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ENZYMOLOGY AND COMPARTMENTATION OF POLYMETHYLATED FLAVONOL GLUCOSIDES IN *CHRYSOSPLENIUM AMERICANUM*

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Key Word Index—*Chrysosplenium americanum*; Saxifragaceae; *O*-methyltransferase; *O*-glucosyltransferase; compartmentation; polymethylated flavonol glucosides; localization.

Abstract—The enzymatic synthesis of polymethylated flavonol glucosides in *Chrysosplenium americanum* is catalysed by a number of distinct, position-specific *O*-methyltransferases and *O*-glucosyltransferases. These enzymes seem to exist in the form of an aggregated, membrane-associated, multienzyme system. This tissue represents an ideal material for the study of these enzymatic steps, their regulation and compartmentation, as well as the intracellular localization of the final metabolites.

INTRODUCTION

Nature abounds with a multitude of secondary plant products which contain one or more methyl groups. These methylations are catalysed by a class of enzymes, the methyltransferases (EC 2.1.1.–) and involve the transfer of a methyl group to a suitable acceptor molecule. Since the discovery of *S*-adenosyl-L-methionine as the methyl donor, it became evident that methyltransferases may exhibit strict specificity toward one group of substrates, e.g. alkaloids [1], flavonoids [2, 3] or lignins [4, 5]. Substrate specificity may be even restricted to one or a limited number of closely related compounds [6] or a specific position on the substrate molecule [7]. Likewise, recent reports on enzymatic *O*-glucosylation tend to indicate the high substrate and position specificity of glucosyltransferases towards their flavonoid acceptors [8, 9]. The physiological significance of *O*-methylation and/or *O*-glucosylation of secondary metabolites is believed to reduce the chemical reactivity of the phenolic hydroxyl groups [10]. The former reaction may also direct the partially methylated intermediates towards specific metabolic pathways (e.g. lignin or flavonoid biosynthesis [11]). Furthermore, both enzymatic reactions may influence the degree of solubility of the final products, and hence, determine their compartmentation within plant cells and tissues [12].

This review describes the multienzyme system which catalyses the methylation and glucosylation sequence of flavonoids in *Chrysosplenium americanum* (Fig. 1); its regulation, compartmentation and the intracellular localization of the final metabolites.

ENZYMOLOGY

O-*Methylation of flavonoid compounds*

Whereas enzymatic *O*-methylation has earlier been discovered in animal tissues with simple phenolics [13] and later with flavonoids [14], it was only in 1972 that a flavonoid *O*-methyltransferase was reported from parsley cell suspension culture [15]. Since then, a number of flavonoid-specific enzymes have been reported to catalyse single methylation steps of positions 3′ of flavones or flavonols [2, 3, 15], 3′/5′ of anthocyanins [16], 4′ of isoflavones [17] and flavones [18], 7 of flavonols [19, 20] and *C*-glycoflavones [21] and 8 of flavonols [22]. None of these enzymes has been reported to accept partially *O*-methylated substrates for further *O*-methylation. Some of these *O*-methyltransferases have been reviewed earlier [23].

Stepwise O-*methylation of flavonoids*

The increasingly reported common occurrence of partially and fully methylated flavonoids in plants [24–26] raised the question as to whether multiple *O*-methylations, within one type of flavonoid structure, are catalysed by one or several, position-specific *O*-methyltransferases.

Using cell-free extracts of Calamondin orange (*Citrus mitis*) Brunet *et al.* [27] were the first to report the stepwise *O*-methylation of flavonoids. Enzyme extracts of seedling root, fruit peel or seed callus tissues catalysed the *O*-methylation of quercetin to 3-methylquercetin, rhamnetin, isorhamnetin and rhamnazin. When rhamnetin and isorhamnetin were used as substrates, they were further methylated to rhamnazin and a number of di- and trimethyl ethers [27]. These results indicated the presence, in Calamondin orange, of several *O*-methyltransferases and were consistent with the accumulation of a number of highly methylated flavonoids in the intact [28] and cultured [29] tissues. Similar results were obtained with

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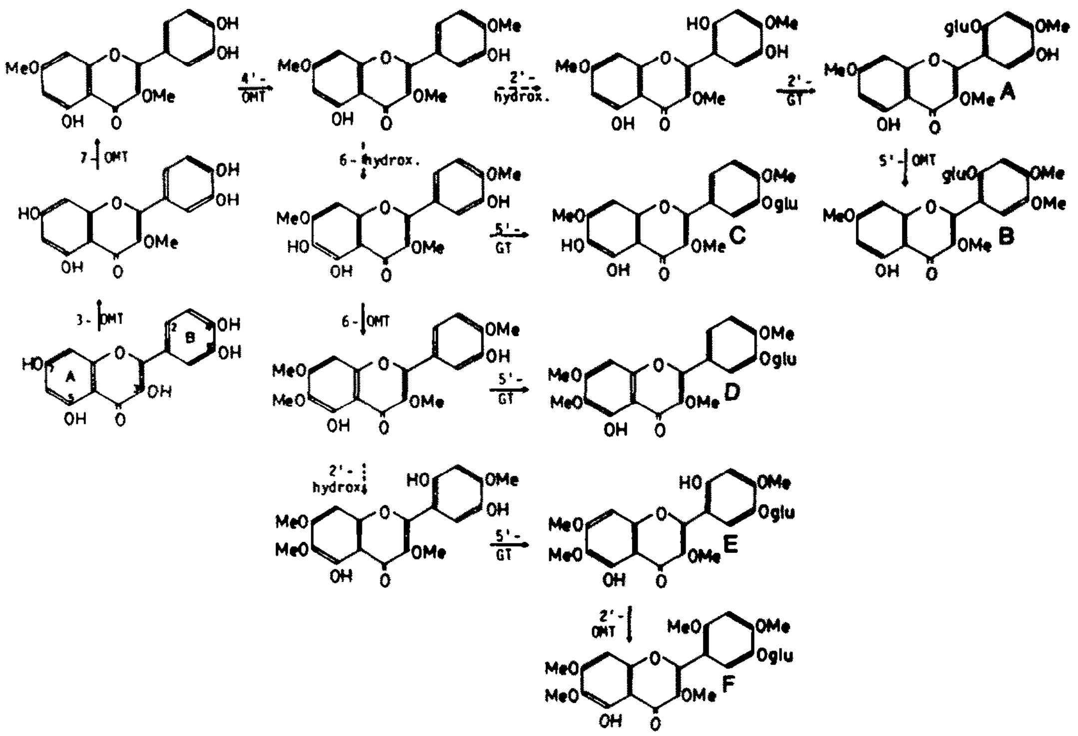

Fig. 1. Proposed pathway for the enzymatic synthesis of polymethylated flavonol glucosides in *Chrysosplenium americanum*. A to F are the only compounds which accumulate in this tissue. GT, *O*-glucosyltransferase; OMT, *O*-methyltransferase.

Chrysosplenium leaves [30] and cell suspension cultures of tobacco [31] and apple [32].

A later study [33] of the enzymatic *O*-methylation of 23 flavonoids with different hydroxylation patterns demonstrated the *O*-methylation of almost all hydroxyl groups on the flavonoid ring, including the 3-position; thus suggesting the presence of several, distinct *O*-methyltransferases. Whereas the precise order of *in vitro* *O*-methylation could not be established due to the limited number of flavonoids tested, there appeared, however, some interesting relationship between the hydroxyl substitution pattern of the substrates, their methyl acceptor ability, and the negative electron density (NED) distribution, as determined by complete neglect of differential overlap (CNDO/2) method [34]. Flavonoids with vicinal hydroxyl groups at positions 5, 6, 7 or 8 and lacking ring B substitution exhibited the highest *O*-methylation and NED for positions 6 and 8. Ring B substitution seemed to affect the nucleophilicity of ring A hydroxyls and, hence, their reactivity towards *O*-methylation. Hydroxylation at the 4'-position may result in *para*-quinoid, rigid structure and concomitant resonance of the flavonoid skeleton that may limit methylation (as in apigenin and kaempferol), except where vicinal hydroxyl groups exist at positions 6 and/or 8. Hydroxylation at 3',4' as in luteolin or quercetin, on the other hand, resulted in *ortho*-quinoid structure which seemed to enhance *O*-methylation at both positions. Furthermore, 3-hydroxylation, as in galangin and quercetin, appeared to stabilize the flavonoid ring system and enhance the reactivity of both ring A and ring B hydroxyls [33].

Chrysosplenium americanum *as a model system to study the enzymatic synthesis of partially methylated flavonol glucosides*

Of the various plants containing partially *O*-methylated flavones/flavonols, *C. americanum* leaves accumulate a variety of tri- to penta-*O*-methylated flavonol glucosides (Fig. 1), which are derived from 2'-hydroxyquercetin and its 6-hydroxy analogue (2'-hydroxyquercetagetin). These compounds, which were identified by spectroscopic methods [30], were found to occur as 2'-*O*-glucosides (A, B) and 5'-*O*-glucosides (C–F, Fig. 1). It is interesting to note that none of the low methylated intermediates (mono-, di- or trimethylquercetin) accumulate in this tissue. However, their enzymatic synthesis can only be demonstrated *in vitro* using cell-free extracts and quercetin as substrate [30, 35]. The fact that the six partially methylated flavonol glucosides were readily labelled from [2-^{14}C]cinnamate, within 5–10 min pulse [36] indicated the existence, in *Chrysosplenium*, of the enzyme complement necessary for the biosynthesis of these metabolites. Therefore, this system was considered ideal to study the individual enzymes involved in the biosynthesis of partially methylated flavonol glucosides.

Sequential O*-methylation of flavonoids is catalysed by distinct, position-specific* O*-methyltransferases*

Enzyme purification. Using conventional, open-column chromatography (gel filtration, hydroxyapatite and chromatofocusing), alternatively with fast protein liquid chromatography [35, 37, 38], it was possible to partially purify five, distinct *O*-methyltransferases; taking advantage of the inherent differences in their apparent isoelectric points (pI). Some properties of these enzymes are listed in Table 1.

Substrate specificity. The first enzyme which focused at pH 4.8 was specific for the 3-position of flavonols with 3',4' (or 4',5') hydroxylation pattern such as quercetin, but not quercetagetin, and was not inhibited by substrate concentrations up to 80 μM. Introduction of a methyl group at the 7-position of quercetin (as in rhamnetin) resulted in a 70% drop in activity; whereas methyl substitution at the 3'-position (as in isorhamnetin) or the 4'-position (as in tamarixetin) resulted in a complete loss of activity [37]. Furthermore, the former compounds were effective product inhibitors, with K_i values of the same order of magnitude as for 3-methylquercetin (Table 2). Such strict position specificity indicates that

Table 1. Properties of *Chrysosplenium* OMTs[*] and GTs[*]

	OMTs					GTs	
Property	3-	7-	4'-	6-[†]	2'-/5'-	2'-	5'-
Substrate[‡]	Q	3Q	3, 7Q	3,7,3'/3,7,4' Qg	PMF-Glc E and A	2'-OH PMF	5'OH PMF
Product	3Q	3, 7-Q	3,7,4'-Q	3,6,7,3'/ 3,6,7,4'-Qg	F and B	2'-*O*- Glucosides	5'-*O*-
Purification (-fold)	85	30[§]	164	92	420[‖]	1230[‖]	1200[‖]
pH optimum	7.8	8.2	8.8	9.0	7.0	7.5	7.5
pI value	4.8	<5	5.4	5.7	<5	5.1	5.1
Mol. wt (k)	57	57	57	57	57	42	42

[*] OMT, *O*-methyltransferase; GT, *O*-glucosyltransferase.

[†] Required Mg^{2+} for activity ($K_a = 30\ \mu M$) and was inhibited by EDTA.

[‡] Q, quercetin; Qg, quercetagetin; PMF, partially methylated flavonol; numbers denote *O*-methylated positions of both Q and Qg.

[§] Before chromatofocusing.

[‖] By fast protein liquid chromatography.

Table 2. Kinetic constants of *Chrysosplenium* OMTs and GTs

			Parameter	
Enzyme	Substrate	Product	K_m (μM)	K_i (μM)
3-OMT	SAM[*]		114	
	Q		12	
		SAH[*]		4.5
		3MeQ		128[§]
6-OMT	SAM		51	
	3,7,3'-MeQg		18	
		SAH		16
		3,6,7,3'-MeQg		167
4'-OMT	SAM		130	
	3,7-MeQ		15	
		SAH		4.4
		3,7,4'-MeQ		10[§]
2'-GT	UDPG		250	
	2'-OH-substrate[†]		5	
		UDP		1000
		2'-glucoside		25
5'-GT	UDPG		250	
	5'-OH-substrate[‡]		10	
		UDP		1000
		5'-glucoside		20

[*] SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine.

[†] 5,2'-Dihydroxy-3,7,4',5'-tetramethoxyflavone.

[‡] 5,5'-Dihydroxy-3,6,7,2',4'-pentamethoxyflavone.

[§] Apparent K_i values.

quercetin is the natural substrate for this enzyme and that 3-methylation is the first step in the sequence of methyl transfers.

The 7-*O*-methyltransferase accepted 3-methylquercetin for further methylation at the 7-position. This enzyme was unable to methylate any of the mono-, di- or trimethyl derivatives tested. The specificity of this enzyme toward the 7-position of quercetin suggests that 7-*O*-methylation is the next step in the sequential methyl transfers of *Chrysosplenium* flavonoids.

The 4′-*O*-methyltransferase converted both 3,7-dimethylquercetin and its 6-hydroxy analogue, equally well, to their respective 4′-methyl derivatives. The 6-*O*-methyltransferase accepted either 3,7-dimethyl- or 3,7,3′- (or 3,7,4′-) trimethylquercetagetin and produced their respective 6-methyl derivatives at a ratio of 2:1. However, the fact that the 4′-*O*-methyltransferase did not react with trimethyl substrates (such as 3,6,7-trimethylquercetagetin) suggests that 4′-*O*-methylation may precede that at position 6; thus this establishes the third and fourth steps in the methylation sequence of these flavonoids [37] (Fig. 1).

In contrast with the above mentioned *O*-methyltransferases which accepted only hydroxylated or partially methylated aglycones, the two remaining methylation steps (at the 2′- and 5′-positions, Fig. 1) seem to take place at the glucoside level. A partially purified enzyme preparation was found to catalyse the 5′-*O*-methylation of A to B and the 2′-*O*-methylation of E to F (Fig. 1), with equal efficiency [38]. In view of the strict position-specificity of the 3-, 6-, 7- and 4′-*O*-methyltransferases, it seems reasonable to assume that the 2′- and 5′-*O*-methylating activities may be catalysed by two distinct enzymes which remain to be resolved.

To-date, only one other system has been characterized where multiple methylation reactions have been shown to be catalysed by distinct, highly specific enzymes [1]. Of the eight different enzymes involved in the biosynthesis of the benzylisoquinoline alkaloid, berberine, four of these are position-specific methyltransferases [1]. The degree of methylation of the functional hydroxyl groups seems to regulate the entry of a precursor, such as *S*-norlaudanosoline, into the biosynthetic pathway; therefore resulting in a variety of structural types characteristic of this group of alkaloids [1].

Kinetics of O-*methylation*

The steady state kinetic properties of three enzymes, the 3-,4′- and 6-*O*-methyltransferases [39], were consistent with a sequential, ordered reaction mechanism in which *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine were leading reaction partners, and included an abortive EQB complex (Fig. 2). Furthermore, the three enzymes exhibited competitive patterns between *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine, whereas the other patterns were either noncompetitive or uncompetitive (Table 3). The 6-*O*-methyltransferase was severely inhibited by its flavonoid substrate at concentrations close to K_m, whereas the other two enzymes were less affected by their respective substrates. The low inhibition constants for *S*-adenosyl-L-homocyteine (Table 2) suggest that the enzymes involved in earlier steps of methylation may regulate the rate of synthesis of the final products [39].

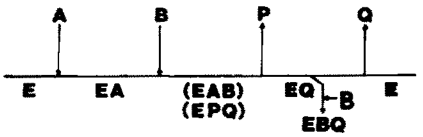

Fig. 2. Kinetic mechanism for the *O*-methyltransferases and *O*-glucosyltransferases involved in the biosynthesis of polymethylated flavonol glucosides. The mechanism for OMTs includes an abortive EQB complex. A, SAM/UDPG; B, flavonol substrate; P, flavonol product; Q, SAH/UDP (for OMTs/GTs, respectively).

O-*Glucosylation of partially methylated flavonols*

Previous studies [40] indicated the presence, in *Chrysosplenium*, of a novel, ring B-specific *O*-glucosyltransferase. This enzyme attacked the 2′- and 5′-positions of partially *O*-methylated flavonols and required two, *para*-oriented substituents on ring B for optimum activity (e.g. aglycones of compounds A and E, respectively, Fig. 1). Its properties are listed in Table 1. The fact that the 2′- and 5′-glucosylating activities could not be separated by conventional chromatography on several columns [40] as well as their similarities in reaction mechanism and kinetic constants [41], suggested that both glucosylation steps may be catalysed by a single enzyme.

Very recently, however, we have achieved the separation of the two *O*-glucosyltransferase activities using a fast protein liquid chromatography (FPLC) system and a combination of UDP-glucuronic acid agarose and brown-10 agarose columns [42]. The fact that UDP is a competitive inhibitor of the glucosyltransferase with respect to UDP-glucose (Table 3 [41]) allowed the use of the former support as an affinity chromatography column. However, separation of the two activities was achieved on the brown dye ligand column using a linear gradient between pH 6.5 and 8.0. The combined chromatographic steps resulted in 1200-fold purification of either activity, as compared with the crude extract. Each of the glucosyltransferase activities gave a single glucosylated product when reacted against its respective substrate, as indicated by autoradiography of the reaction products. This clearly indicates that the 2′- and 5′-glucosylation reactions in *Chrysosplenium* are catalysed by two distinct enzymes which differed only in their elution properties from the dye ligand affinity support [42].

Kinetics of O-*glucosylation*

The detailed kinetic analysis of the partially purified glucosyltransferase [41] was consistent with an ordered bi bi reaction mechanism, where UDP-glucose binds to the enzyme first followed by the flavonoid aglycone, and the release of the flavonoid glucoside followed by UDP (Fig. 2). The high inhibition constants for the glucosylated products, as compared with those of the flavonoid substrates (Table 2) indicated that glucosylation of *Chrysosplenium* flavonoids is not inhibited by the glucosylated products formed [41], and is consistent with the accumulation of compounds A and E (Fig. 1) as major flavonoid constituents of this tissue.

Table 3. Kinetic patterns of *Chrysosplenium* OMTs and GTs

Enzyme	Substrate	Product	Kinetic pattern*
3-OMT	SAM	SAH	C
	SAM	3MeQ	NC
	Q	SAH	UC
	Q	3MeQ	NC
6-OMT	SAM	SAH	C
	SAM	3,6,7,3'-MeQg	NC
	3,7,3'-MeQg	SAH	UC
	3,7,3'-MeQg	3,6,7,3'-MeQg	UC
4'-OMT	SAM	SAH	C
	SAM	3,7,4'-MeQ	NC
	3,7-MeQ	SAH	UC
	3,7-MeQ	3,7,4'-MeQ	NC
2'-GT	UDPG	UDP	C
	UDPG	2'-Glucoside†	NC
	2'-OH-substrate†	UDP	NC
	2'-OH-substrate†	2'-Glucoside†	NC
5'-GT	UDPG	UDP	C
	UDPG	5'-Glucoside‡	NC
	5'-OH-substrate‡	UDP	NC
	5'-OH-substrate‡	5'-Glucoside‡	NC

*C, competitive; NC, noncompetitive; UC, uncompetitive.

†5,2'-Dihydroxy-3,7,4'5'-tetramethoxyflavone and its 2'-*O*-glucoside.

‡5,5'-Dihydroxy-3,6,7,2',4'-pentamethoxyflavone and its 5'-*O*-glucoside.

Regulation of polymethylated flavonol glucoside synthesis in C. americanum

Metabolic regulation depends largely on the compartmentation of enzymes and the channelling of metabolic precursors, intermediates and final products from the site of activity to the site of accumulation. Apart from the hydroxylation steps at the 2'- and 6-position (Fig. 1) which remain to be investigated, the pathway of polymethylated flavonol glucoside synthesis consists of six *O*-methylation and two *O*-glucosylation reactions which were studied in some detail. This pathway seems to be under tight regulation, not only by the substrate specificity of the enzymes involved, but also by a number of other factors, namely (a) the strict position-specificity of these enzymes towards their hydroxylated or partially methylated substrates; (b) the apparent difference in compartmentation of the methylating enzymes, whereby *O*-methyltransferases earlier in the pathway utilized aglycones as substrates, whereas later enzymes accepted only glucosides; (c) the subtle, characteristic differences in the properties of the *O*-methyltransferases, with respect to their pH optima, pI values and requirement for Mg^{2+}, despite their similar molecular weights (Table 1); and (d) the sequential order of the multiple enzymes involved in the biosynthesis of polymethylated flavonol glucosides in this tissue.

Another important aspect of the regulation of partially methylated flavonol glucoside synthesis derives from the detailed kinetic analysis of a number of enzymes [39, 41]. The similarity of the kinetic mechanisms of the *O*-methyltransferases and *O*-glucosyltransferases studied (Fig. 2) and their regulation by a very specific range of substrate and product concentrations (Table 2) attest to this view. Despite the fact that the enzymes involved in the early steps of methylation had K_m values in the same range as those of glucosylation (Table 2) the apparent K_i values for the latter reaction were in the mM range as compared with the μM values for *O*-methylation, and are consistent with glucoside accumulation as the final products.

Another means of regulation of the methylation sequence involves the differential affinities of the different enzymes for *S*-adenosyl-L-methionine and *S*-adenosyl-L-homocysteine [39]. Whereas the three enzymes studied displayed similar affinities for their respective substrates (K_b values), the affinity for *S*-adenosyl-L-methione (K_a) was similar for the 3- and 4'-*O*-methyltransferases, whereas that attacking position 6 was two times greater (Table 2). Furthermore, the two former enzymes were subject to inhibition by low concentrations of *S*-adenosyl-L-homocysteine, since the apparent K_i for the latter was 25 times smaller than the K_m for *S*-adenosyl-L-methionine (as compared with 3 times for the 6-*O*-methyltransferase). These characteristics suggest that the enzymes earlier in the methylation sequence may regulate the rate of synthesis of the final products [39].

COMPARTMENTATION

Localization of partially methylated flavonol glucosides

It is generally accepted that hydrophilic secondary metabolites may be located in non-cytoplasmic compartments, mostly soluble in the cellular vacuole [43 and refs. therein]. On the other hand, lipophilic compounds are located either within membranes, or in the free state in cytoplasmic or non-cytoplasmic compartments [44 and refs. therein]. They may also be excreted on plant surfaces as farinose [45–48] or gummy [49] exudates. Whereas *Chrysosplenium* flavonoids possess some hydrophilic properties (due to the 2′-/5′-*O*-glucosyl residue), they are quite lipophilic since they contain between three and five methoxyl groups (Fig. 1). This dual solubility property prompted the study of their intracellular localization. Localization of flavonoids has been investigated using *in situ* microscopic (cytochemical) methods, including UV fluorescence microscopy, and the formation of precipitates with suitable reagents for non-fluorescent compounds (for review see [44, 48 and refs. therein]). Despite the variety of techniques used in the separation of component tissues and different cellular compartments [9, 44], it is often difficult to interpret the data on the localization of flavonoid glycosides due to the varying degree of purity of the isolated tissue/organelle, as well as its possible contamination with the metabolites released during the experimental procedures. Such problems have been compounded by the lack of specific histochemical tests for the detection of phenolic/flavonoid glycosides, as well as their solubility in the reagents normally used for microscopic preparations [9].

A novel approach, recently employed in our laboratory, is the use of immunological methods for the *in situ* intracellular localization of *Chrysosplenium* flavonoids. Compound A, a major constituent of this tissue, was conjugated to bovine serum albumin by the diazo reaction (Fig. 3A) in good yield [50]. An antibody raised against the latter conjugate [51] was found to be specific for the 2′-*O*-glucosides of tri- and tetramethoxyflavones (compounds A and B), with some cross reactivity against the pentamethoxyflavone 5′-*O*-glucoside (compound F); but none with quercetin or any of its methylated (3,7,4′-tri- or 3,7,3′,4′-tetramethyl-) aglycone derivatives [51]. Using this antibody with an indirect immunofluorescence technique allowed the localization of flavonoid glucosides within the walls of epidermal cells and, to a lesser extent, in mesophyll cell walls [52]. The weak fluorescence observed in mesophyll protoplast vacuoles suggested a minor role of this compartment in flavonoid accumulation. Further unequivocal evidence was obtained using a post-imbedding, immunogold labelling technique [53] which demonstrated that the partially methylated flavonol glucosides of *Chrysosplenium* were exclusively associated with the walls of epidermal and mesophyll cells (Fig. 3B and [54]), although the nature of such association is yet to be determined.

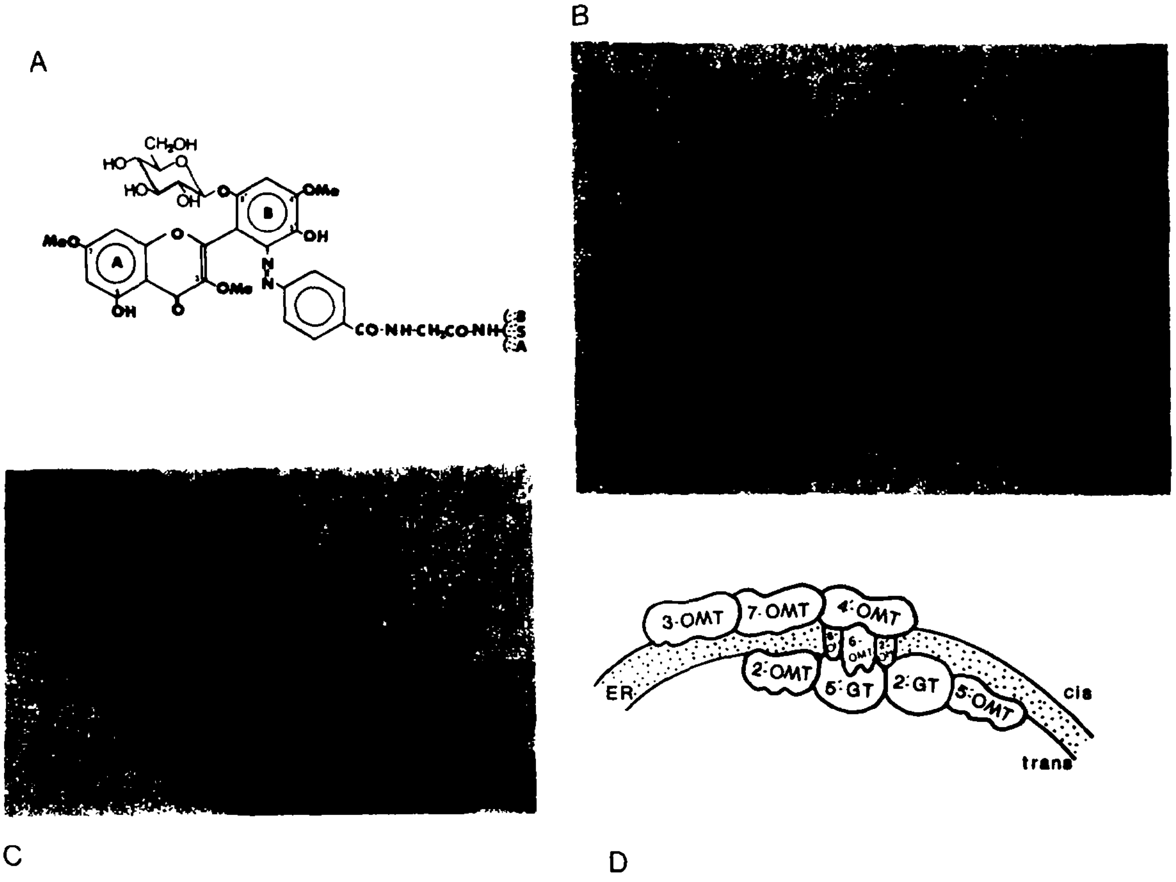

Fig. 3. A, Proposed structure of the flavonoid-BSA conjugate; B, electron micrograph of a section in *C. americanum* leaf showing immunogold labelling of the walls of epidermal cells; C, electron micrograph of a section in caffeine-treated leaf tissue showing the cell wall and periplasmic area of an epidermal cell (note the electron-dense deposits on the wall (arrow) and the membrane vesicles); D, a proposed model for the compartmentation of the enzymes involved in the biosynthesis of polymethylated flavonol glucosides in *C. americanum* (refer to the insert in Fig. 3C).

Electron microscopy has frequently been used to study the localization of phenolic polymers [e.g. 55–57]. The ultrastructural features of flavonoid accumulation in *Chrysosplenium* were also studied using caffeine as stabilizing [55–57] and visualizing agent. These studies [58] revealed the presence of electron-dense depositions within the cell walls of epidermal and mesophyll cells (Fig. 3C). These wall depositions disappeared when leaf segments were dipped for 1–2 sec intervals in organic solvents [48]. HPLC analysis of these effusates indicated the recovery of the major glucosides of this tissue [58]. Furthermore, various membrane profiles and associated vesicles in the periplasmic area were filled with darkly stained material (Fig. 3C). The fact that most of these vesicles were observed fused with the plasmalemma indicated the secretory nature of this tissue [58]. However, there was no evidence to indicate that the Golgi apparatus was involved in the packaging or channelling of the partially methylated flavonol glucosides in *Chrysosplenium* [54, 58].

Compartmentation of the partially methylated flavonol glucoside pathway

Whereas the enzymes studied in this pathway were found, after cell disintegration, in the cytosolic fraction, it is difficult to determine whether they are actually soluble, or easily solubilized, enzymes. They may be located in the living cell close to membranes where the protein concentration is much higher than in the cytoplasmic matrix, or loosely attached to a membrane (e.g. the ER) by weak ionic bonds. Such microenvironments may be destroyed during cell disintegration and protein isolation [12, 44]. However, in view of the concerted, spatially coupled, multistep sequence of *O*-methylations and *O*-glucosylations, it is tempting to postulate that the synthesis of partially methylated flavonol glucosides in *Chrysosplenium* may take place on the surface of an aggregated, membrane-associated, multienzyme system [59]. It is evident that if such system exists *in vivo*, its component enzymes appear to be loosely associated, or bound together by non-covalent forces, unlike the membrane-bound pathway of cyanogenic glycoside biosynthesis [60], or the multifunctional protein of the arom complex [61]. Such 'surface model' compartmentation [59] facilitates the formation of metabolic chains of intermediates and allows for increased catalytic activity. Furthermore, it permits the physical differences inherent to the microenvironments of the different enzymes of the pathway, such as changes in pH, changes in solubility of substrates and products, especially if the last steps in biosynthesis involves further methylation and/or glucosylation of relatively lipophilic intermediates [44], as is the case in *Chrysosplenium*. We have not yet been able to isolate a multienzyme system catalysing the methylation–glucosylation sequence in this tissue, since attempts to fractionate tissue homogenates by differential or equilibrium density centrifugation resulted in the recovery of both sets of enzymes from the supernatant fraction. However, various lines of evidence tend to support the concept of a multienzyme system; these are: (a) the incorporation of [^{14}C]cinnamate into the final products, after 5–10 min pulse, without labelling of the low methylated intermediates [36]; (b) the absence of low methylated intermediates among the products which accumulate in this tissue; (c) the sequential multistep *O*-methylation of positions 3, 7 and 4′ by cell-free extracts, and of other *O*-methylated intermediates at positions 6, 2′ and 5′; (d) the similarity of the kinetic mechanisms of the enzymes studied; (e) the regulation of these enzymes by very specific range of substrate and product concentrations; (f) the varied microenvironment of the different *O*-methyltransferases with respect to their pH optima and utilization of aglycones or glucosides as substrates; and (g) inhibition of the *O*-methyltransferases and *O*-glucosyltransferases studied by their flavonoid substrates. Whereas this indirect evidence does not unequivocally demonstrate the existence of a multienzyme system, we propose a model for the compartmentation of this pathway (Fig. 3D) in which the 3-, 7- and 4′-*O*-methyltransferases are loosely associated with the *cis* 'forming' face [62] of the ER membrane. Partially *O*-methylated intermediates could easily be hydroxylated at positions 6 and/or 2′ by specific hydroxylases, which are probably embedded in the membrane [44, 59], and further methylated at position 6. Due to differences in their microenvironment (pH, solubility), the 2′- and 5′-*O*-glucosyltransferases as well as the 2′- and 5′-*O*-methyltransferases are located on the *trans* 'maturing' face [62] of the ER membrane. Partially *O*-methylated intermediates could easily be transported ascross the membrane for further glucosylation and accumulation at the *trans* face, since they are sufficiently lipid-soluble. Since the cell wall is the site of accumulation of the final metabolites [52, 54, 58], the latter are usually packaged for secretion, in the non-cytoplasmic lumen of these membranes, across the plasmalemma [44]. Therefore, it may be envisaged that in a secretory system, such as *Chrysosplenium* [58], the *trans* face of the membrane invaginates to form cisternal channels or membrane-surrounded vesicles [63] in order to sequester the partially methylated flavonol glucosides, in a manner similar to that of the shikonin secretory system [64]. Such 'flavonoid vesicles' were observed in the periplasmic area of epidermal and mesophyll cells (Fig. 3C) [58]. These appeared to move towards, and fuse with, the plasmalemma for the discharge of their flavonoid content within the cell wall material. The fashion by which the flavonol glucosides are associated with the cell wall is, as yet, unknown. However, the fact that these compounds were readily recovered from leaf effusates [58] suggests that they may be adsorbed on cell wall proteins. Despite the attractive features of this proposed model, however, the use of immunocytochemical techniques, especially immunogold labelling, should provide unequivocal evidence for the compartmentation of the enzymes involved in this pathway.

Ecophysiological significance of partially methylated flavonol glucosides

Whereas *C. americanum* grows in a semi-aquatic habitat and low light intensity, it accumulates a number of highly methylated flavonoids of the type characteristic of semi-arid plants. The localization of these metabolites in the walls of epidermal and mesophyll cells may be considered as a means of eliminating such cytotoxic agents from the cell symplast, as well as protection of the plant against pathogens, predators and ultraviolet radiation [10], especially in the absence of lignified tissues, as is the case in *Chrysosplenium*. A similar situation exists in many aquatic and semi-aquatic plants which are known to accumulate a variety of sulphated flavonoids [65]. Nevertheless, some particular ecological factor ought to be sought to explain the accumulation and intracellular localization of *Chrysosplenium* flavonoids.

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