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REVIEW

ENZYMIC REGULATION OF PROCYANIDIN BIOSYNTHESIS; LACK OF A FLAV-3-EN-3-OL INTERMEDIATE

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Key Word Index—Procyanidins; condensed tannins; flav-3-en-3-ol; catechin; epicatechin; stereochemistry; biosynthesis; multienzyme complexes.

Abstract—A speculative scheme for procyanidin (condensed tannin) biosynthesis involving multienzyme complexes is presented in which the stereochemistry at C-3 and C-4 of (+)-dihydroquercetin is maintained in (+)-catechin and its oligomeric products. This is in contrast to Haslam's speculation involving a symmetrical flav-3-en-3-ol intermediate, which requires the re-introduction of the stereochemistry at the terminal reduction steps. In the proposed scheme, the origin of the 2,3-*cis* stereochemistry of (−)-epicatechin from (+)-dihydroquercetin would require an epimerase, in addition to two reductases and a condensing enzyme neeeded for (+)-catechin stereochemistry.

INTRODUCTION

In a recent review [1], Haslam supports his earlier speculative biosynthetic scheme in which the symmetrical flav-3-en-3-ol (**8**) is an intermediate leading to flavan-3-ols (**7**) and their oligomeric procyanidins (Schemes 1 and 2). According to this hypothesis, the final 2,3-*trans* and 2,3-*cis* stereochemical forms are introduced only at the terminal reduction steps to produce (+)-catechin (**9**) and (−)-epicatechin (**10**) and their respective carbocations (**6**) [2]. Recent enzymic and non-enzymic data, however, support a scheme in which the 2,3-*trans* stereochemistry of (+)-dihydroquercetin (DHQ) (**4**) is retained in the synthesis of (+)-catechin and its carbocation [3, 4]. Another route will be necessary to produce the 2,3-*cis*-stereochemistry of (−)-epicatechin (**10**).

Haslam's evidence with intact shoots cited for support of a symmetrical flav-3-en-3-ol can have other interpretations. The negative 'cold trapping' experiments may indicate that the diol intermediate exists *in vivo* only on the surface of an enzyme complex. The loss of ^3H label of the middle carbon of cinnamate which becomes the C-3 position in the procyanidin products might occur at another step such as the dioxygenase catalysed hydroxylation step at C-3 [5, 6]. Haslam also postulated that a 2-hydroxychalcone rather than DHQ might be the intermediate leading to the symmetrical intermediate. However, this pathway starting with cinnamate would be highly unlikely because it would require phenylalanine ammonia-lyase to function reversibly *in vivo*.

ENZYMIC EVIDENCE OF ASYMMETRIC INTERMEDIATES

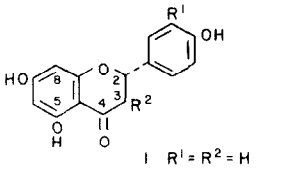
The more likely central intermediate at the flavonoid level leading to at least the 2,3-*trans* stereochemistry of (+)-catechin is (+)-DHQ (**4**) [6–8]. The cell-free enzymology of the steps from the first C-15 intermediate to DHQ have been demonstrated [9], although the sequence of hydroxylation steps is not known for most tissues. There are three possible routes to DHQ (Scheme 3); there is evidence for route I via naringenin (NAR) (**1**) and DHK (**3**) in parsley [6] and route II via NAR and eriodictyol (ERIO) (**2**) in Douglas fir (8), either route involving only 2,3-*trans* intermediates.

Extracts from cell suspension cultures of Douglas fir have recently been shown to contain an NADPH-dependent reductase capable of converting (+)-DHQ to a flavan-3,4-diol (**5**), a monomeric procyanidin. The accumulated diol product was assumed to be the all-*trans* isomer since it produced cyanidin upon heat treatment in acid, and could be condensed non-enzymically with added (+)-catechin and H^+ to form the all-*trans* catechin–catechin dimer [4]. Recently, in the presence of 2-mercaptoethanol, extracts have been found to form catechin in addition to the diol, due to a double step involving a second reductase [unpublished data]. This second reductase activity might be expected to involve a stoichiometric reduction by a thiol-containing hydrogen-carrying protein, analogous to the conversion of ribose to deoxyribose [10].

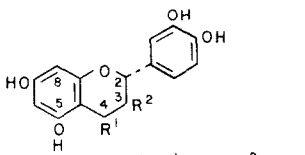
NON-ENZYMIC EVIDENCE OF RETENTION OF THE ASYMMETRY AT C-3

These enzymic results indicating the accumulation of only the all-*trans* flavan-3,4-diol are supported by the prior work of Roux and co-workers who demonstrated that the stereochemistry of the (+)-DHQ precursor was maintained when (+)-DHQ was first reduced with sodium borohydride and the diol product was immediately condensed in acid with added (+)-catechin to

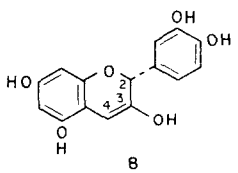
Abbreviations: DHQ, dihydroquercetin (**4**); DHK, dihydrokaempferol (**3**); NAR, naringenin; ERIO, eriodictyol.



1 $R^1 = R^2 = H$
2 $R^1 = OH$; $R^2 = H$
3 $R^1 = H$; $R^2 = OH$
4 $R^1 = OH$; $R^2 = OH$



5 $R^1 = OH$; $R^2 = OH$
6 $R^1 = +$; $R^2 = OH$
7 $R^1 = H$; $R^2 = OH$



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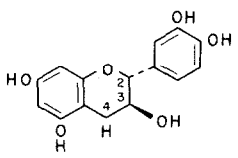

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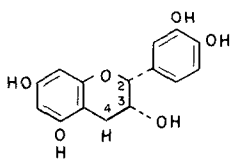

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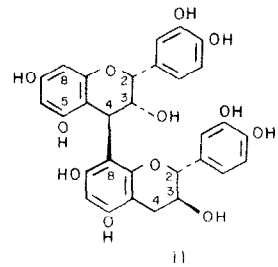
Scheme 1. Formulas for potential intermediates in the biosynthetic pathway to flavan-3-ols (**7**) and their oligomeric procyanidins: flavanones, NAR (**1**) and ERIO (**2**); 3-hydroxyflavanones, DHK (**3**) and DHQ (**4**); flavan-3,4-diols (**5**); flavanyl-4-carbocations (**6**); flav-3-en-3-ol (**8**); (+)-catechin (2,3-*trans*) (**9**); (−)-epicatechin (2,3-*cis*) (**10**). The stereochemistry at C-2 is the same in all cases, but differs at C-3 and C-4 to give *cis* and *trans* isomers.



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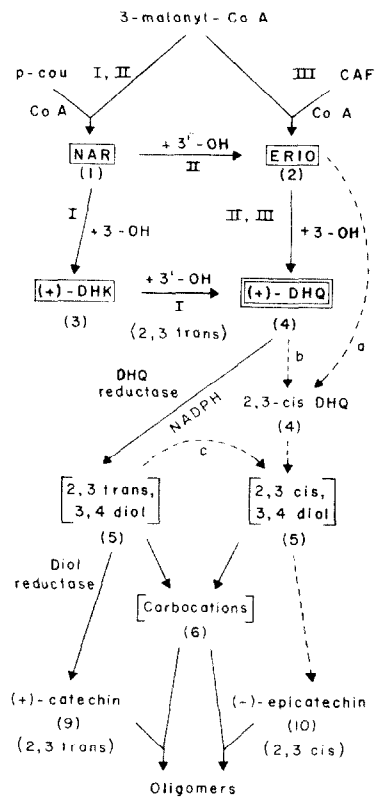
Scheme 2. Example of a dimer formed by the condensation of a carbocation having the 2,3-*cis* stereochemistry of (−)-epicatechin ('upper' unit) with a flavan-3-ol having the 2,3-*trans* stereochemistry of (+)-catechin ('lower' unit) to form a 4 → 8 linkage (3,4-*trans*).



Scheme 3. Postulated biosynthetic pathways to procyanidins.

form the all-*trans* dimer of catechin. The latter was identified by NMR [3].

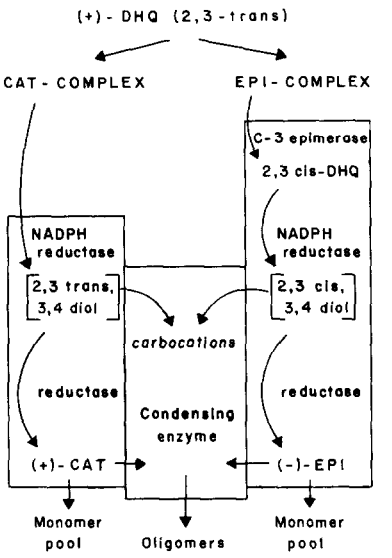
Roux's group was unable to purify the diol intermediate, but we repeated the non-enzymic reduction and isolated two 2,3-*trans*-3,4-diol products by HPLC and PC at neutral pH, presumably the expected 3,4-*trans* and 3,4-*cis* isomers [4]. The 3,4-*cis* isomer [diol 2 in ref. 4] could be converted to the 3,4-*trans* isomer [diol 1 in ref. 4] by acid. This was in agreement with earlier chemical work of Clark-Lewis *et al.* with diols lacking a 5-hydroxyl group. They postulated that the 3,4-*trans*-diol was less stable because it was more readily converted to the 4-carbocation (**6**) that tends to condense with electrophilic groups [11]. Porter's group also repeated the non-enzymic reduction of DHQ by sodium borohydride with a modified procedure and identified the major isomer by NMR as the all-*trans*-diol (2,3-*trans*-3,4-*trans* diol) [12]. All of these reports of non-enzymic-reduction of (+)-DHQ support the earlier brief report by Creasy and Swain [13].

CONDENSATION STEP TO FORM OLIGOMERS

There is general agreement that oligomeric proanthocyanidins are formed by the addition of a stereospecific

flavanyl-4-carbocation (6) to one of the two stereospecific flavan-3-ols or to an existing chain rather than via self-condensation of the carbocation [1, 3]. The interflavan bond linkage is generally 4 → 8, but some 4 → 6 linkages can also occur (Scheme 2). *In vivo* support for this condensation from two separate metabolic pools was demonstrated originally by Haslam's group; they showed that in angiosperm shoots the extension or 'upper' units of a dimer contained several times the radioactivity as the 'lower' initiating flavan-3-ol unit [2]. This asymmetry of labelling was corroborated with cell suspension cultures of Douglas fir for both dimers and higher molecular weight oligomers [7].

The above stereospecific carbocation can be derived non-enzymically from dimers by mild acid treatment anaerobically [2, 7], or from flavan-3,4-diols merely by the addition of acid [3, 4, 13]. While the condensation of the stereospecific carbocations with added nucleophilic groups such as the flavan-3-ols occurs easily non-enzymically, it is still possible and preferable from a regulatory point of view, that *in vivo* the condensation is enzymically controlled.



Scheme 4. Possible multienzyme complexes in procyanidin-biosynthesis.

REGULATION OF FLOW INTO FLAVAN-3-OL MONOMERS AND OLIGOMERIC PROCYANIDINS

Since both the flavan-3-ol (+)-catechin and its stereospecific carbocation are derived biosynthetically from (+)-DHQ (2,3-*trans*), and there is a greater incorporation of radioactive label into the 'upper' units of an oligomer than into the 'lower' unit [2, 3], competition between these pathways would be expected. Haslam's scheme to control this partitioning between production of the monomers and carbocations was based on an NADPH requirement for the final reduction to the monomer [1, 2]. However, since this coenzyme is required in the preceding reductase step leading to both monomer and the carbocation, this mechanism is no longer possible.

An alternative hypothesis would involve enzyme complexes with control depending on the amount and activity of diol reductase and the presence of an active condensing enzyme to accept directly the stereospecific carbocations (Scheme 4). However, since a diol product did accumulate in Douglas fir extracts [4], the diol reductase activity producing (+)-catechin must have been limiting, and the postulated condensing enzyme was inactive, permitting the diol to leave the surface of the enzyme. Another possibility to consider is that both 3,4-diol epimers might be produced, since both occur together in tissues containing the non-5-hydroxy diols [14]. The 3,4-*trans* diol which forms a carbocation more easily [11] might be transferred directly to the condensing enzyme, while the 3,4-*cis* diol might be converted directly to (+)-catechin.

The above regulatory mechanism must also account for the greater labelling of the free monomer flavan-3-ol pools of catechin and epicatechin than that of the 'lower' or initiating unit of the same stereochemistry when ^{14}C phenylalanine was fed to cells of Douglas fir [7]. One possibility is that there may be only a limited number of sites on the condensing enzyme to which flavan-3-ols such as catechin are anchored non-covalently, and to which carbocations are added covalently [7]. If at the time of the addition of the isotope the majority of the initiating sites were already filled, relatively little label would be found in these lower units.

ORIGIN OF 2,3-*CIS* STEREOCHEMISTRY

Further speculation is needed to form the 2,3-*cis* stereochemistry of (−)-epicatechin. Three possible enzymic routes are shown in Scheme 3. Eriodictyol could be hydroxylated at the 3-position to form 2,3-*cis* DHQ by a second stereospecific hydroxylating enzyme (a), or an epimerase could convert either 2,3-*trans* DHQ (b) or its diol (c) to the 2,3-*cis* stereochemistry. No natural 2,3-*cis*-DHQ has ever been identified as a plant product and the aglycone is considered unstable [14]. It could be protected, however, as a glycoside or by remaining on the surface of an enzyme complex. However, since 2,3-*trans* DHQ does accumulate as a glycoside in many plants, the likely pathway as shown in Scheme 4 is via an epimerase that converts 2,3-*trans* DHQ to the 2,3-*cis* form on the surface of an enzyme, followed immediately by the action of two reductases acting in sequence.

CONCLUSION

In summary, it is postulated that the key intermediate (+)-DHQ can be converted by two multienzyme complexes to either (+)-catechin or (−)-epicatechin; the one consisting of two reductases; the other has an epimerase in addition. Under still undefined conditions, the diol intermediates on the surface of the complex can be converted instead to their respective carbocations which are directly transferred to the surface of a condensing enzyme where condensation with associated flavan-3-ols occurs to form oligomeric chains of varying length. More than one type of condensing enzyme might be required to permit 4 → 6 as well as 4 → 8 interflavan linkages. Variations in the relative amount of these two complexes and regulation of the diol reductase activity could account for the varied patterns of monomers and oligomers of procyanidins in plants.

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