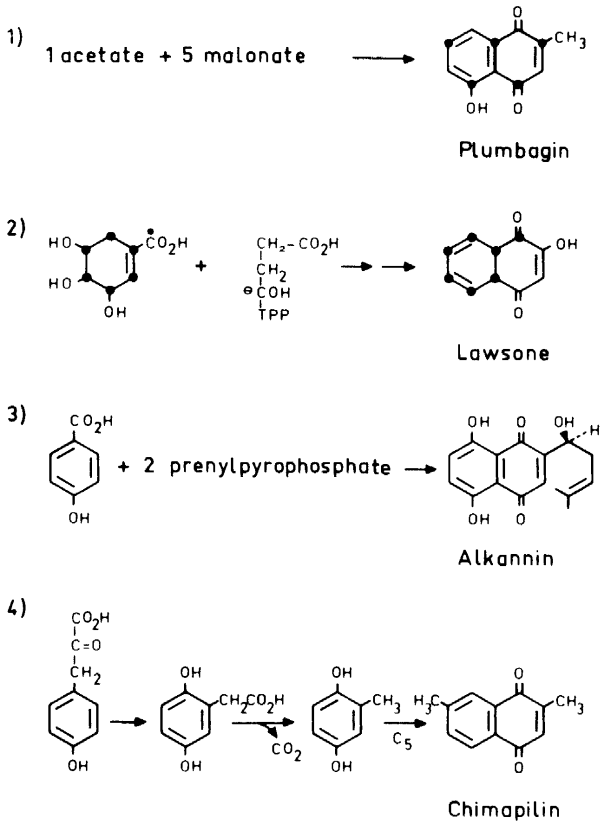


Figure 1. Four different biosynthetic routes to naphthoquinones.

Anthraquinones can also be formed by two—and maybe more—different routes. Chrysophanol (1) and emodin (2) (*Figure 2*) are formed in leaves and roots of *Rheum* and *Rumex* (Polygonaceae) as well as in branches of *Rhamnus* (Rhamnaceae) by the acetate pathway[2]. Similar results have been obtained with fungi[3]. However, tracer experiments with *Rubia tinctorium* have shown that shikimic acid and mevalonic acid, rather than acetic acid, serve as primary precursors for alizarin (3) and purpurin (4)[4]. The three remaining

C-atoms of the anthraquinone nucleus are very probably derived from α-oxoglutarate via succinyl-semialdehyde[4–6] (*Figure 2*). On the basis of these findings it seems that a relationship exists between substitution pattern and biosynthesis of anthraquinones, but more work has to be done to verify such a conclusion, which would help to classify the anthraquinones for the taxonomist.

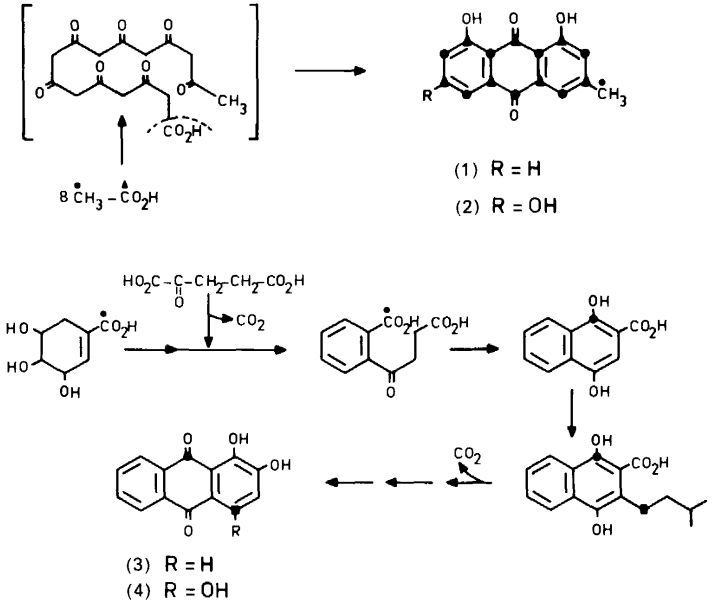


Figure 2. Different routes to anthraquinones. (1) chrysophanol; (2) emodin; (3) alizarin; (4) purpurin.

Different biosynthetic routes also exist for the 9-phenylperinaphthenone pigments, found so far only in plants of the monocotyledenous family *Haemodoraceae*, and for the mould perinaphthenones[7, 8]. The former are formed through combination of one acetate residue with two shikimate-derived C_6–C_3 units, whereas the latter are acetate-derived (*Figure 3*). However, in this case the 9-phenyl substituent allows easy differentiation between these two groups.

C_6–C_1 compounds can be formed in higher plants by the following different biosynthetic pathways: the acetate pathway; direct formation from shikimic acid; and formation from phenylpropane compounds through loss of two carbon atoms[9].

The biosynthesis of 4-hydroxy-, 3,4-dihydroxy- and 2-hydroxybenzoic acids and of related C_6–C_1 compounds occurs in higher plants mainly by degradation of the corresponding cinnamic acids[9, 10]. However, when the incorporation of ^{14}C-labelled shikimic acid into benzoic acids of *Quercus*

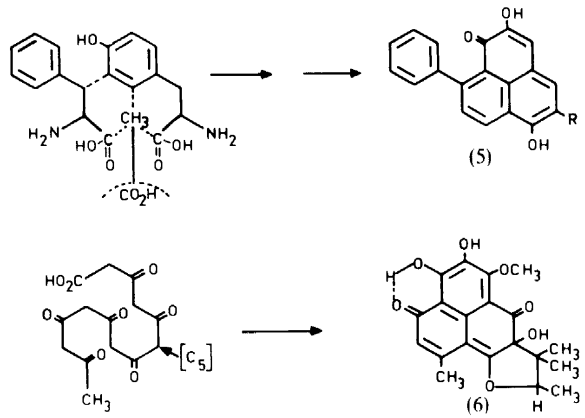


Figure 3. Different biosynthetic routes to perinaphthenones. (5) algycone of haemocorin from *Haemodorum* spp.; (6) herqueinone from *Penicillium herquei*.

pedunculata was compared with the incorporation of L-[U-^{14}C]-phenylalanine, good incorporation of radioactivity from shikimic acid into protocatechuic acid and gallic acid as well as into 5-dehydroshikimic acid was found, whereas only very little radioactivity appeared in these acids with phenylalanine as precursor[11]. It therefore seems that protocatechuic acid can be formed in higher plants directly from 5-dehydroshikimic acid, a reaction which was first observed in a mutant of *Neurospora crassa* and also occurs in other microorganisms[12]. The biosynthesis of gallic acid occurs mainly or exclusively by dehydrogenation of dehydroshikimic acid[13] (*Figure 4*).

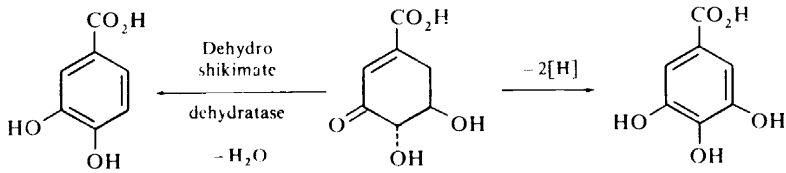


Figure 4. Biosynthesis of protocatechuic and gallic acids

How complicated the determination of a biosynthetic pathway can be, is exemplified by an experiment carried out by Kindl[22] in which he infused a mixture of [U-$^{14}C_7$]-shikimic acid and D,L-[3-^{14}C]-phenylalanine into leaves of *Astilbe chinensis* and *Sinapis alba*. With *A. chinensis*, the isolated salicyclic acid and *p*-hydroxybenzoic acid contained respectively 77 per cent and 8 per cent of their radioactivity in the carboxyl group, whereas with *S. alba* the corresponding figures were 75 per cent and 47 per cent (*Figure 5*). When [U-$^{14}C_7$]-shikimic acid alone was fed to the leaves less than 2 per cent

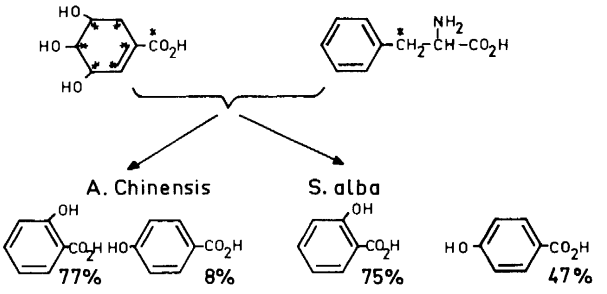


Figure 5. Incorporation of [U-^{14}C]-shikimic acid and [3-^{14}C]-phenylalanine into salicylic and *p*-hydroxybenzoic acid in different plants.

of the total radioactivity of the benzoic acids was located in their carboxyl group. Since the carboxyl group of shikimic acid is lost when a benzoic acid is formed by the pathway phenylalanine → cinnamic acid → *p*-coumaric acid the benzoic acids in both plants are obviously not formed directly from shikimic acid. The low incorporation of radioactivity from D,L-[3-^{14}C]-phenylalanine into the carboxyl group of *p*-hydroxybenzoic acid in *S. alba* could be explained by assuming that synthesis takes place by the pathway shikimic acid → tyrosine → *p*-coumaric acid. In several higher plants phenylalanine cannot be converted into tyrosine. Since, however, cinnamic acid can be hydroxylated to *p*-coumaric acid, this explanation is not completely satisfactory.

As a final example I should like to mention the biosynthesis of L-querbrachitol (9). In *Acer pseudoplatanus* (9) is formed from myo-inositol (7) via D-bornesitol (8), whereas in *Artemisia vulgaris* L-inositol (10) is the immediate precursor of (9)[14] (*Figure 6*).

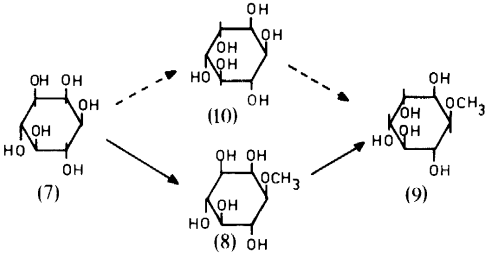
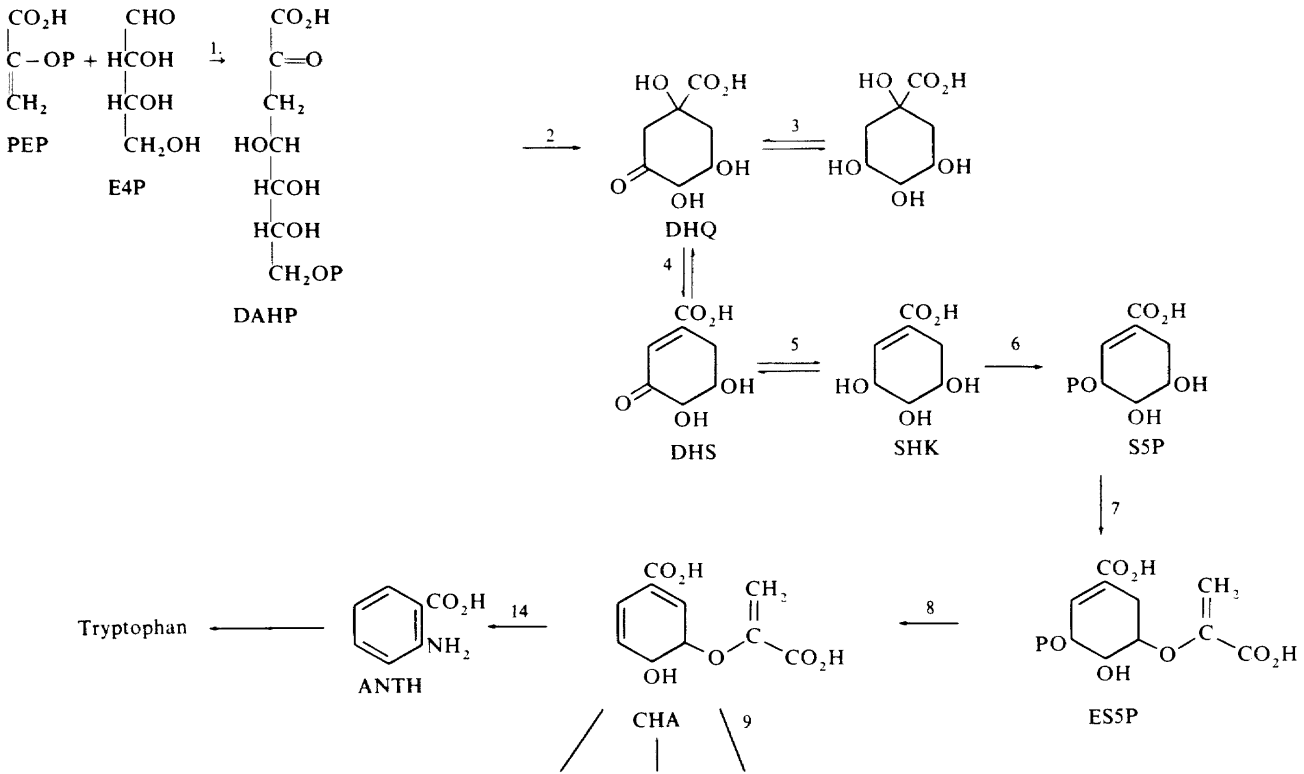


Figure 6. Different biosynthetic routes to L-querbrachitol (9) ———, in *Acer pseudoplatanus*; – – – –, in *Artemisia vulgaris*: (7) myo-inositol; (8) D-bornesitol; (10) L-inositol.

If the situation were as complex in all cases as it is in naphthoquinone, anthraquinone, or benzoic acid biosynthesis, chemical plant taxonomists would be in a difficult position. Fortunately, however, only one biosynthetic route seems to exist for most major classes of secondary plant products. Nevertheless, the above-mentioned examples should serve as a warning against careless generalizations.


PEP
E4P
DAHP
1
2
DHQ
3
4
DHS
5
SHK
6
S5P
7
ES5P
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CHA
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ANTH
14
Tryptophan

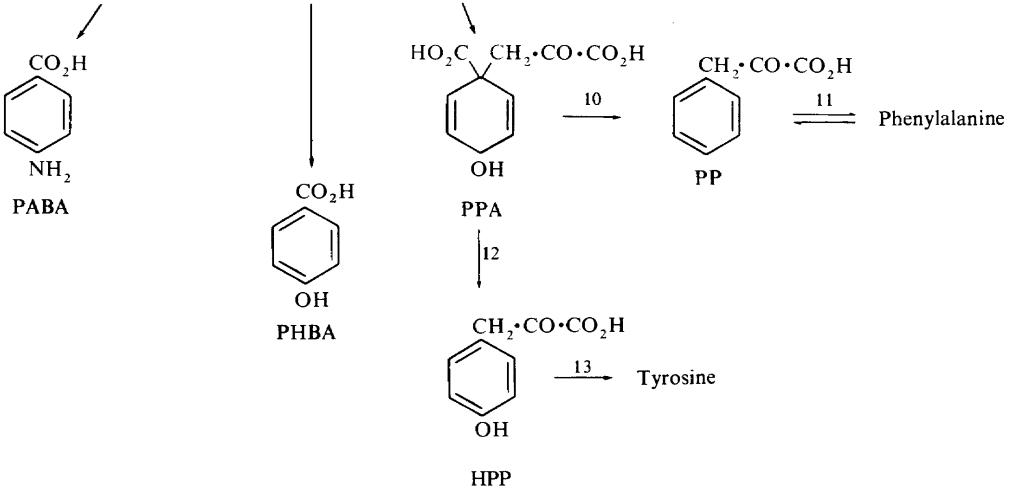


Figure 7. The shikimic acid pathway. 1—DAHP synthetase; 2—dehydroquinate (DHQ) synthetase; 3—quinate dehydrogenase (in higher plants); 4—5-dehydroquinase; 5—5-dehydroshikimate (DHS) reductase; 6—shikimate kinase; 7—3-enol-pyruvylshikimate-5-phosphate (ES5P) synthetase; 8—chorismate (CHA) synthetase; 9—chorismate mutase; 10—prephenate (PPA) dehydratase; 11—phenylalanine transaminase; 12—prephenate dehydrogenase; 13—tyrosine transaminase; 14—anthranilate (ANTH) synthetase; PABA, *p*-aminobenzoic acid; PHBA, *p*-hydroxybenzoic acid.

THE SHIKIMIC ACID PATHWAY

One of the major routes to secondary plant products is the shikimic acid pathway[15]. This pathway has been evolved in prokaryotes and eukaryotes, and the comparative biochemistry of this pathway and of the products arising from it is one of the best examples of what can be expected from such an approach to evolution and systematics.

The primary end products of this pathway, originating from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P), are the aromatic amino acids phenylalanine, tyrosine, and tryptophan (*Figure 7*). Condensation of PEP with E4P leads to the formation of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP). This compound is converted to chorismic acid through a series of reactions which constitute the common segment of the aromatic pathway. From chorismate one metabolic branch diverges to tryptophan and the second branch bifurcates to form phenylalanine and tyrosine.

Important controlling enzymes for the regulation of this pathway are 1) DAHP synthetase, 2) anthranilate synthetase (14; *Figure 7*), the first enzyme of the tryptophan branch and 3) chorismate mutase, catalyzing the conversion of chorismic to prephenic acid.

The regulation of this complex pathway has been studied extensively mainly in bacteria, *Neurospora* and *Saccharomyces*[15]. Only a few important points relevant to the subject should be mentioned here. Jensen *et al.*[16] examined thirty-two genera and different species of microorganisms and could classify them according to the pattern of regulation of the activity of DAHP synthetase. In general, a total of six patterns of regulation of DAHP synthetase were shown to exist: isoenzyme feedback, sequential feedback, cumulative feedback, and specific inhibition by tyrosine, phenylalanine, and tryptophan, respectively. Since the regulatory patterns for the control of this enzyme within a genus were uniform, he could utilize this character to determine the taxonomic relationship of two genera whose classification was uncertain—*Sporosarcina urea* and *Aeromonas*. This type of approach might also be fruitful with higher plants, but nothing is known at present about the regulation of DAHP synthetase in these plants[18]. A comparative study on the regulation of DAHP synthetase activity has been made only in blue-green and green algae[17]. In blue-green algae the activity of this enzyme is consistently effected via end product inhibition by tyrosine and phenylalanine, whereas DAHP synthetase activity of two obligate autotrophic green algae does not seem to be regulated at all.

In bacteria the chief control point of the aromatic linear segment consists of DAHP synthetase. The second point of regulation is located at the chorismate branch point. In *Neurospora* and the other fungi studied, on the other hand, the five enzymes of the linear segment are organized into a multienzyme complex catalyzing the reactions which lead from DAHP to 3-enolpyruvylshikimate-5-phosphate[19]. A similar multienzyme complex seems to exist in *Euglena gracilis*[20]. An indication that in higher plants as well regulation of the linear segment of the aromatic pathway might be achieved through formation of a multienzyme complex was obtained by studies with extracts of roots of *Quercus penduculata* in which the enzymes 5-dehydroquinase and 5-dehydroshikimate reductase could not be separated

by several methods. They form an aggregate with a molecular weight of about 55 000 daltons[21].

The results on the regulation of tyrosine, phenylalanine and tryptophan pathways beyond the chorismate branch-point will not be discussed here because again very little is known with respect to higher plants. It is, however, clear from the few examples mentioned that a further examination of the aromatic pathway in higher plants will be profitable in the study of evolutionary relationships.

Some secondary plant products branching off from the aromatic pathway have already been mentioned. I now should like to discuss several other secondary products originating from this pathway.

Meta-CARBOXY-SUBSTITUTED AROMATIC AMINO ACIDS

Four aromatic amino acids (*Figure 8*, (11)–(14)), possessing a carboxyl group *meta* to the sidechain, have been isolated in recent years from higher plants[23]. So far, representatives of this group of amino acids have been found in four families: the monocotyledonous *Iridaceae*, the dicotyledonous *Curcubitaceae* and the closely related *Cruciferae* and *Resedaceae*. It seems, therefore, that the occurrence of this type of amino acid might be of taxonomic value.

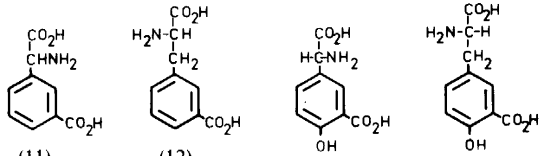


Figure 8. meta-Carboxy aromatic amino acids from higher plants.

When [U-^{14}C]-shikimic acid was fed to *Reseda* species, a high incorporation of radioactivity into the amino acids (12), (13) and (14) occurred. Degradation of these acids demonstrated that $\frac{1}{7}$ of the total radioactivity was located in the aromatic carboxyl group and the residual $\frac{6}{7}$ in the rest of the molecule[23].

These results can be best interpreted by the assumption that isochorismic acid ((15), *Figure 9*) is an intermediate in the biosynthesis of these amino acids. Isochorismic acid has been isolated from a mutant of *Aerobacter aerogenes*[24]. In this organism it is a precursor of 2,3-dihydroxybenzoic acid. Isochorismic acid can be formed from chorismic acid by an enzymatic reaction requiring Mg^{2+}. The chemical transformation at pH 7.0 of isochorismic acid to 3-carboxyphenylpyruvic acid (17) has been demonstrated[24]. One can therefore postulate the pathway shown in *Figure 9* for the biosynthesis of *meta*-carboxy-substituted aromatic amino acids.

The transformation of the still hypothetical *meta*-carboxyprephenic acid (16) to 3-carboxyphenylpyruvic acid or 3-carboxy-4-hydroxyphenylpyruvic acid (18) would be analogous to the prephenate dehydratase and prephenate dehydrogenase reactions, respectively.

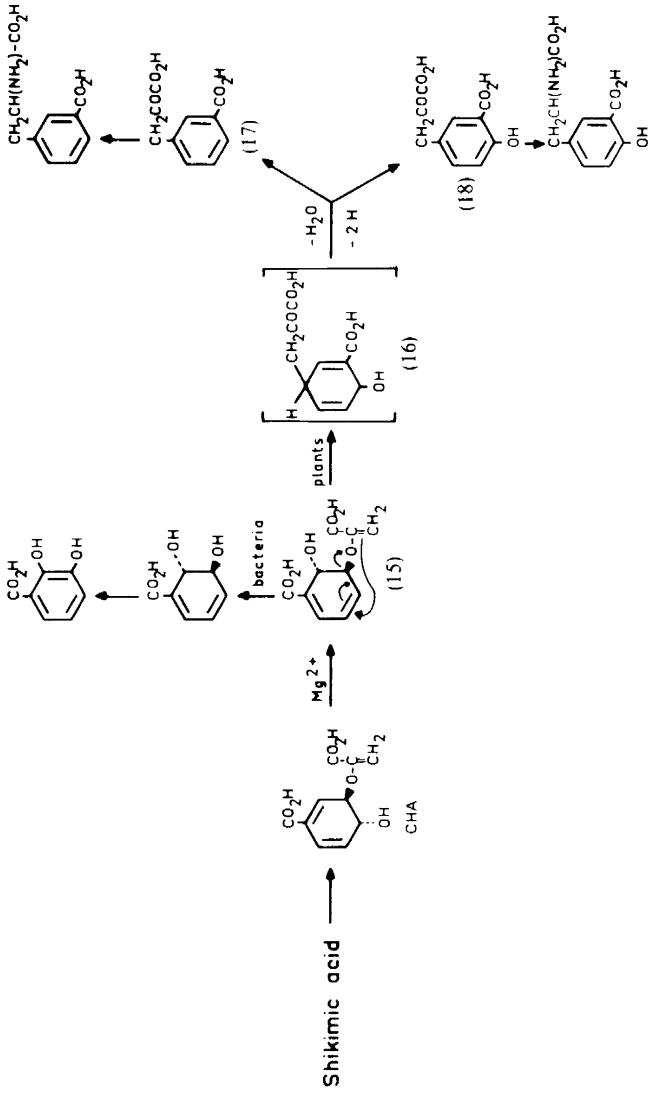


Figure 9. Biosynthesis of *meta*-carboxy aromatic amino acids. (15) isochorismic acid; (16) *meta*-carboxyprephenic acid; (17) 3-carboxyphenylpyruvic acid; (18) 3-carboxy-4-hydroxyphenylpyruvic acid.

BIOSYNTHESIS OF LIGNIN

The ability to form lignin has played an important role in the evolution of land plants. No lignins have been found in plants lower than mosses and it is still uncertain whether the mosses contain substances which can be regarded as true lignins[25].

Lignin is formed via the shikimic acid–phenyl propanoid pathway[26]. The pathway shown in *Figure 10* is assumed for lignification predominantly on the basis of tracer studies. Precursors which could be diverted to flavonoids (see below) are also indicated in this figure. The enzyme L-phenylalanine ammonia lyase (PAL) (E.C. 4.1.3.5), catalyzing the deamination of L-phenylalanine to *trans*-cinnamic acid, plays an important role in this pathway. It is still a matter of dispute whether a second enzyme responsible for the deamination of L-tyrosine (E.C. 4.1.3) exists or whether PAL has a more or less broad specificity for L-tyrosine depending on the plant source[22, 27].

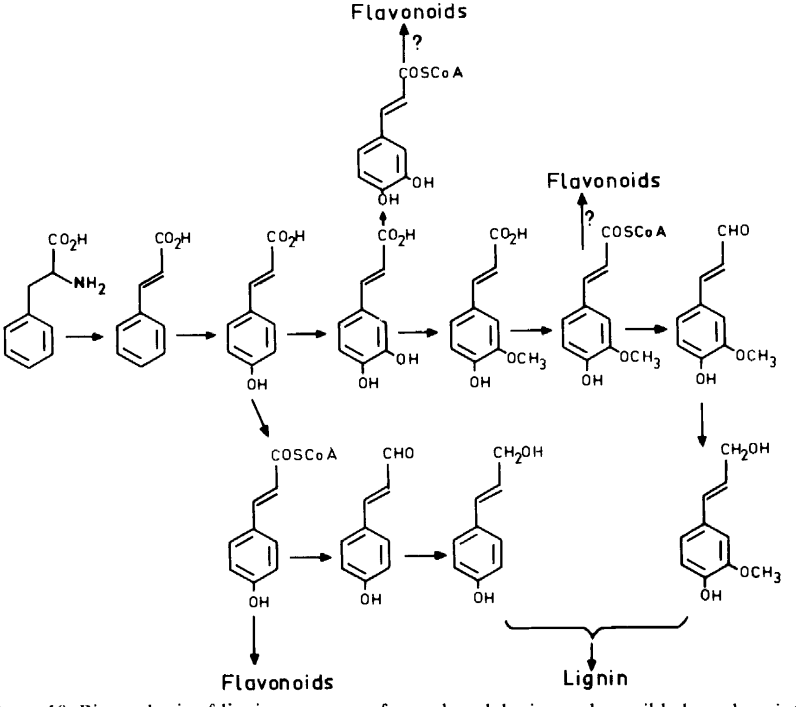


Figure 10. Biosynthesis of lignin precursors from phenylalanine and possible branch points to flavonoids. Formation of the syringyl precursor has been omitted. The precursors for lignin and flavonoid biosynthesis could be present in different cell compartments.

In 1966 Young *et al.*[28] published a study on the taxonomic distribution of ammonia lyases for L-phenylalanine and L-tyrosine in relation to lignification. They came to the following conclusion: 'In general, it may be said that ammonia-lyases for phenylalanine and tyrosine are unique to the plant kingdom and are predominant in vascular plants. In other words, the distribution of these enzymes is correlated with the ability of the plant to form tracheids and other cells with lignified walls'.

In the meantime the situation has changed. PAL has now been found in several Basidiomycetes, *Streptomyces versicularis* and algae (e.g. *Porphyridium sp.*)[22]. It therefore seems questionable whether a correlation exists between the occurrence of PAL and lignification, and one must look for other enzymes which could function as control points in the regulation of lignin biosynthesis. Certainly a correlation between the distribution of phenolases or peroxidases and lignification does not exist because these enzymes, which are involved in the polymerization process[26], are widely distributed in lower plants, which do not contain lignin. However, a possible control point could be the reduction of cinnamic acids to their corresponding cinnamyl alcohols (*Figure 10*), which are the primary building stones of lignin[26]. Evidence for the reduction of ferulic acid to coniferyl alcohol via coniferylaldehyde has been obtained from tracer experiments[26, 29]. For thermodynamic reasons it must be assumed that this reduction occurs with an activated form of the cinnamic acid, e.g. with the CoA esters. The formation of coenzyme A esters of cinnamic acids with enzyme preparations from plants[30] or cell cultures of plants[31, 32] has recently been shown, but the reduction of these esters to the corresponding cinnamylaldehydes or alcohols† has not yet been demonstrated. It can be expected that studies on the regulation of such an enzyme(s) would give important clues to the regulation of lignification.

In this connection it is interesting to note that some species of fungi can reduce the carboxyl group of a number of aromatic acids to the corresponding aldehyde or alcohol. Gross and Zenk[33] were able to purify an aryl aldehyde: NAPD oxidoreductase from mycelia of *Neurospora crassa*, catalyzing the following reaction:

$$R\text{–}CO_2H + ATP + NADPH + H^+ \rightarrow R\text{–}CHO + NADP^+ + ADP$$

Coenzyme A is not required for the reduction. The enzyme shows a broad range of substrate specificity with respect to aromatic acids, especially phenyl carboxylic and phenyl acrylic acids. The activity with cinnamic acid is high but with *p*-coumaric and ferulic acids relatively low.

Considering the properties of this enzyme from *Neurospora*, I would expect that higher plants contain a different enzyme(s) for the reduction of cinnamic acids to the lignin precursors. A new alcohol dehydrogenase acting preferentially on aromatic primary alcohols (or aldehydes) has also been isolated from *N. crassa*[34]. In *Neurospora* the reduction of cinnamic acids to cinnamyl alcohols therefore occurs in two stages catalyzed by two different enzymes.

FLAVONOID BIOSYNTHESIS

In flavonoid biosynthesis the shikimic acid pathway and acetate–malonate pathway are combined[35]. This combination apparently cannot take place in bacteria, fungi and algae since flavonoids have never been found in any of

† Such a reduction could occur either by action of one enzyme without release of the intermediate aldehyde or by action of two enzymes, an aldehyde oxidase and an alcohol dehydrogenase

these organisms†. Until quite recently it was considered that flavonoids do not occur in plants more primitive than the mosses, but flavone *O*- and *C*-glycosides have now been found to occur in liverworts (Hepaticae)[36].

Both lignification and flavonoid biosynthesis depend on *p*-coumaric acid as precursor and the points at which lignin and flavonoid biosynthesis diverge have already been indicated in *Figure 10*. It is still uncertain whether flavonoids with different substitution patterns in ring B (e.g. 4′-hydroxy-; 3′,4′-dihydroxy-; 3′-methoxy-4′-hydroxy-substitution etc.) all originate from *p*-coumaric acid or whether other substituted cinnamic acids such as caffeic acid, ferulic acid, etc., can also serve as direct precursors. Some recent results on the enzymatic level pertinent to this question are discussed below.

The biogenetic relationships of the flavonoids as they can be postulated from tracer studies and studies with some individual enzymes are summarized in *Figure 11*. Some recent results on the biosynthesis of flavonoids and its regulation will now be discussed.

THE FLAVONE GLYCOSIDE PATHWAY IN PARSLEY

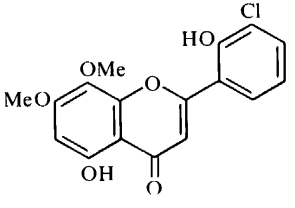
Studies in our laboratory on the flavone glycoside pathway in parsley seedlings and in cell culture of parsley (*Petroselinum hortense*) have led to the detection of most enzymes involved in this pathway and have also furnished some interesting results on their regulation.

The flavone glycoside pathway and the enzymes involved are shown in *Figure 12*. Only enzymes 4 ('chalcone synthetase') and 6 ('flavanone dehydrogenase') have not yet been characterized.

The activation of cinnamic acid as CoA esters has already been mentioned, but a study on the substrate specificity of the CoA-ligase in plants containing flavonoids with different substitution patterns in ring B must await further purification of this enzyme. Such a study together with knowledge of the substrate specificity of the 'chalcone synthetase' may finally settle the question whether the substitution pattern of the B-ring of flavonoids is already determined at the cinnamic acid stage[37] through selection of a certain cinnamic acid by a specific 'chalcone synthetase', or whether this substitution pattern is determined predominantly after the chalcone stage.

The chalcone–flavanone isomerase (enzyme 4) from parsley has a high substrate specificity for A-ring substitution (flavanone numbering)[38]. Of

† The isolation of chloroflavonin, a flavone of the structure shown below from cultures of *Aspergillus candidus* has recently been reported[55]. As long as it is not proved that this flavone is



a genuine metabolite of the fungus and does not originate from the medium one must be cautious about the taxonomic significance of this finding.

The isolation of flavonoid *c*-glycosides from the green alga *Nitella hookeri* (Characeae)[69] has not yet been confirmed.

p-Coumaroyl-CoA + 3 malonyl-CoA
Aurone
Dihydrochalcone
Flavone
Isoflavone
Dihydroflavonol

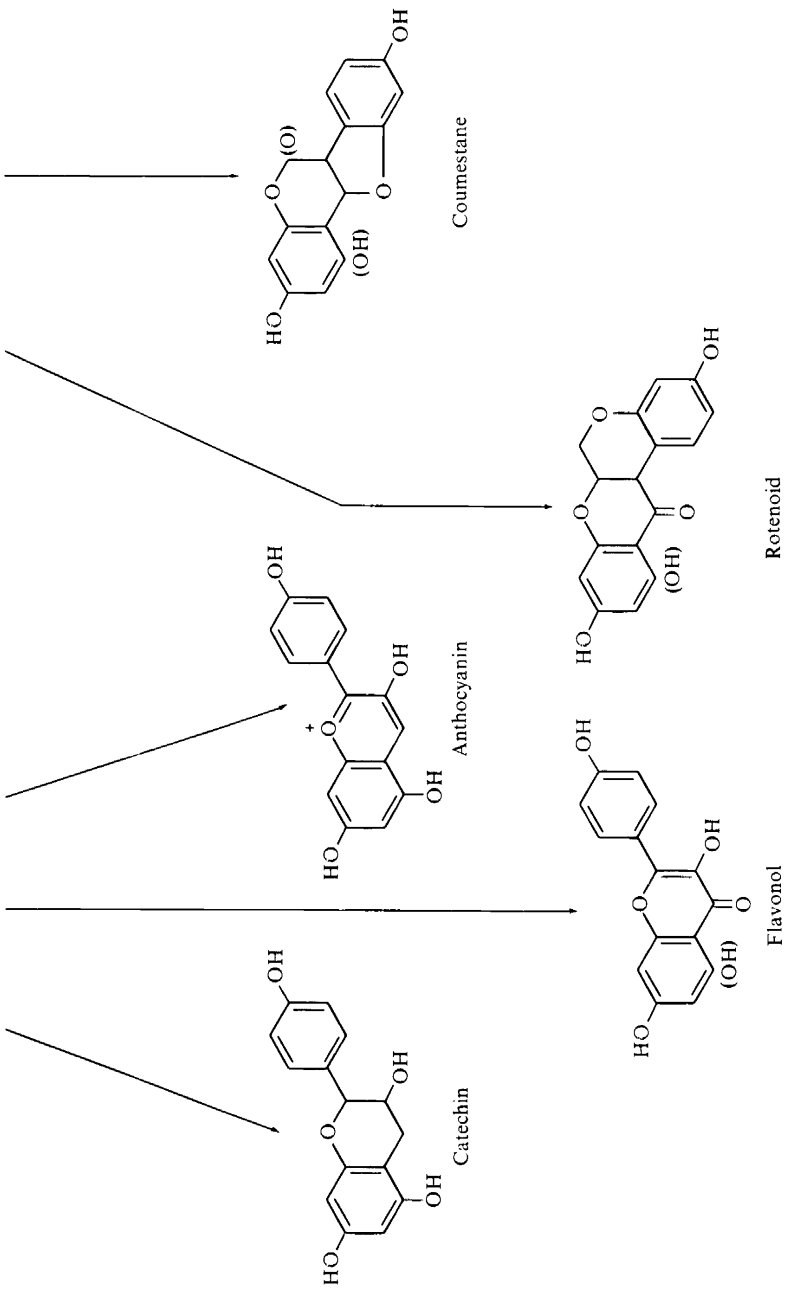


Figure 11. Biogenetic relationship of flavonoids as they have been established mainly by tracer experiments.

HOOC
NH2
1
HOOC
2
HOOC
3
CoAS—CO
OH
4
Mal-CoA
5
6
UDP-glucose
7
8
apiose-Glc—O
UDP-apiose
9
UDP-glucuronic acid
Glc—O
APHN

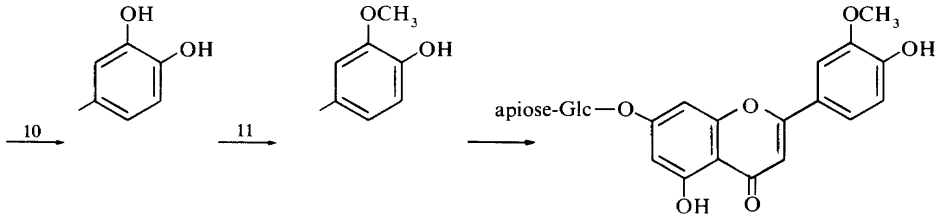


Figure 12. The flavone glycoside pathway in parsley. APHN, apiin.

Enzymes involved (indicated by numbers 1–11) are the following

Systematic name	*Trivial name or abbreviation*
1. L-Phenylalanine ammonia-lyase	Ammonia-lyase
2. *trans*-Cinnamic acid 4-hydroxylase	Cinnamic acid hydroxylase
3. *p*-Coumarate:CoA ligase	
4.	'Chalcone synthetase'
5. Chalcone-flavanone isomerase	Isomerase
6.	'Dehydrogenase'
7. UDP-glucose:apigenin 7-*O*-glucosyltransferase	Glucosyltransferase
8. UDP-apiose:7-*O*-glucosylapigenin [1→2]-apiosyltransferase	Apiosyltransferase
9.	UDP-apiose synthetase
10.	3 (or 3')-Hydroxylase
11. *S*-Adenosylmethionine:luteolin 3'-*O*-methyltransferase	Methyltransferase

the seven chalcones tested only 2',4,4',6'-tetrahydroxychalcone with a phloroglucinol type of substitution in ring A was found to serve as substrate. This is consistent with the fact that only flavonoids of this substitution pattern in ring A have been found in parsley. The same specificity for ring A substitution has been found for the isomerase(s) from *Datisca cannabina*[39]. In contrast, isomerases from mung bean seedlings (*Phaseolus aureus* Roxb.) and garbanzo bean seedlings (*Cicer arietinum* L.) catalyse the isomerization of chalcones with phloroglucinol and resorcinol substitution pattern in ring A, and these plants also contain these two groups of flavonoids[38].

Chalcone–flavanone isomerases from mung bean seedlings, garbanzo bean seedlings and parsley leaves have been separated into a number of isoenzymes[38]. The potential value of isoenzyme analysis in taxonomy has been shown in algae systematics. Isoenzyme patterns of malate dehydrogenase, leucine aminopeptidase and other enzymes revealed a close similarity between *Chlamydomonas eugametos* and *C. moewusii* but a distant relationship between the two and *C. reinhardtii*[56]. Since isoenzymes of chalcone–flavanone isomerase are easily detectable, the analysis of isoenzyme patterns of this enzyme in different plants might be of taxonomic value.

The conversion of the chalcone–flavanone to apigenin (enzyme 6) was achieved with a cell-free extract from parsley seedlings (*Table 1*). This reaction is O_2-dependent, but whether it is a dehydrogenation of a flavanone or an oxidation of a chalcone is not yet known.

Table 1. Conversion of naringenin to apigenin with parsley seedlings cell-free extract

Extract from	Apigenin dpm	Conversion %
Leaf buds	37 650	41
Boiled control from buds	36	
Primary leaves (6 weeks old)	388	0.4
Extract from leaf buds		
with O_2	20 560	
without O_2	3 110	

Incubation with cell free extract (50000 × g supernatant) in 0.2 M ethylenediamine-acetate buffer (pH 7.5) containing 10^{-3} M mercaptoethanol and 10µl 2-^{14}C-naringenin. Time 2 h at 30°C.

The question whether the introduction of the 3'-hydroxyl group (enzyme 10) is catalyzed by a phenolase of relatively broad substrate specificity[40] or whether there exists a specific 3'-hydroxylase is still an open question.

A 3'-*O*-methyltransferase has been purified about 82-fold from parsley cell cultures[41]. Only *ortho*-dihydric phenols can serve as substrates and methylation takes place exclusively in the *meta*-position of 1-substituted 3,4-dihydric phenols. Of the compounds tested luteolin (5,7,3',4'-tetrahydroxyflavone) and its 7-*O*-glucoside have the highest affinity for the enzyme with apparent *Km*-values of 4.6×10^{-5} and 3.1×10^{-5} M, respectively, whereas the *Km* for caffeic acid is 1.6×10^{-3} M. One can therefore assume that the flavone or its glucoside but not ferulic acid are the natural

precursors for 3′-*O*-methylated flavone glycosides. Luteolin-7-*O*-glucoside has recently been detected in extracts from illuminated cell cultures from this plant[42].

Apiin and 3′-methoxyapiin (chrysoeriol) occur in parsley and in cell cultures of this plant as 7-*O*-apiosyl glucosides with a 1 → 2 linkage between the sugars. The two sugar transferases 7 and 8 catalyzing the transfer of D-glucose and D-apiose, respectively, could be completely separated on a hydroxylapatite column[43]. The glucosyl transferase was purified about 89-fold and was investigated in respect to glucosyl donor and glucosyl acceptor[43]. Besides UDP-glucose ($Km = 1.2 \times 10^{-4}$ M) TDP-glucose can serve as glycosyl donor ($Km = 2.6 \times 10^{-4}$ M). A number of flavones, flavanones and flavonols can function as glucosyl acceptors, whereas isoflavones, cyanidin, *p*-coumaric acid, and some other phenols are inactive as acceptors. The best

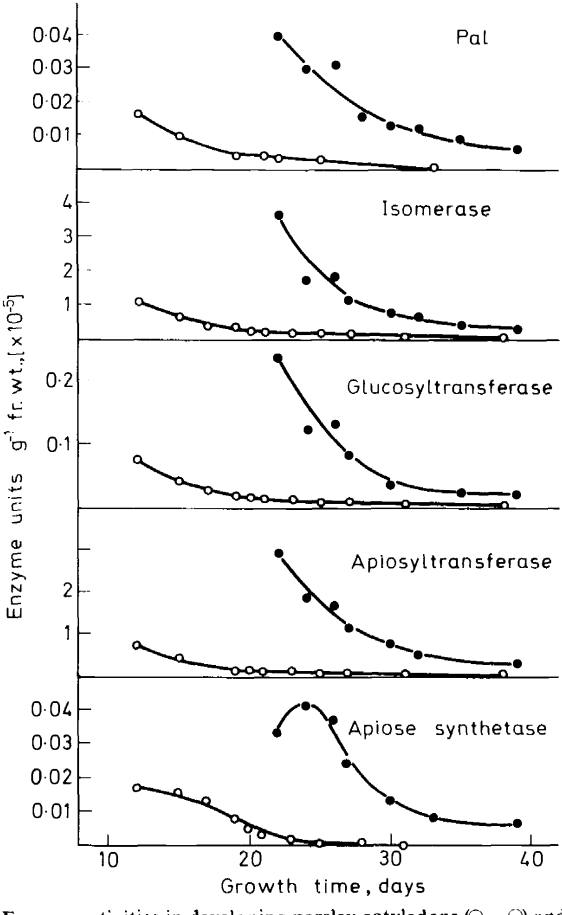


Figure 13. Enzyme activities in developing parsley cotyledons (○ – ○) and leaves (● – ●).

acceptors are luteolin, apigenin, chrysoeriol and naringenin, with relative activities of 210:100:63:45. Glucose is transferred to the 7-hydroxyl group of these flavonoids. The 3-*O*-glucosides of flavonols later discovered in parsley cell cultures[42] are formed by a different UDP-glucosyl transferase separable from the above enzyme by chromatography on DEAE cellulose[44].

The UDP-apiosyl transferase was purified about 123-fold[45]. The enzyme is specific for UDP-apiose as glycosyl donor. 7-*O*-Glucosides of flavones, flavanones, isoflavones, apigenin-7-*O*-glucuronide and glucosides of *p*-substituted phenols can function as acceptors. The best acceptors found are apigenin-7-*O*-β-D-glucoside, formed by the action of the glucosyl transferase mentioned above, and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone)-7-*O*-glucoside. No reaction was observed with flavonol-3-glucoside, apigenin-8-*C*-glucoside, aglycones of flavonoids or free glucose.

According to these results, glucose and apiose are transferred to apigenin in a stepwise manner to form apiin. The transferases involved are quite specific for the glycosyl donor and glycosyl acceptor. It can therefore be expected that a number of different glycosyl transferases involved in flavonoid biosynthesis occur in plants and that a comparative study of this group of enzymes might be of taxonomic value.

The level of enzyme activities changes with the development of parsley seedlings[46] (*Figure 13*). In cell cultures of this plant extractable enzyme activities increased greatly upon illumination of the cells. Two groups of

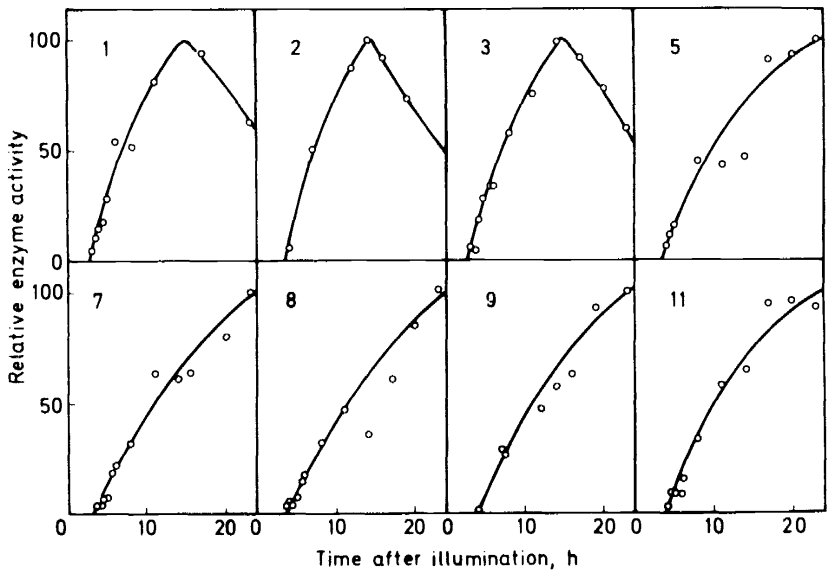


Figure 14. Changes in the activities of phenylalanine ammonia-lyase (1); cinnamic acid hydroxylase (2); *p*-coumarate–CoA ligase (3); chalcone–flavanone isomerase (5); glycosyl transferase (7); apiosyltransferase (8); UDP-apiose synthetase (9); and methyl transferase (11) during 24 h of continuous illumination of cell suspension cultures from parsley. The reactions catalyzed by these enzymes are shown in *Figure 12*.

enzymes can be distinguished on the basis of their responses to light (*Figure 14*). The first group comprises the three enzymes acting on substrates of the phenylpropanoid type; the second group includes all those enzymes involved exclusively in the formation of flavonoid compounds and their glycosides[47]. The question can therefore be asked, whether the synthesis of these enzymes is under coordinate control on the gene level?

In contrast to the above enzymes of secondary metabolism the activity of a number of enzymes of primary metabolic processes were not influenced by illumination of the cells. The enzymes which have so far been investigated in this respect and which showed equal activity in light and dark are: acetate-CoA ligase[31], glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NAD and NADP-dependent phosphoglyceraldehyde dehydrogenase, catalase, peroxidase and UDP-glucuronate carboxylase[48].

ANTHOCYANINS AND BETALAINS

One of the most impressive examples of the restricted occurrence of secondary plant products is the restriction of betalains to ten families of the order Centrospermae and the mutual exclusiveness of betalains and anthocyanins[49, 50]. What could be the biochemical basis for these taxonomically important findings?

According to tracer studies the best precursor which has so far been found for cyanidin is dihydrokaempferol. Earlier results with buckwheat seedlings[51] have now been confirmed and extended with cell suspension cultures of *Haplopappus gracilis*[52]. The incorporation rates into cyanidin and dilution values obtained with different precursors under identical conditions are shown in *Table 2*.

Table 2. Incorporation of precursors into cyanidin in cell cultures of *Haplopappus gracilis.*

Precursor	Incubation time h.	Incorporation %	Dilution
D,L-[2-^{14}C]-Phenylalanine	1	3.3*	128
	3	3.1*	136
[β-^{14}C]-4,2',4',6'-Tetrahydroxychalcone-2'-glucoside	1	0.26	540
	3	0.26	617
	6	0.31	541
[G-^{3}H]-Dihydrokaempferol	1	9.5†	51
	3	10.8†	27
	6	12.5†	20

* Related to the L-isomer; † corrected for loss of tritium.

3',4'-Dihydroxyphenylalanine (dopa) is the precursor for both the cyclo dopa portion and the hydropyridine ring of betanidine (*Figure 15*). Betalamic acid, which is also the precursor for all other betalains[53], is formed by *meta*-cleavage of the aromatic ring of dopa[54].

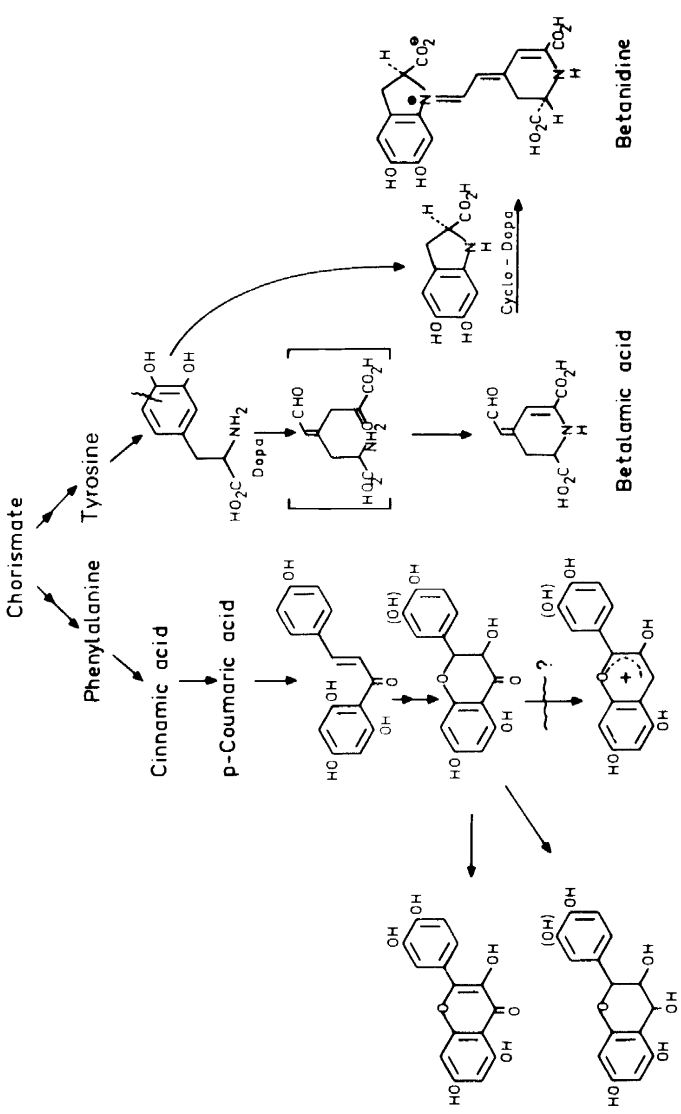


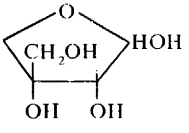
Figure 15. Biogenetic relationship between betalains and flavonoids. It can be postulated that betalains or an intermediate in their synthesis inhibits the conversion of dihydroflavonol to anthocyanins.

According to these results, the biosynthetic pathways to anthocyanins and betalains can be formulated as shown in *Figure 15*. Since a phenylalanine hydroxylase seems to be absent in higher plants, the first common intermediate for both pathways is chorismic acid. It can be postulated that the reason why betalains occur only in Centrospermae is that only these plants contain the enzyme for the *meta*-fission of the aromatic ring of dopa to form betalamic acid. However, the fact that the pathway to anthocyanins is blocked whereas leucoanthocyanins[50], flavones and flavonols can be formed is difficult to interpret as long as the enzyme(s) for the formation of anthocyanins from dihydrokaempferol is unknown.

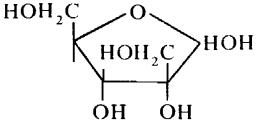
BRANCHED-CHAIN SUGARS

I should now like to examine the question whether studies on the distribution and biosynthesis of branched-chain sugars can contribute to the topics of this symposium.

Sugars with a methyl-, hydroxymethyl-, formyl-, hydroxy-ethyl-, oxoethyl- and glycoloyl-branch occur in microorganisms[57], but in higher plants only the hydroxymethyl-branched sugars D-apiose (19) and D-hamamelose (20) have been found so far.



(19)



(20)

Whereas the distribution of D-apiose in glycosidic form is widespread and obviously has no taxonomic significance[57–59], the occurrence of this sugar as a component of polysaccharides of the cell wall seems to be restricted predominantly to the families of Helobiae and Spathiflorae. According to earlier investigations and a recent survey by Beusekom, besides Lemnaceae only species of *Hydrocharitaceae*, *Potamogetonaceae* and *Zannichelliaceae* which grow in the sea or in brackish water contain appreciable amounts of apiose in their polysaccharide fraction (*Table 3*). According to these results apiose-rich cell walls seem to occur mainly in extreme hydrophytes. An exception is the occurrence of apiose in a polysaccharide from *Tilia vulgaris*, but this sugar may also occur in minor quantities in the hemicellulose fraction of other land plants[59].

The structure shown in *Figure 16* with an apiobiose linked to the 2 or 3 position of an α-(1 → 4)-polygalacturonane has been established for the polysaccharide from the cell wall of *Lemna minor*[60]. The pectic substances of *Zosteraceae* (Zosterines) seem to have a similar structure[63].

Investigations in our laboratory have shown that uridine diphospho apiose is formed from UDP-D-glucuronic acid with expulsion of C-3 by an NAD^+ dependent reaction[57] (*Figure 17*). The enzymatic activity for the formation of UDP-D-xylose could not yet be separated from the UDP-apiose-forming enzyme, though an approximately 1000-fold purification of the enzyme was achieved[61].

Table 3. Aquatic plants in which apiose-containing polysaccharides have been found.

Family, subfamily tribe	Species	Literature references
Helobiae		
Thalassioideae	*Thalassia hemprichii* (Ehrenb.) Aschers.	68
Potamogetonaceae	*Potamogeton pectinatus* L.	68
Potamogetoneae	*Ruppia spiralis* L. ex Dum.	68
Posidonieae	*Posidonia australis*	65, 66
	P. oceanica Del.	68
Zostereae	*Zostera marina*	59, 66, 67
	Z. nana	58
	Z. pacifica	67
	Z. spec. indet.	68
	Phyllospadix spec. indet.	67, 68
Zannichelliaceae		
Cymodoceae	*Cymodocea nodosa* Aschers.	68
Spathiflorae		
Lemnaceae	*Lemna gibba* L.	64, 68
	L. minor L.	64, 68
	Spirodela polyrhiza (L.) Schleiden	68
	Wolffia arrhiza (L.) Wimm.	58

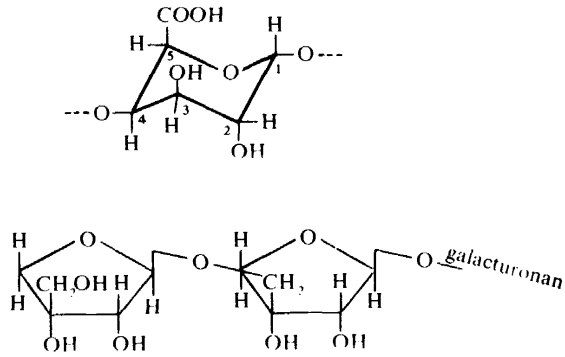


Figure 16. Proposed structure for the polysaccharide from the cell wall of *Lemna minor*. The apiobiose is attached to the 2- or 3-hydroxyl group of the polygalacturonic acid chain.

From the results published on the biosynthesis of L-streptose[62] it is obvious that there exists a close biogenetic relationship between the biosynthesis of L-streptose from *Streptomyces* and D-apiose from higher plants, a finding which is of phylogenetic interest (*Figure 17*).

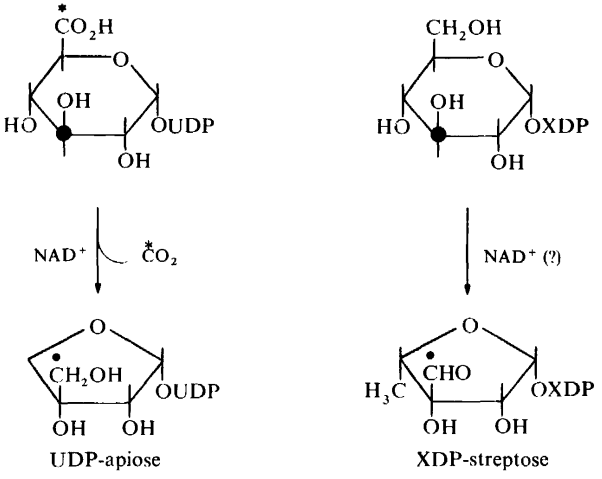


Figure 17. Biosynthesis of D-apiose and L-streptose.

CONCLUSION

The comparative analysis of secondary plant constituents in a great number of plants by means of modern analytical methods is now almost a routine procedure. This approach to systematics and evolution should, however, only be applied when the biosynthetic routes to the compounds investigated are known. Furthermore, the dependence of the presence and concentration of a given substance on the stage of development of the plant and on external factors must not be neglected. This analytical approach can be supplemented by comparing not only the end products of a gene reaction sequence but also the biosynthetic pathways or, even better, the enzymes involved in these pathways and in the turnover and regulation of these compounds.

The enzymology of secondary plant products is just in its infancy. The principal difficulties in working with such enzymes have now been overcome, and the recent successes obtained in this field allow us to expect that many new enzymes for secondary metabolism will be discovered in the coming years. After a long period in which tracer studies *in vivo* dominated the field of research, the study of biosynthetic pathways in higher plants now enters a new era in which enzyme studies will lead to new and exciting discoveries.

ACKNOWLEDGEMENTS

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