Enzymology of Flavonoid Methylation:
Purification and Kinetics of a
Number of Novel O-Methyltransferases

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ABSTRACT

Enzymology Of Flavonoid Methylation: Purification And Kinetics
Of a Number Of Novel O-Methyltransferases

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Four novel flavonol-O-methyltransferases (3-, 6-, 7- and 4'-OMTs)
from Chrysosplenium americanum and an 8-OMT from Lotus corniculatus
were partially purified by precipitation with ammonium sulphate and
successive chromatography on gel filtration column, hydroxyapatite,
S-adenosyl-L-homocysteine (SAH)-Agarose and chromatofocusing on
Polybuffer ion exchanger. They exhibited strict position specificity
for positions 3 of quercetin; 7 of 3-methylquercetin; 4' of
3,7-dimethylquercetin and 6 of 3,7,3'-trimethylquercetagetin and for
the 8-OMT, position 8 of 8-hydroxykaempferol. None of these enzymes
reacted with phenylpropanoids, flavones, dihydroflavonols or any of
their glucosides.

Except for the 7-OMT whose activity was lost during
chromatofocusing, the other enzymes had apparent pI values of 4.8,
5.4, 5.77 and 5.8 for 3-, 4'-, 6- and 8-OMTs respectively. They had
similar molecular weights (M 57,000) and their pH optima varied
between 7.5 and 8.8. Unlike the 3- and 4'- enzymes, the 6- and 8-OMTs
showed an absolute requirement for Mg^{2+} whose activation was saturable
and was inhibited by EDTA.

The steady state kinetic behavior of the 3-, 4'-, 6- and 8-OMTs suggested that all enzymes studied proceeded by a sequential ordered binding mechanism, whereby both flavonol substrate and the methyl donor S-adenosyl-L-methionine (SAM), had to bind to the enzyme prior to any product release. The three enzymes from Chrysosplenium had SAM and SAH as leading reaction partners and included an abortive EQB complex. The Lotus 8-OMT might also have SAM and SAH as leading reaction partners with an isomerisation of free enzyme or alternatively it could bind kaempferol before SAM, followed by the release of SAH and 8 methoxykaempferol.

The in vitro stepwise O-methylation of quercetin 3-methylquercetin → 3,7-dimethylquercetin → 3,7,4'-trimethylquercetin, tends to suggest a coordinated sequence of methyl transfers in Chrysosplenium. Furthermore, these reactions could be controlled by the respective K_{mSAM}/K_{iSAH} ratio for each enzyme in the sequence, whereas in the case of the 8-OMT of Lotus the regulation of enzyme activity could be controlled solely by the concentration of SAH in the cell.

D.E.D.I.C.A.T.I.O.N.

To my parents for their constant

encouragement

To Liliana for her

love and patience

To Erica and Antonio who are

my inspiration

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A. INTRODUCTION

Flavonoid compounds are widely distributed in the plant kingdom and may be characterized by methyl ether substitution at various positions of the flavonoid ring system. Enzymatic O-methylation, which is catalysed by O-methyltransferase (E.C.2.1.1.6.), will reduce the chemical reactivity of the phenolic hydroxyl groups and render them lipophilic.

The flavonoid O-methyltransferases, so far reported, are known to mediate the transer of methyl groups from S-adenosyl-L-methionine to the meta or para hydroxyl groups of ring B of flavones/flavonols and isoflavones. More recently, an ortho and A-ring-para directing O-methyltransferase were reported from Glycyrrhiza echinata and tobacco cell cultures, respectively. However, these enzymes catalysed specific, single methylations and did not accept partially methylated substrates for further O-methylation. Recent work in this laboratory demonstrated the stepwise methylation of flavonoid substrates, including quercetin, to various methylated derivatives by partially purified enzyme preparations of Calamondin orange. These enzyme extracts did not lend themselves to further purification and raised the question as to whether sequential methylation of flavonoids is catalysed by one or several distinct enzymes.

Chrysosplenium americanum accumulates several partially methylated flavonol glucosides which have interesting methyl substitutions at positions 3, 6, 7, 2', 4' and/or 5'. Our current interest in the O-methylation of flavonoid compounds prompted us to use this tissue as a source of O-methyltransferases in order to

study the sequential methylation of flavonols, to establish the order of this O-methylation and to isolate and purify these enzymes. In addition, studies with the O-methyltransferase of <u>Lotus corniculatus</u> which catalyses the 8-O-methylation of flavonols, would complement the studies on the <u>Chrysosplenium</u> system which lacks substitution at this position.

Although several flavonoid O-methyltransferases have been isolated from different tissues, very few studies have dealt with the kinetics of these reactions. The only well known kinetic analysis from plant tissues is that of the isoflavone 4'-O-methyltransferase from <u>Cicer arietinum</u> and very recently, there was a report on the vitexin 2"-rhamnoside-7-O-methyltransferase from <u>Avena sativa</u>. These enzymes were postulated to proceed by an ordered bi bi mechanism with S-adenosyl-L-methionine and S-adenosyl-L-homocysteine as leading reaction partners. The highly purified 3-, 6- and 4'-O-methyl-transferases from <u>Chrysosplenium</u> as well as the 8-O-methyltransferase from <u>Lotus</u> were submitted to substrate interaction and product inhibition kinetics in order to establish the kinetic mechanisms of these reactions, in relation to the sequential methylation of <u>Chrysosplenium</u> flavonoids versus the final methylation step in <u>Lotus</u>.

B. REVIEW OF LITERATURE

Our knowledge of the biosynthesis of flavonoids has expanded very rapidly in the last decade. In particular, a great deal has been learned about the enzymology and regulation of flavone and flavonol glycoside biosynthesis. Therefore, the aim of this review will be to describe some of the novel enzymes involved in the assembly and modification of the flavonoid ring with special emphasis on how these steps may pertain to the biosynthesis of polymethylated flavonoids.

B.1. Enzymatic synthesis of flavonoids

Grisebach (1) postulated in 1962 that flavonoids were synthesized by the successive condensation of a cinnamoyl-CoA ester with three molecules of malonyl-CoA and the simultaneous liberation of 3 mol of CO_2 (Fig. 1e). This hypothesis, primarily based on feeding experiments with ^{14}C-labelled acetate and phenylalanine (see ref. 2 for review),has been widely accepted as a result of more recent work at the enzymatic level (see ref. 3 for review).

Much of the enzymology based on the work of the Freiburg group (2, 3) was carried out using parsley cell suspension cultures (Petroselinum hortense). The enzymes of flavonoid biosynthesis shown in Fig. 1 can be classified into two groups. The first (group I) which converts L-phenylalanine to 4-coumaroyl-CoA provides a pool of precursors, not only for flavonoid biosynthesis, but also for most other phenylpropanoid compounds such as lignins (4) and cinnamoyl esters (5). The second (group II) consists of at least 13 enzymes and converts phenylpropanoid precursors into complete acylated flavonol

Group I

Group II

Phenylalanine

Cinnamate

4-Coumarate

4-Coumaroyl-CoA

CoAS

Acetate ⟶ $\xrightarrow{d}$ Malonyl-CoA $\diagdown$ e

Naringenin chalcone

Naringenin
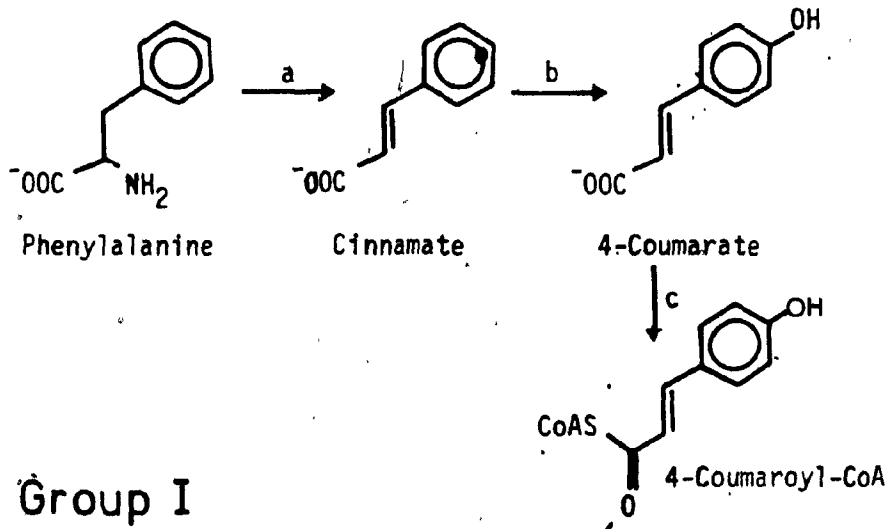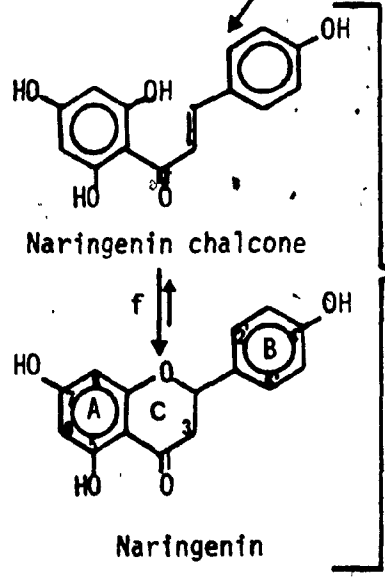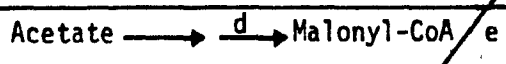
flavones,
flavonols,
anthocyanins,
isoflavonoids, etc.

Figure 1.   Common steps in the biosynthesis of flavonoids (3).

(a) Phenylalanine ammonia-lyase (E.C.4.3.1.5.)

(b) Cinnamate-4-hydroxylase (E.C.1.14.13.11)

(c) 4-Coumarate-CoA ligase (E.C.1.12)

(d) Acetyl-CoA carboxylase (E.C.6.4.1.2.)

(e) Chalcone synthase _____

(f) Chalcone isomerase (E.C.5.5.1.6.)

glucosides (3). These enzymes have been extensively reviewed in 1975 (2), 1979 (6) and most recently in 1981 (3).

B.1.1. Chalcone synthase

Chalcone synthase (Fig. 1e), the key enzyme of flavonoid biosynthesis, was previously thought to be a flavanone synthase (7). Recently however, a naringenin chalcone was identified (8) as the immediate product of the reaction. This important enzyme catalyses the sequential condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA (7, 9).

The substrate specificity of the purified enzyme for 4-coumaroyl-CoA, rather than caffeoyl (3,4-dihydroxy- cinnamoyl) or feruloyl (3-methoxy-4-hydroxycinnamoyl)-CoA esters (10), indicates that ring B substitution of flavonoids may be determined at the C_{15} stage and not at the cinnamoyl level. This was found true for both the enzymes from parsley (10) and Haplopappus gracilis (11) cell cultures. The substrate specificity of chalcone synthase also indicates that further hydroxylation of positions other than 5-, 7- and 4'- as well as subsequent O-methylation, may be catalysed by specific hydroxylases and O-methyltransferases (OMTs).

B.1.2. Hydroxylases

The hydroxyl groups which arise during the assembly of flavonoid rings from their natural precursors are usually located at 5-, 7- and 4'- positions (Fig. 1). Others (6-, 8-, 2'- and 3'- or 5'-) may be substituted prior to the synthesis of the C_{15} chalcone, according to the "cinnamic acid starter hypothesis" of Hess (12). However, recent

evidence showed that modifications in the substitution pattern may also take place after the assembly of the basic flavonoid molecule.

There are two alternative strategies for the introduction of a hydroxyl group into a substrate during metabolism. The first involves the addition of a preformed hydroxyl group originating from water to an electron deficient center. The second type, catalyzed by oxygenases (13), occurs with oxygen-deficient lipophilic substrates and involves de novo synthesis of a hydroxyl group due to oxygenation of a substrate with molecular oxygen.

B.1.2.a. Cinnamate-4-hydroxylase (E.C.1.14.13.11) (Fig. 1b)

The second step in the general phenylpropanoid pathway involves the hydroxylation of trans-cinnamic acid to its 4-hydroxy derivative, p-coumaric acid, by the action of cinnamate-4-hydroxylase. This enzyme which was reviewed recently (14), has been established as a member of the cytochrome P-450 dependent oxygenases (15-19). It utilizes 1 mole of NADPH and 1 mole of O_2 for each mole of cinnamic acid as required for a mixed function oxidase (16). Cinnamate-4-hydroxylase seems to be membrane-bound (16, 18-20) and co-migrates with marker enzymes on sucrose density gradients corresponding with the endoplasmic reticulum. Unfortunately, the range of substrate specificity for the microsomal preparations from various plant species that catalyse the 4-hydroxylation of trans-cinnamate has not been extensively investigated (21).

B.1.2.b. Flavonoïd-specific hydroxylases

The determination of the substitution pattern of the B-ring of flavonoids remained for a long time an unsolved question in flavonoid biosynthesis (2). Early work using a spinach beet phenolase (22, 23) demonstrated the 3'-hydroxylation of naringenin (5,7,4'-trihydroxy-flavanone), dihydrokaempferol and kaempferol (3,5,7,4'-tetrahydroxy-flavone). However, this enzyme exhibited broad specificity and accepted p-coumaric and caffeic acids as substrates.

More recently, two laboratories working in collaboration (24), and separately (25, 26) seem to have solved this perplexing question. Extracts from Matthiola incana (24), Antirrhinum majus (snapdragon) flowers (25) and parsely cell cultures (26) were shown to catalyse the 3'-hydroxylation of the B-ring of flavanones and dihydroflavonols. Furthermore, enzymes from flowers of Verbena hydrida (27), not only catalysed the 3'-hydroxylation of flavanones and dihydroflavonols, but also at the 5'-position of these compounds. Enzymes from each source were found in the microsomal fraction, required NADPH as cofactor and could be cytochrome P-450 dependent.

Flavanone   Flavanonol

Flavone   Flavonol
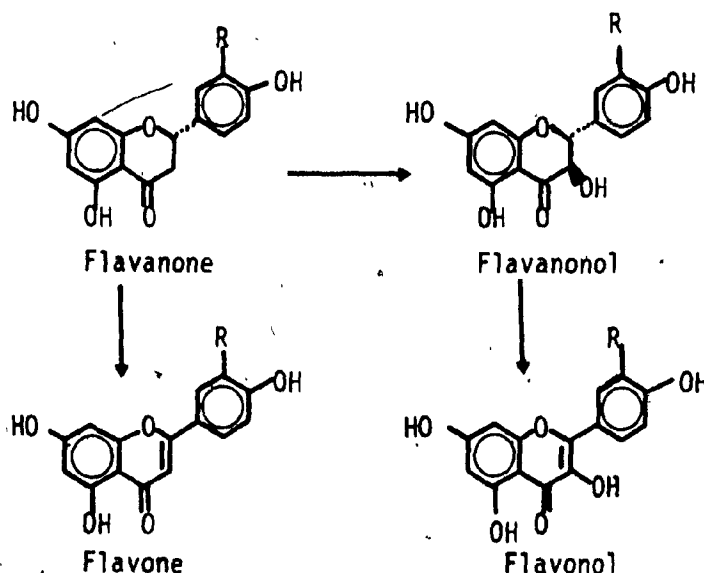
A. majus (25) and parsely cell cultures (26) were also shown to contain a flavanone oxidase which catalysed the oxidation of flavanone to flavone. The enzyme preparation from A. majus which catalysed the conversion of flavanone to flavone and of dihydroflavonol to flavonol (25) was microsomal and required NADPH as cofactor; whereas in parsley (26), this reaction was soluble and was dependent on 2-oxoglutarate, ascorbate and Fe^{2+} for activity. Thus, two distinctly different enzymes, an NADPH-dependent monooxygenase and a 2-oxoglutarate-dependent dioxygenase appear to mediate the oxidation of flavanones and flavonols. Surprisingly, Matthiola incana flowers (24), were shown to contain a soluble flavanone 3-hydroxylase which also required 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors. This preparation catalysed the 3-hydroxylation of naringenin and eriodictyol (5,7,3'4'-tetrahydroxyflavanone) to their respective dihydroflavonols, but did not mediate the oxidation to flavonols.

Further characterisation of these preparations is necessary to establish their specificity for flavonoid compounds in comparison with hydroxycinnamic acids. It would be interesting to compare the specificity of cinnamate-4-hydroxylase in microsomal preparations with that of the flavonoid B-ring-specific hydroxylases (24-27) from these tissues. Such studies may serve to test the hypothesis that multiple forms of plant cytochrome P-450 may confer substrate specificity to the hydroxylase reaction (14, 16).

Nothing is known about the introduction of hydroxyl groups at positions 6 and/or 8 of the flavonoid ring A. Although substitution at these positions was believed to be of less common occurrence

(2), many reports of 6- and 8- substituted flavonoids have appeared recently in the literature (28).

The known example of a flavonoid 8-hydroxylase was reported from the bacterium Pseudomonas putida (29). This bacterium was grown on quercetin (3,5,7,3',4'-pentahydroxyflavone) as the sole carbon source, thus inducing the production of enzymes involved in the degradation of this substrate. The first enzyme in the sequence of degradation was shown to be a mixed function oxygenase (29) which introduced a hydroxyl at C_8 of ring A. This enzyme had properties typical of a flavoprotein and required NADH as a cofactor in the reaction. This hydroxylation step was necessary for ring cleavage between C_8 and the flavonoid C-ring by a dioxygenase, the second enzymatic step in quercetin degradation. It is interesting to speculate whether a similar type of enzyme(s) is responsible for ring A hydroxylation in plants.

B.1.3. Flavonoid-O-methyltransferases (E.C.2.1.1)

Methyl transfer reactions are catalysed by methyltransferases which have been shown to be of widespread occurrence (30-32) in nature. Transmethylation has been demonstrated with DNA, proteins, polysaccharides, tRNA as well as other small molecules in animal systems (30, 31). In plants (32) transmethylation seems to be even more widespread as evidenced by the vast number of secondary products which contain one or more methyl groups. This section of the review will concern itself with the O-methylation of flavonoids and the reader is referred to several excellent reviews on the methylation of other substrates (30-33).

The study of flavonoid OMTs has long been complicated by the fact
that plant tissues contain another OMT which transforms caffeic acid
(3,4-dihydroxycinnamic) to ferulic acid (3-methoxy-4-hydroxycinnamic).
After a hypothetical 5-hydroxylation of the latter to sinapic acid
(3,5-dimethoxy-4-hydroxycinnamic) (32), both methoxy acids undergo
reduction to their corresponding alcohols before being polymerized to
lignin. Caffeic OMT which is found in most plant tissues has been
reported to possess broad substrate specificity and has often been
compared to the catechol OMT of animal tissues (34-35). Caffeic OMTs
which did not accept flavonoids as substrates were recently isolated
from bamboo shoots, Japanese black pine, soybean tissue culture and
tobacco cell culture (32). The specificity of this enzyme for
phenylpropanoids was recognized; however, unlike catechol OMT, it has
never been purified to homogeneity (36, 37) or studied kinetically.

Similarly, flavonoid-specific OMTs free of contaminating caffeic
OMT have seldom been prepared. Therefore, it was not certain whether
methyl groups were introduced into flavonoids at the phenylpropanoid
level, at a later stage during flavonoid biosynthesis, or both. It is
certain, however, that flavonoid-specific OMTs can only be studied
once contaminating caffeic OMT has been eliminated through
purification.

B.1.3.a. Substrate specificity

Until recently, our knowledge of O-methylation by
SAM-flavonoid-OMTs had been restricted to the formation of monomethyl
ether derivatives. These methylations involved the _meta_-directing
3'-OMT of flavones and flavonols in parsley (38) and soybean cell

cultures (39) and _Tulipa_ anthers (40), the para-directing OMT which
attacked the 4'-position of isoflavones in chick pea cell cultures
(41) and the 7-position of quercetin in tobacco cell culture (42, 43)
and more recently, the ortho-directing OMT of the retrochalcone,
licodione in cultured cells of _Glycyrrhiza echinata_ (44). On the
other hand, O-methylations of position 3 of the heterocyclic ring, as
well as positions 6 and 8, by specific O-methyltransferases have not
unequivocally been demonstrated. Most of these enzymes (38-43) have
recently been reviewed by Poulton (32) and were shown to catalyse
single, position-specific methylations on the flavonoid ring.
However, none of these enzymes has been shown to accept partially
methylated compounds for further O-methylation. The common occurrence
of partially and fully methylated flavonoids (28, 45, 46) raises the
question as to whether multiple methylations within one type of
flavonoid structure are mediated by one or several distinct enzymes.

Recent work in this laboratory demonstrated the stepwise
methylation of a number of flavonoid substrates, including quercetin,
to various methylated derivatives by partially purified preparations
of Calamondin orange (47, 48). Furthermore, these enzyme preparations
accepted partially methylated flavonoid substrates and transformed
them to the next higher order of methylation (48), thus indicating the
presence of several OMTs. Unfortunately, Calamondin enzyme extracts
did not lend themselves to further purification.

More recently, it was demonstrated that the stepwise methylation
of phosphatidylethanolamine to phosphatidylcholine in rat liver
microsomes was mediated by two distinct N-methyltransferases; the

first of which was rate-limiting and required Mg^{2+} for activity (49).
Furthermore, _Ruta graveolens_ cell extracts were shown to contain a
soluble OMT system which methylated the 5- and 8-hydroxyl groups of
linear furanocoumarins (50). One enzyme methylated the 8-hydroxyl
groups of xanthotoxol (8-hydroxyfuranocoumarin) and 8-hydroxybergapten
(5-methoxy-8-hydroxyfuranocoumarin) and the other methylated the
5-hydroxyl groups of bergaptol (5-hydroxyfuranocoumarin) and
5-hydroxyanthotoxin (8-methoxy-5-hydroxyfuranocoumarin) (51). These
two examples demonstrate that methylation at different positions of
the same molecule may be catalysed by distinct, position-specific
enzymes.

B.1.3.b. Regulation

The _in vitro_ inhibition of methyltransferases by S-adenosyl-L-
homocysteine (SAH) has been extensively studied as a potential
regulator of biological methylation. Mudd et al. (52) were the first
to show that transmethylation reactions which utilize S-adenosyl-L-
methionine (SAM) as methyl donor are sensitive to inhibition by SAH.
Since 1963, the list of methyltransferases that are known to be
inhibited by SAH has grown considerably. It is now well established
that, with the exception of DNA methylation, all transmethylation
reactions that utilize SAM as a substrate are inhibited by SAH
(32, 53).

Cantoni et al. (53), in describing the sensitivity of different
methyltransferases to changes in the SAM/SAH ratio, found that these
changes did not correlate with either the K_m's for SAM or the K_i's for
SAH, but with the ratio K_{mSAM}/K_{iSAH}. Furthermore, it was

demonstrated that this type of calculation gave a hierarchy of

methyltransferases, some of which were very sensititive to

$K_m SAM/K_i SAH$ and others which were less sensitive to this ratio.

Poulton and Butt (54) were the first to suggest that

transmethylation reactions in plants might be controlled by the

intracellular SAM/SAH ratio. However, the question of which factors

control the intracellular level of SAM and SAH is much more difficult

to resolve. These two important metabolites have been measured in

plants (55, 56). Their levels, which were similar to those of animal

systems (53), were low and within the same orders of magnitude

(SAM/SAH = 1.5, SAM concentration = 0.04 μmoles/g fw). These

observations, as well as the potent inhibition observed with SAH for

most methyltransferases (32, 53), demonstrate the requirement for

strict regulation of SAH within the cell.

Cleavage of SAH by SAH-hydrolase (E.C.3.3.1.1.) is the only known

pathway for SAH metabolism in plants (57). This enzyme has been

demonstrated in several plant tissues (56) and has been partially

purified from spinach·beet (58) and to homogeneity from yellow-lupin

seeds (59). It has been suggested (32, 53) that SAH concentration and

thus, the rates of methylation could be regulated by SAH hydrolase.

The evidence for this is very convincing in animal systems (53),

whereas it has not unequivocally been proven in plants (32).

SAH hydrolase from lupin seeds (59) resembled the enzymes from

rat liver (60) and yeast (61) in showing high specificity for both

L-homocysteine and adenosine. In addition, all SAH hydrolases studied

were shown to strongly favor SAH synthesis (58-61). The equilibrium

could be shifted towards the hydrolysis of SAH if the concentration of products were kept low (58, 60). It has been suggested that the in vivo levels of these products could be decreased via remethylation of homocysteine to methionine by tetrahydro-pteroylglutamate methyl-transferase (62, 63) and the conversion of adenosine to ADP by the successive action of adenosine kinase and adenylate kinase (58). However, this hypothesis has not been tested in plants, and there is no clear indication of its contribution to the regulatory mechanisms by which the cell catalyses different methylation reactions.

Whereas several OMTs have been shown to transfer methyl groups from SAM to flavonoids (38-46), inhibition of the reaction by methylated products has not been extensively studied. The determination of inhibition constants for the methylated products, as compared to the K_m for the flavonoid substrate, could be important in assessing the contribution of these terms to the overall rates of methylation observed. For this reason, it is difficult to understand why these types of studies have not been performed (32).

B.1.3.c. Localisation studies: the SAM pathway

The subcellular localisation of the enzymes responsible for the conversion of aspartate to o-phosphohomoserine (Fig. 2) has been shown to occur within the chloroplast (64), though it was not known whether these enzymes occured in other cell fractions. Conclusive evidence for the localisation of these enzymes, including homoserine kinase, cystathionine γ-synthase and cystathionine β-lyase (Fig. 2) in the chloroplast was presented by Wallsgrove et al. (65). However, the enzymes responsible for further metabolism of homocysteine to
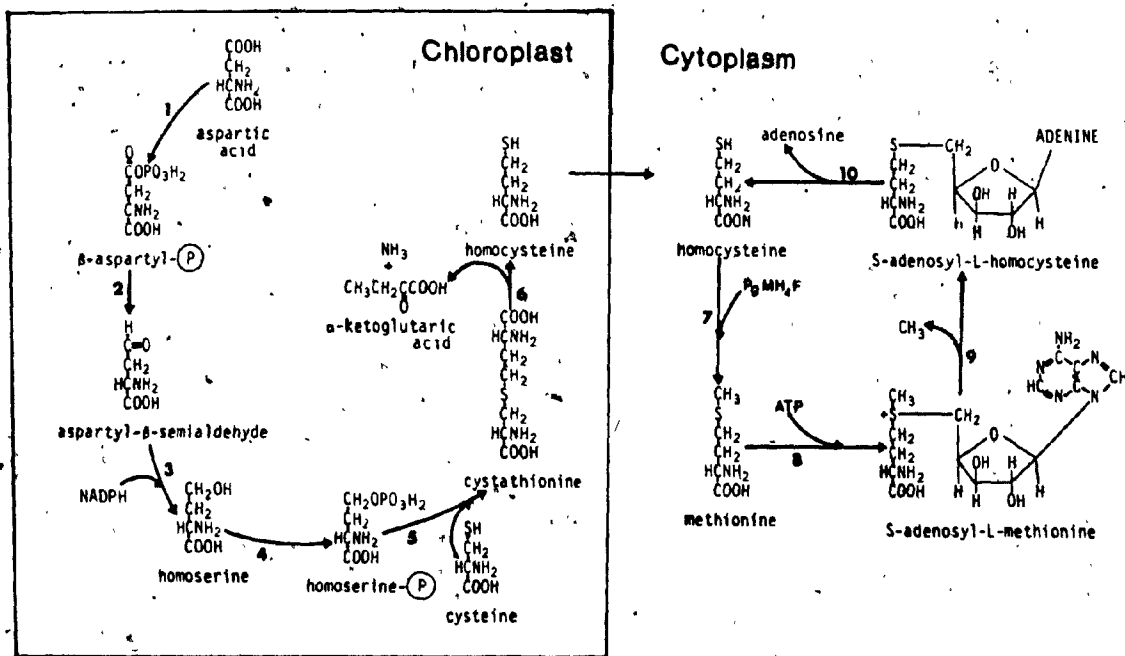
Figure 2.   Localisation of the pathway of S-adenosyl-L
             methionine biosynthesis (65).

             (1) Aspartate kinase, (2) Aspartyl semialdehyde
             dehydrogenase, (3) Homoserine dehydrogenase,
             (4) Homoserine kinase, (5) Cystathionine-γ
             -synthase, (6) Cystathionine-β-lyase, (7)
             Methionine synthase (8) Methionine adenosyl
             transferase, (9) Methyltransferase, (10) S-
             Adenosyl-L-homocysteine hydrolase. PgMeH$_4$F is
             Polyglutamyl-5-methyltetrahydrofolate

methionine and SAM (Fig. 2) (65) could not be localised within the
chloroplast. The inability of plastids to synthesize SAM was also
demonstrated when isolated cucumber etioplasts (66), which are
dependent on exogenous SAM for the methylation of Mg-protoporphyrin X,
could not carry out this reaction in the presence of methionine and
ATP.

Figure 2 shows the suggested localisation of the SAM pathway.
All the enzymes necessary for homocysteine biosynthesis are contained
in the chloroplast, which is also the site of incorporation of
inorganic sulfur into cysteine (56). However, the synthesis of
methionine and SAM takes place in the cytoplasm. It is interesting to
note that the only enzymes found in animal cells, methionine synthase
and methionine adenosyltransferase (Fig. 2), are precisely those not
located in the chloroplast (65).

B.1.3.d. Localisation studies: enzymes of the flavonoid pathway.

The enzymes of the phenylpropanoid pathway and flavonoid
biosynthesis have been postulated to occur within the plastids (67-68)
with the subsequent transport of completed flavonoids to the vacuole
(69). On the other hand, the cytosol was shown to be the main site of
chalcone synthase, chalcone-flavanone isomerase and UDP-glucose:
anthocyanidin 3-O-glucosyltransferase activities in Hyppeastrum and
Tulipa spp. (70). Similarly, the activities of SAM:caffeic acid OMT,
flavanone synthase, UDP:flavonoid 3-O-glucosyltransferase and
SAM:quercetin OMT could only be detected in the cytoplasm of peas,
green beans, spinach and red cabbage (71). These workers concluded
that chloroplasts are not involved in flavonoid synthesis.

A recent study by Charriere-Ladreix et al. (72) claimed that the stepwise O-methylation of quercetin and quercetagetin (6-hydroxy-quercetin) was catalysed by spinach chloroplast envelope, whereas the chloroplast stroma contained a soluble caffeic OMT. However, spinach contains only trace amounts of methylated flavonoids (73, 74) and low levels of caffeic-OMT and quercetin-OMT (71). Furthermore, the claim that the chloroplast envelope was the site (72) of multiple methylation of flavonoids was based on assays containing 700 µg protein which converted about 4 nmoles of quercetin or 8 nmoles of caffeic acid to their methylated products within 30 min. This represented less than one and two percent of the respective quercetin and caffeic OMT activities, present within the whole tissue (71). These results could easily be attributed to cytoplasmic OMTs contaminating the 'purified' chloroplasts and therefore, could be artifacts of the preparation. Furthermore, these workers (72) failed to give convincing evidence for the identity of the radiolabelled products which were synthesized by this system.

B.2. Enzyme kinetics·

Very few studies have dealt with the complete kinetic analysis of enzymes catalysing methyl transfer reactions, most of which were reported·from animal systems (75-79). The best characterised of the methyltransferases, catechol-OMT, catalyzed the conversion of epinephrine to metanephrine and paranephrine. The kinetic mechanism derived for the catechol-OMT involves random addition of substrates and release of products. Furthermore, the formation of a dead-end

complex between the enzyme, methyl acceptor and methylated product
was postulated in order to explain the product inhibition patterns
observed (75). A ping pong mechanism for histamine-N-methyl-
transferase (76) was proposed on the basis that substrate interaction
kinetics gave parallel lines in Lineweaver-Burke plots. However,
these studies alone cannot exclude a sequential binding mechanism
(79). Similarly, two out of five charge-isoenzymes of rabbit adrenal
norepinephrine N-methyltransferases (77, 78) gave parallel lines in
substrate interaction kinetics. However, these authors postulated an
ordered bi bi mechanism with SAM and SAH as leading reaction partners
and containing one or more dead-end complexes, based on product
inhibition studies (78).

Although plant systems catalyse various types of methylation by
specific methyltransferases, no complete kinetic analysis involving
initial velocity and product inhibition studies has been reported.
Most of the work carried out on plant enzymes demonstrated that SAH
was a potent competitive inhibitor versus SAM at physiological
concentrations (32).

The isoflavone-4'-O-methyltransferase isolated from Cicer
arietinum (41) was postulated to proceed by an ordered bi bi mechanism
with SAM and SAH as leading reaction partners. However, product
inhibition studies using the methylated isoflavonoid product were not
carried out because of poor solubility of this product. Evidence for
a sequential binding mechanism was also obtained for SAM:Mg-
protoporphyrin methyltransferase isolated from Euglena (80), although
the nature of binding was not determined. Indirect evidence for an

ordered binding mechanism was shown by Sharma and Brown (51) for the 5- and 8-hydroxyfuranoconmarin methyltransferases as well as caffeic-OMT from _Ruta graveolens_ cell cultures. It was found that the latter enzymes could only bind to xanthotoxin- and ferulic acid-immobilized Sepharose columns, if SAM or SAH was present in the irrigant buffer. It was concluded that each of these enzymes had to bind SAM or SAH before it could bind the phenolic substrate and therefore a compulsory, ordered kinetic mechanism was proposed (51).

B.3. The stereochemistry of methylation

Stereochemical studies can contribute significantly to our understanding of the mechanism of enzymatic reactions. Enzymatic processes, unlike non-biological reactions, are almost always stereospecific since the enzyme must orient reaction partners within a rigid conformation in order to achieve high rates of reaction and high degree of substrate specificity. This fact permits the use of chiral groups to determine the orientation of transfer and thus, to distinguish between sequential and ping pong mechanisms (81).

Two laboratories, those of Arigoni (Zurich) and Floss (Ohio State), have recently initiated studies on the stereochemistry of the enzymatic transfer of chiral methyl (H,D,T) groups from SAM to various substrates. Six methyltransferases were studied (82-86) and in each case, the transfer occurred with inversion of configuration of the methyl group; thus precluding a ping pong mechanism and methylated-enzyme intermediate. This suggested a common SN_2 mechanism where the methyl donor and methyl acceptor would have to bind to the enzyme

active site prior to any product release. This is consistent with a sequential mechanism in which a ternary-SAM-methyl acceptor-complex was formed.

Thus, in the case of methyltransferases several enzymes which catalyse methyl group transfer to carbon (84-86), sulphur (86), nitrogen (84) or oxygen (82, 83, 86) nucleophiles do so with the same stereochemistry. This increases the possibility that the steric course of methyl.transfer is dictated by its mechanism and is in agreement with the sequential binding of substrates postulated for several methyltransferases through steady state enzyme kinetics (41, 51, 75, 77, 78, 80).

B.4: The regulation of flavonoids in plants

It is apparent from previous reviews (eg 87) that flavonoids may function in higher plants as attractants of agents involved in pollination. Some have speculated that flavonoids act as protective substances against UV light (88) or infection by phytopathogenic organisms (89). It is also well established that flavonoid levels may vary greatly during different stages of plant development (90), thus providing evidence that the plant actively controls the biosynthesis and degradation of these compounds.

Irradiation with ultraviolet light has been shown to induce the enzymes of general phenylpropanoid metabolism (Group I, Fig. 1) and of the flavonoid glycoside pathway (Group II, Fig. 1) in cultures of parsley cells (91). This coordinated induction of enzyme activities was attribued to de novo synthesis of subunits and assembly of these

enzymes (92-94) and not to an activation of preformed inactive proteins. These results have been extended to Phaseolus vulgaris (95) as well as other systems (96) indicating that the phenomena observed in parsley cell cultures may be widespread. It is believed that methyltransferases, which belong to Group II, would also be induced in a similar fashion (3), although this has not been studied in these systems.

The enzymes of Group I could be induced more rapidly when parsley cells were treated with an 'elicitor' from the phytopathogen, Phytophthora megasperma (97). The enzymes of group II were not induced under these conditions. Instead, the accumulation of linear furanocoumarins (phytoalexins) in response to the elicitor treatment (97, 98) suggested the existence of a third group of enzymes which might be involved in an elicitor-specific, light-independent pathway.

Thus, each signal (UV irradiation or treatment with elicitor) appears to trigger the formation of one class of compounds (UV protective flavonoids or antimicrobial phytoalexins) whose potential function is to protect the plant against possible damage by the inducing agent. The nature of these triggers is not well understood; however, their elucidation should contribute to the understanding of these phenomena, as well as the controls regulating secondary metabolite synthesis in plants.

C. MATERIALS AND METHODS

C.1. The plant material

C.I.1. Chrysosplenium americanum

Chrysosplenium comprises 55 species, that are widely distributed in the northern hemisphere. This genus is characterized by a high degree of flavonoid O-methylation and, in several species, the occurrence of unusual substitution with hydroxyl and/or methoxyl groups at positions 6 and/or 2' (99-101).

C. americanum, which occurs in eastern North America, accumulates a variety of tri-, tetra- and penta-O-methylated flavonols which are derived from quercetin and quercetagetin (6-hydroxy-quercetin) both with 2'-substitution (102) as shown in Figure 3. These compounds, which are of relatively rare distribution occur as 2'-/5'-monoglucosides, together with smaller amounts of their free algycones. Conspicuous by their absence, are the early methylated intermediates (mono- and dimethylquercetin and -quercetagetin); indicating that these are rapidly transformed into final products in vivo (102).

Our current interest in the O-methylation of flavonoid compounds (42, 43, 47, 48) prompted us to use C. americanum as a source of OMTs because of its unusual methyl substitution at positions 3,6,7,2',4' and/or 5' (Fig. 3). Early work in this laboratory demonstrated that when [2-^{14}C]-cinnamic acid was administered to leaves, the polymethylated flavonol glucosides were labelled within 30 min. (Fig. 3 and Appendix Fig. 1). This demonstrated the presence of the whole pathway and indicated that all the enzymes catalysing these reactions

(1)

(2)

(3)

(4)

(5)

(6)
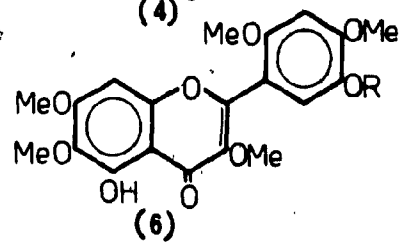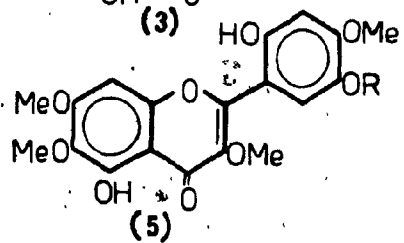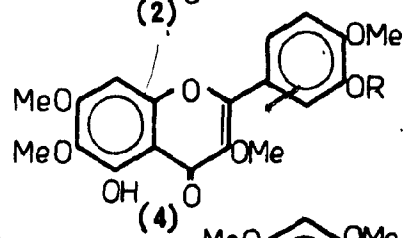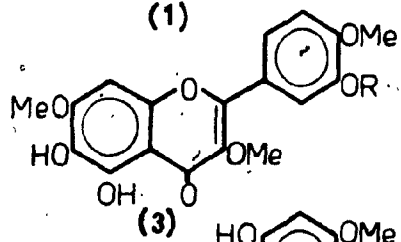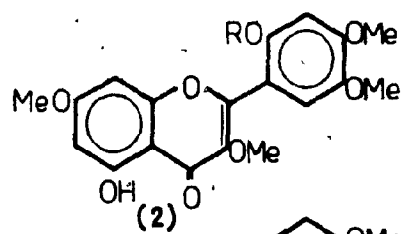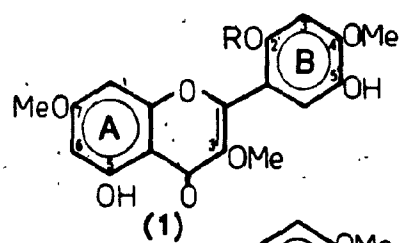
Figure 3. Structural formulae of the six highly O-

methylated flavonol glucosides of

<u>Chrysosplenium</u> <u>americanum</u> (102).

This tissue contains two 2'-β-0-D-glucosides of

partially methylated 2'-hydroxyquercetin and

four 5'-β-0-D-glucosides of partially

methylated 6-hydroxy and 6,2'-dihydroxy-

quercetin. Note that since the 2'-position is

substituted, then numbering of the <u>o</u>-dihydroxy

grouping on ring B becomes 4', 5' instead of

3',4' since both 3'- and 5'- positions are

identical.

were present in this tissue. The potential of this system was recently demonstrated by the characterization of a glucosyltransferase (103, 104) that catalysed the last step in the biosynthesis of these compounds (Fig. 3).

C.1.2. Lotus corniculatus

Lotus corniculatus, a common legume, is widely distributed in the pastures of temperate regions and has been the subject of many studies with the aim of improving its fodder quality (105). It represents an interesting example of the ontogeny of 8-substituted flavonols in its vegetative and flowering parts. The leaves contain the flavonol aglycones: kaempferol, quercetin and isorhamnetin (3'-methyl-quercetin), whereas the flowers accumulate their 8-methoxy-derivatives: 8-methoxykaempferol, 8-methoxyquercetin and 8-methoxy-isorhamnetin (Fig. 4) (106).

Since the methoxyl groups at position 8 and 3' of Lotus flavonols represent meta-methylation of rings A and B, respectively, it was interesting to find out whether both reactions are catalysed by one or two distinct OMTs. Furthermore, the study of the 8-OMT in Lotus complements the work on Chrysosplenium which lacks substitution at the 8-position.

C.1.3. Growth conditions of plant material

C. americanum was collected from Sutton Junction, Province of Quebec, where it thrives in wet bogs, under diffuse light and cool temperature. In order to simulate its natural habitat, the plants
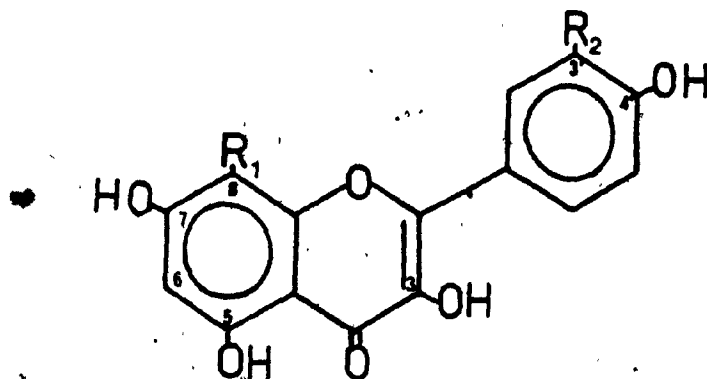
R_1	R_2	
H	H	Kaempferol
H	OH	Quercetin
OH	OH	Gossypetin
OCH_3	H	8-methoxykaempferol
H	OCH_3	isorhamnetin
OCH_3	OCH_3	8-methoxyisorhamnetin

Figure 4.   Structural formulae of the flavonol

derivatives of _Lotus corniculatus_.

were installed in a compartment of the glass house, equipped with a netting material designed to decrease the light intensity by more than half during the summer months (May-September) and by 25% in the winter months. The temperature ranged between 15-20°C (September-May) and 20-25°C during the summmer months. The plants (Fig. 5) were grown in flat plastic containers (25 x 36 cm) that were filled with a potting soil-peat moss mixture (3:1) and were kept flooded with water at all times.

Both vegetative and reproductive parts of L. corniculatus were obtained from glasshouse-raised plants as well as the Agriculture Experimental Farm of Macdonald College, McGill University. The plant material was collected in liquid nitrogen until required for enzyme extraction.

C.2. Chemicals

S-Adenosyl-L-[$^{14}CH_3$] methionine (60 mCi/mmol) was purchased from Amersham (Oakville, Ontario); cold SAM and SAH were obtained from Boehringer Mannheim (Germany). Most commercially unavailable flavonoid compounds were generous gifts from Dr. M. Jay, University of Lyon and Prof. E. Wollenweber, Darmstadt. Other flavonoid substrates were purchased from Roth (Karlsruhe, Germany) and Sarsyntex (Bordeaux, France) and were further purified by recrystallization or preparative TLC. Sephacryl S-200, Sephadex G-100, Polybuffer ion exchanger (PBE-94) and Polybuffer (PB-74) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite (Biogel HT), Bio-Rad protein reagent, Acrylamide (99%) and bis Acrylamide were from

Figure 5.   Photograph of <u>Chrysosplenium</u> <u>Americanum</u>

This tissue was grown under glass house

conditions as described in section C.1.3.

Bio-Rad (Richmond, CA). SAH-Agarose was from Bethesda Research Labs (Gaithersburg, MD), sodium dodecyl sulfate from BDH chemicals, commercial Polyamide 6-MN TLC plates and Polyamide powder from Brinkmann Instrument (Mississauga, Ont.). All column chromatographic supports were prepared according to manufacturers' specifications for subsequent use with enzyme purification. Imidazole was recrystallized from ethyl acetate. All other chemicals were of analytical grade.

C.3. Characterization of 3-, 6-, 7- and 4'-OMTs from Chrysosplenium

C.3.1. Preparation of crude extracts

Unless stated otherwise, all purification steps were carried out at 2-4°C. Young shoot tips (ca 90 g) were frozen in liquid N_2 and mixed with Polyclar AT (10% w/w), then homogenized in a blender with 500 ml of 0.1 M phosphate buffer, pH 7.6, containing 5 mM EDTA, 10 mM DIECA (diethyl ammonium diethyl-dithiocarbamate) and 14 mM 2-mercaptoethanol. The slurry was filtered through nylon mesh and the filtrate was centrifuged for 15 min at 20,000 g. The supernatant was stirred with Dowex 1 X 2 (10% w/v) which had previously been equilibrated with 0.1 M phosphate, pH 7.6. The filtrate was fractionated with solid $(NH_4)_2SO_4$ and the protein fraction which precipitated between 30-70% was collected by centrifugation at 20,000 g for 10 min.

C.3.2. Chromatography on Sephacryl S-200

The protein pellet was resuspended in 10.5 ml of 25 mM imidazole-HCl, pH 7.4 containing 10% glycerol, 14 mM 2-mercaptoethanol

and 0.5 M NaCl (buffer B) and 10 ml were chromatographed on a
Sephacryl S-200 column pre-equilibrated with buffer B. The column was
developed for 20 hours using buffer B at a flow rate of 29 ml/hr and
145 fractions were collected (4 ml/fraction). Enzyme assays were
conducted against the five flavonoid substrates shown in Figure 6.

The remaining 0.5 ml was desalted on a Sephadex-G25 column (1.5 x
25 cm) pre-equilibrated with buffer C (buffer B without NaCl) prior to
determining the enzyme activity of the partially purified preparation.
Preparations could not be assayed before Sephadex-G25 chromatography
because of the high background counts obtained in control assays.
However after passage on Sephadex-G25, this high background was
totally eliminated. It was concluded that DIECA was the cause of this
problem (Table 1), since its inclusion in assays with desalted enzyme
resulted in the appearance of high background radioactivity.

C.3.3. Fractionation on hydroxyapatite

The OMT activity (40 ml) recovered from the Sephacryl S-200
column was applied onto hydroxyapatite (3 x 3 cm) which had been
pre-equilibrated with buffer C. The column was washed with buffer C
(25 ml) until the optical density at 280 returned to base level.
Enzyme activity was eluted (32 ml/hr) from the hydroxyapatite column
using a linear gradient of phosphate (0-100 mM) in buffer C. Seventy
fractions were collected (2.8 ml/fraction) and assayed against the
five different substrates.
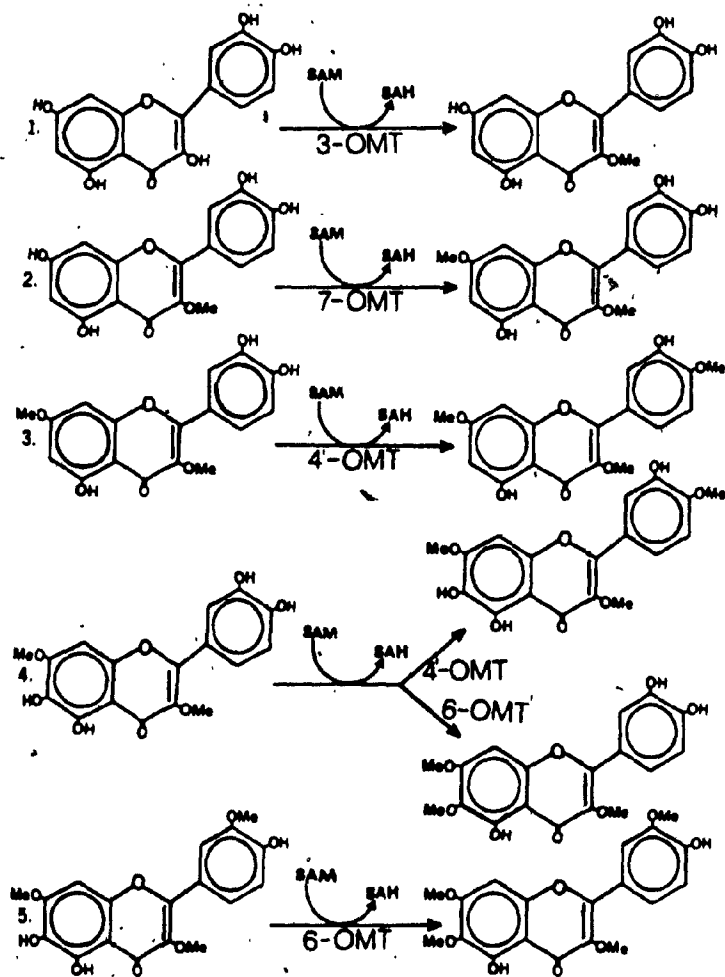
Figure 6.   Substrates used to assay for the 3-, 6-, 7-

and 4'-OMTs of <u>Chrysosplenium</u>

1.   Quercetin (Q) to 3-methyl Q

2.   3-methylquercetin to 3,7-dimethyl Q

3.   3,7-dimethyl Q to 3,7,4'-trimethyl Q

4.   3,7-dimethylquercetagetin to 3,7,4'

trimethylquercetagetin or 3,6,7-trimethyl-

quercetagetin

5.   3,7,3'-trimethylquercetagetin to 3,6,7,3'-

trimethylquercetagetin
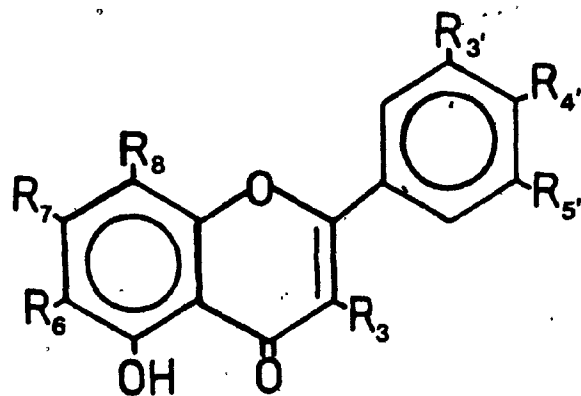
R_3	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$	$R_{5'}$	
OH	H	OH	H	OH	OH	H	Quercetin
OH	OH	OH	H	OH	OH	H	Quercetagetin
OH	H	OH	H	H	OH	H	Kaempferol
OH	H	OH	H	OH	OH	OH	Myricetin
OH	H	OH	OH	OH	OH	H	Gossypetin
H	H	OH	H	OH	OH	H	Luteolin
H	OH	OH	H	H	OH	H	6-Hydroxyapigenin

Figure 6A. Structural formulae of other potential substrates for O-methylation by _Chrysosplenium_ and _Lotus_ enzymes.

Table 1. Effect of DIECA on the assay of <u>Chrysosplenium</u> OMTs [a]

Substrate	Enzyme activity (dpm/assay)[b]	
	plus 1mM DIECA	minus DIECA
None	7800	290
Quercetin (Q)	6000	4500
3-Methyl-Q	1600	750
3,7-Dimethyl-Q	5300	3600
Quercetagetin (Qg)	4000	500
3,7-Dimethyl-Qg	8400	6700
3,7,3'-Trimethyl-Qg	6200	4400

[a]Partially purified enzyme preparations (Section C.3.1.) were desalted on Sephadex G-25 in order to remove DIECA that was present in the grinding buffer.

[b]Standard enzyme assays were performed as described in the Materials & Methods section.

C.3.4. Chromatofocusing on Polybuffer ion exchanger. -

Each of the two major OMT peaks eluted from hydroxyapatite were
subjected to chromatofocusing on a Polybuffer ion exchanger column
(1 x 20 cm) which had been pre-equilibrated with buffer C. The enzyme
activity was eluted with Polybuffer-94 which generated a linear, 200
ml gradient of pH 7-4. The eluting buffer consisted of
Polybuffer-94:water (1:8) containing 20% glycerol and 10 mM
2-mercaptoethanol and its pH was adjusted to 4 with dilute HCl. A
total of 70 fractions (3 ml/fraction) were collected at a rate of 70
ml/hr and assayed for OMT activity against the different substrates.

C.3.5. Removal of contaminating polybuffer

After chromatofocusing on Polybuffer ion exchanger, each purified
enzyme (38 to 50 ml) was subsequently applied to hydroxyapatite as
described in C.3.3., in order to eliminate contaminating polybuffer
and to concentrate each OMT. After enzyme application, the column was
washed with buffer C (25 ml) and the enzyme activity was eluted
batchwise with 0.2M phosphate buffer pH 8, containing 20% glycerol and
5 mM DTT. Enzyme activity was recovered in a total volume of 6 ml.

C.4. Characterization of Flavonol 8- and 3'- OMTs from Lotus
C.4.1.a. OMT activity in different organs

Leaves, apical meristems or flower buds were obtained from Lotus
in order to characterize their OMT activities. One gram of each
material was pulverized in liquid nitrogen and the enzymes were
extracted using 3 ml of buffer A as described in section C.3.1.

However, these extracts were not submitted to $(NH_4)_2SO_4$ fractionation; instead, they were desalted on Sephadex G-25, as in section C.3.2., and were subsequently used for enzyme assays.

C.4.2. Preparation of crude extracts

Flower buds (2 g) were frozen in liquid N_2, ground to a fine powder with Polyclar AT (20% w/w), then homogenized in buffer A (1:5 w/v). All subsequent procedures were performed as described in section C.3.1.

C.4.3. Chromatography on Sephadex G-100

The protein pellet (section C.3.1.) was resuspended in buffer C and chromatographed on a Sephadex G-100 column which was preequilibrated in the same buffer. Enzyme activity was eluted with buffer C at a flow rate of 20 ml/hr and 100, 3-ml fractions were collected. Enzyme assays were performed with 8-hydroxykaempferol and quercetin as substrates for the 8- and 3'-OMTs, respectively.

C.4.4. Chromatofocusing

The enzyme activity from the Sephadex G-100 column (35 ml) was applied on a Polybuffer ion exchanger as was described in section C.3.4. The 8- and 3'-OMTs were freed from contaminating polybuffer and concentrated as described in section C.3.5.

C.5. Purification of 8-OMT from Lotus

Two thousand young flower buds (ca 20 g) were used in order to purify enough 8-OMT for kinetic studies. The protocol followed was

similar to that described in section C.4. up to and including the
Sephadex G-100 step.

C.5.1. SAH-Agarose chromatography

The protein fractions from Sephadex G-100 containing high OMT
activity were pooled and chromatographed on a SAH-Agarose column
(1 x 5 cm) which was pre-equilibrated with buffer C. The column was
washed with 100 ml of buffer C and the enzyme activity was eluted in 3
ml fractions using a linear gradient of NaCl (0-1M) in buffer C.

C.5.2. Chromatography on hydroxyapatite

The active fractions from the SAH-Agarose column were pooled and
applied to hydroxyapatite as described in section C.3.3.

C.5.3. Chromatofocusing

The enzyme protein from the hydroxyapatite column was
subsequently bound to Polybuffer ion exchanger as described in section
C.3.4. However, this column was pre-equilibrated in 25 mM histidine,
pH 6.4, 10% glycerol and 10 mM-2-mercapteothanol, instead of buffer
C. In addition, the pH of Polybuffer was adjusted to 4.8 instead of
4 and a linear gradient of pH 6-4.8 was generated. Active fractions
were pooled and concentrated as in section C.3.5.

C.6. OMT assay

The assay for OMT activity was performed as described previously
by Tsang and Ibrahim (42) using S-adenosyl-L-[^{14}CH$_3$]-methionine as
methyl donor. The standard assay mixture consisted of 1 nmole of the
phenolic substrate (dissolved in 10 μl of dimethylsulfoxide), 0.7

nmole of SAM (containing 25 nCi), 1.4 μmole 2-mercapteothanol and the enzyme protein. The assay was carried out in a final volume of 100 μl containing 0.1 M phosphate buffer, pH 8, and the reaction was started by the addition of enzyme. The reaction mixture was incubated at 30°C for 30 min in a constant temperature water bath and the reaction was terminated by the addition of 20 μl of 6 N HCl.

The O-methylated products were separated from unreacted SAM by extraction with 250 μl of benzene-ethyl acetate (1:1) and shaking in an Eppendorf rotary shaker for 2 min. The organic phase containing the methylated products was separated from the aqueous layer by a 2 min centrifugation using an Eppendorf centrifuge. An aliquot (100 μl) of the organic layer was transferred to plastic scintillation vials (Beckman Bio Vials) together with 2.5 ml scintillation fluid (5 g PPO/l of toluene) and was counted for radioactivity using a Wallac LKB model 1215 Rackbeta Liquid Scintillation Spectrometer which was programmed with a quench correction curve.

C.6.1. Efficiency of extraction of reaction products

In order to estimate the percentage recovery of the methylated flavonoid product after extraction with ethyl acetate-benzene mixture, several assays were performed with the purified 3-, 6-, 8- and and 4'-OMTs, and their radioactive products counted. Therefore, 3*-methylquercetin, 3,6*,7,3'-tetramethylquercetagetin, 8*-methoxy-kaempferol and 3,7,4'*-trimethylquercetin of known radioactivity were placed in separate Eppendorf vials and the organic solvent was allowed to evaporate. DMSO (10 μl, 50% v/v) was added to each vial followed

Table 2. Effect of the extraction procedure on the recovery of
flavonoid products

Volume (µl)[a]	Percent Recovery		
	3-Methyl-quercetin	3,6,7,3'-Tetra-methylquercetagetin	3,7,4'-Tri-methylquercetin
50	95	104	92
100	107	100	99
150	103	99	76
200	104	101	100
250	106	100 —	98
300	103	96	99

[a] Fifty µl of the organic layer contained 1250, 1850 and 1750 dpm
of labelled 3*-methylquercetin, 3,6*,7,3'-tetramethylquercet-
agetin and 3,7,4'-trimethylquercetin, respectively (*, $^{14}CH_3$).

by phosphate buffer (90 µl). Each tube was then processed according to the procedure outlined in section C.6.

Table 2 shows that nearly 100% of the radioactivity was recovered, irrespective of the actual amounts of radioactive product present (marked*) or the position of methylation on the flavonoid ring. Similar results were obtained when 10 µl DMSO-water (50% v/v) containing the respective flavonoid substrate was added to the radioactive product.

C.6.2. Identification of reaction products

The reaction products of each enzyme studied were subjected to similar procedures for identification purposes. Several assays were performed in order to obtain enough radioactive products for autoradiographic analysis. The products were evaporated to dryness and dissolved in 50 µl of methanol, then chromatographed on commercial Polyamide-6 MN TLC plates in different solvent systems (described in figure legends). The identity of methylated products was confirmed by co-chromatography with reference compounds(Table 3), visualisation in UV-light (366 nm) and by autoradiography on X-Ray film.

C.7. Analytical gel electrophoresis

Standard 7.5% polyacrylamide disc gels were prepared according to Davis (107) and were used to establish protein profiles during different steps of purification of OMTs. In addition, some gels were run in the cold room when enzymes were to be extracted for determination of activity profiles.

Table 3. Identification of reaction products of Chrysosplenium

OMT activity[a]

Flavonol substrate	Reaction products[b]		
	Methyl substitution	R_f values $(\times 100)^c$	
		Solvent A	Solvent B
Quercetin	3-	18	52
3-Methylquercetin	3,7-	53	75
3,7-Dimethylquercetin	3,7,4'-	80	75
3,7-Dimethylquercetagetin	3,6,7-	63	80
	3,7,4'-	68	85
3,7,3'-Trimethylquercet-agetin	3,6,7,3'-	84	90

[a] Partially purified enzyme preparation (Section C.3.1.) desalted on Sephadex G-25. The standard enzyme assay was used as was described in the Methods section.

[b] The reaction products were identified by co-chromatography with reference compounds, visualization in UV-light ànd autoradiography.

[c] On Polyamid-6 MN TLC plates in solvents: A, toluene-ethyl formate-butyl acetate- formic acid (25:50:23:2) and B, benzene-methyl ethyl ketone-methanol (4:3:3).

When the latter protocol was used, the gels were sliced with a razor blade into 5 mm segments and each slice was transferred to an Eppendorf tube. Each slice was then extracted with 250 μl of 0.2 M phosphate buffer, containing 10% glycerol and 5 mM DTT. Gel slices were macerated in this buffer using a glass rod until a fine pulp was produced and the gels were stored overnight at 4°C prior to enzyme assay. Enzyme assays were performed as described in section C.2.1. using 100 μl of each extracted fraction.

C.7.1. Sodium dodecyl sulfate PAGE (SDS-PAGE)

SDS-PAGE slab gels (20 cm x 20 cm) were prepared as described by Laemli (108) and were used to establish protein profiles under denaturing conditions at different stages of OMT purificaton. SDS gels containing 11% acrylamide were routinely used for this purpose.

C.8. Molecular weight determination

The molecular weights of both crude and purified enzymes were determined by gel filtration on a calibrated column of Sephacryl S-200 (section C.3.2.). The column was equilibrated in buffer B and proteins were eluted using this buffer. The column was previously calibrated with a number of standard proteins, and the void volume of the column was determined by elution of a sample of blue dextran (108). OMTs were routinely applied together with a standard protein to ensure the reproducibility of the system. The K_{ab} values for the proteins used were plotted against their molecular weights on a

logarithmic scale. The K_{av} of each OMT was then calculated using

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_t = total volume, V_0 = void volume and V_e = elution volume.

C.9. Localisation of OMT activities on sucrose gradients

The method of Beevers et al. (110) was used to isolate organelles on sucrose gradients. Five grams of Chrysosplenium leaves were placed in a petri dish containing five ml of grinding buffer consisting of 150 mM tricine, pH 7.6, 10 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA and 13% (w/v) sucrose (Buffer D). The leaves were chopped until the tissue was finely ground then filtered through a nylon mesh (300 micron mesh size) and the filtrate was centrifuged for 5 min. at 500 g to remove cellular debris. The supernatant was applied on top of a linear sucrose gradient in buffer D (without sucrose). This gradient was constructed in 25 ml Polyalomer centrifuge tubes with a 60% (w/w) sucrose cushion (4 ml) followed by 16-60% (w/w) linear sucrose gradient (18 ml) and 2.5 ml supernatant. The gradients were placed in a 3 x 25 ml swing-out rotor (cat. no. 43127-104) and centrifuged in an MSE Superspeed 75 Ultracentrifuge at 60,000 g at 4°C for 2 hr.

After centrifugation, the bottom of each centrifuge tube was taped with masking tape, then punctured with a 15 gauge needle and drops were collected at the open end. Fractions were collected (0.7 ml) and assayed for OMT and glucosyltransferase activities. Relative chlorophyll absorbance was measured at 670 nm. Each fraction was

subsequently extracted with ethyl acetate-benzene (1:1) and the organic phase was evaporated to dryness then chromatographed on Polyamide TLC plates. The solvent system used permitted the separation of chlorophylls from polymethylated flavonol glucosides. The latter could be visualized under UV-light and thus permitted their localisation on the sucrose gradient.

C.10. Analysis of kinetic data

Kinetic data were analyzed using the nonlinear regression computer program as described by Duggleby and Dennis (111). All of the substrate interaction data were fitted to both of the following equations:

$$v = \frac{V_{max}}{1 + (K_a/[A]) + (K_b/[B]) + (K_{ia}K_b/[A][B])} \qquad I$$

$$v = \frac{V_{max}}{1 + (K_a/[A]) + (K_b/[B])} \qquad II$$

where A and B are the varied substrates, K_a and K_b are the respective limiting Michaelis constants, V_{max} is the maximum velocity and $K_{ia} K_b$ is an interaction term.

All data from inhibition studies were fitted to the equation for non competitive inhibition (III). In addition, the data were fitted to the equation for competitive inhibition (IV) or uncompetitive

inhibition (V) as determined by inspection of the double reciprocal plots.

$$v = \frac{V'_{max}}{1 + (K'_a/[A]) + (K'_a[I]/K_{is}[A]) + ([I]/K_{ii})} \quad \text{III}$$

$$v = \frac{V'_{max}}{1 + (K'_a/[A]) + (K_a[I]/K_{is}[A])} \quad \text{IV}$$

$$v = \frac{V'_{max}}{1 + (K'_a/[A]) + [I]/K_{ii}} \quad \text{V}$$

In these equations, A is the variable substrate, I is the inhibitor, K'_a and V'_{max} are the Michaelis constant and maximum velocity, respectively, in the presence of inhibitor, and K_{is} and K_{ii} are the slope and intercept inhibition constants, respectively.

The data from the kinetic studies of the 3-, 6-, 8- and 4'-OMTs are presented as double reciprocal plots which were fitted by linear regression analysis (method of least squares). However, all the data (3-, 6-, 8- and 4'-OMTs) were also submitted to the above-mentioned non-linear regression analysis and the appropriate model was chosen using the variance ratio test. In most cases the model formed by the computer programme was identical to that generated by fitting the data by the method of least squares. It should also be noted that the kinetic constants given in Tables 15 to 20 were those given by the computer programme of Duggleby and Dennis (111).

In the method of data analysis described by Duggleby and Dennis (111), replicates of individual rates were not performed since it appeared to be more advantageous to increase the number of data points rather than perform replicates. It was also assumed that the measured velocity, v was normally distributed around the predicted velocity, v and the variance in initial velocities was constant. Furthermore, the errors in substrate and inhibitor concentrations were asummed to be constant at all concentrations. Since replicates were not performed, no estimate of pure error could be made. However, the residual sum of squares (RSS_c), which resulted when the data were fitted to equations I and III, gave a measurement of the pure error in the data. When the data were fitted to equations II, IV, and V, the residual sum of squares (RSS_r) was due both to pure error and lack of fit to the equations. The residual sum of squares (RSS_{lf}) due to lack of fit to the equation could be estimated from

$$RSS_{lf} = RSS_r - RSS_c$$

The significance of the lack of fit to equations II, IV and V could be determined by a variance ratio (F) test

$$F = \frac{RSS_{lf}\,(n - p)}{RSS_c} \qquad\qquad VI$$

where n was the number of assays and p (the number of parameters in the complete equation) was 4. In all cases, the null hypothesis was used, i.e. equation II, IV or V is chosen if a significantly better

fit is not found with equation I or III i.e. if the residual sum
squares due to lack of fit to equation II, IV or V is not significant
as compared with the error in the data. Using this analysis, the
significance of the $K_{ia}K_b$, $[I]/K_{ii}$ and $K'_a[I]/K_{is}[A]$ terms in
equation I or III was evaluated by comparision of the experimentally
determined F value with tables of F values at a probability of 0.99.

C.11. Protein determination

Protein was determined according to Bradford (112) (Fig. 7) using
the Bio-Rad protein reagent and bovine serum albumin as a standard
protein.

C.12. Definition of enzyme units

Enzyme units are expressed in Katals as recommended by the
International Union of Biochemists (113). One Kat is define as the
amount of activity which converts one mole of substrate per second
under the assay conditions.
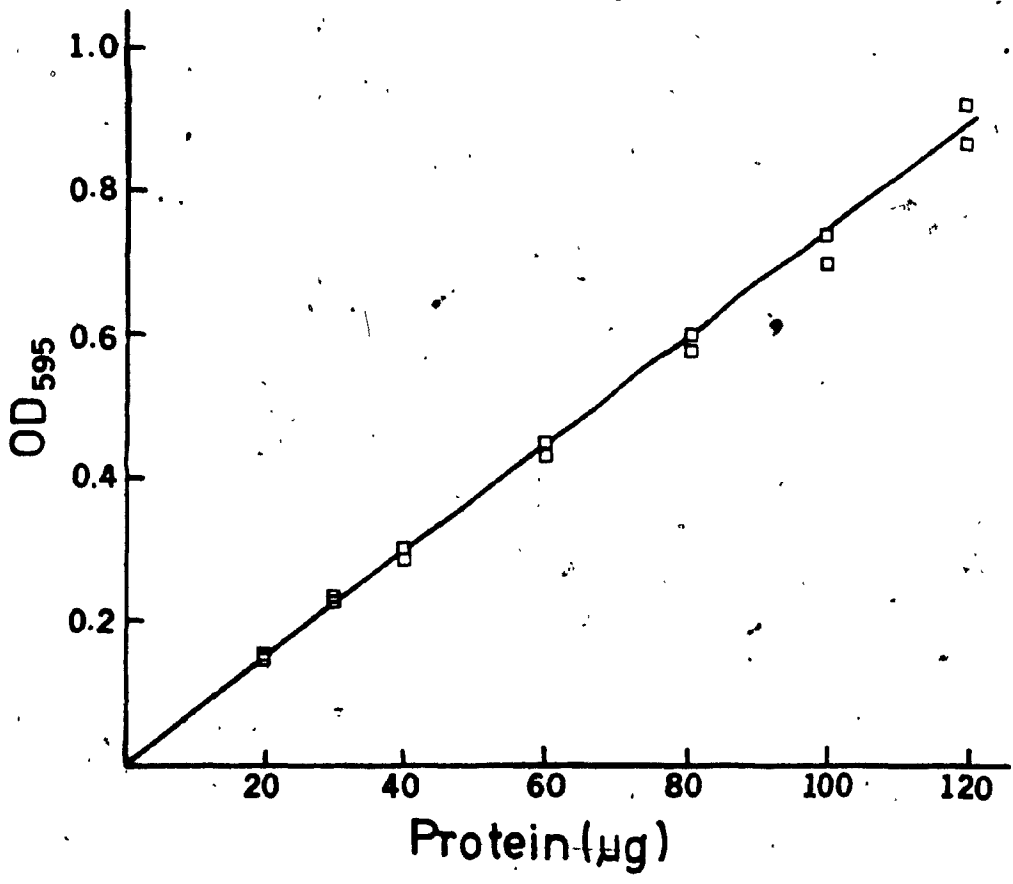
Figure 7.   Standard curve for the Bio-Rad protein

determination (112).

The standard protein used was bovine serum

albumin.

D. RESULTS

D.1. Stabilization of OMTs

Since plant tissues vary in their content of protein-inactivating phenols, no universal procedure for enzyme extraction has gained general acceptance. However, methods designed to remove phenolic compounds during the preparation of tissue extracts are often essential to the isolation of plant enzymes in an active state.

Generally, the removal of polyphenols is achieved by the inclusion of adsorbents such as the widely used Polyclar AT (114) and Dowex 1 X 2 (115) in the homogenization medium. In addition, the presence of the copper chelator, DIECA (116) decreases the formation of protein binding quinones via the copper-dependent polyphenol oxidase.

Preliminary experiments with C. americanum established that in the absence of DIECA, Polyclar AT or Dowex 1 X 2, partially purified preparations were brown in color (indicative of phenol-bound protein) instead of white and showed little or no methylating activity against flavonoid substrates. In addition, loss of enzyme activity of partially purified preparation (C.3.1.) occurred within 24 hr in absence of glycerol. On the other hand, no loss in OMT activity could be observed for several days in the presence of 10% glycerol and storage at 4°C.

D.2. Enzymatic synthesis of methylated flavonoids in Chrysosplenium

Preliminary studies on the OMT system of Chrysosplenium were conducted with partially purified enzyme preparations (C.3.1.).

Figure 8 is a photograph of the autoradiographed reaction products of a Sephadex G-25 OMT preparation that was assayed against a number of flavonoid substrates. Quercetin was readily accepted as substrate and was mainly transformed to 3-methylquercetin, as well as decreasing amounts of 3,7-di- and 3,7,4'-trimethyl derivatives (Fig. 8). Furthermore, partially methylated flavonol intermediates were readily accepted (Table 4) and were transformed to the next higher order of their methyl derivatives (Fig. 8).

D.2.1. Substrate specificity of partially purified preparations

Several flavonoid substrates were tested for their methyl acceptor ability. The results (Table 4) indicate that, unlike quercetagetin, quercetin was the best substrate used and may be considered a possible precursor for the synthesis of polymethylated flavonols in this tissue. When partially methylated quercetagetin derivatives (3,7-dimethyl- and 3,7,3'-trimethyl-) were used as substrates, further methylation resulted in substitution of positions 6 and/or 3' or 4', respectively. This activity was absolutely dependent on Mg^{2+} ions (Fig. 8). However, further O-methylation of 3,6,7-trimethylquercetagetin proceeded poorly and did not require Mg^{2+} ions.

Commercial kaempferol, dihydroquercetin, rhamnetin (7-methyl-quercetin) and myricetin (5'-hydroxyquercetin) all contained varying amounts of quercetin as contaminant (Appendix Fig. 2). Therefore, these substrates were further purified prior to use for substrate specificity studies (C.3.1.). Upon removal of the quercetin
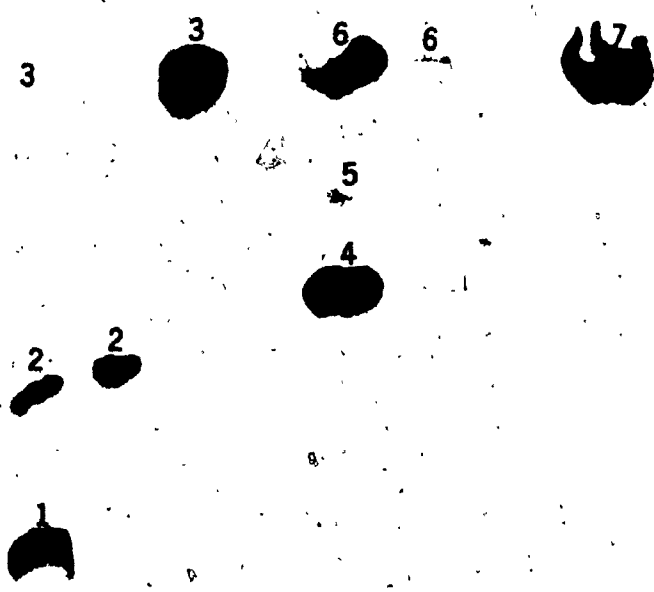
Q   3Q   3,7Q   3,7Qg   3,6,7Qg   3,7,3'Qg
a   b   c   d   e   f   g   h

Figure 8.   Photograph of an autoradiograph of the
            chromatographed reaction products (Solvent A,
            Table 3) of partially purified (desalted
            ammonium sulfate pellet) OMT preparation with
            the indicated substrates. Numbers preceeding Q
            (quercetin) and Qg (quercetagetin) indicate the
            methylated positions of these substrates.   The
            reaction products were identified by
            co-chromatography with reference compounds: (1)
            3-methyl-Q; (2) 3,7-dimethyl-Q; (3) 3,7,4'-
            trimethyl-Q; (4) 3,6,7-trimethyl-Qg;
            (5) 3,7,4'-trimethyl-Qg (6) 3,6,7,4'-
            tetramethyl-Qg; (7) 3,6,7,3'-tetramethyl-Qg.
            Columns a, b, c, e, f and h all contained 10 mM
            $MgCl_2$ in the assay mixture (section C.6.),
            whereas d and g did not contain this divalent
            cation.

Table 4. Methyl acceptor ability of partially purified OMT preparation[a]

Substrate	Relative activity[b] (% of control)
Quercetin	100
3,7-Dimethylquercetagetin	80
3,7-Dimethylquercetin	68
3,7,3'-Trimethylquercetagetin	65
3-Methylquercetin	32
3,6,7-Trimethylquercetagetin	15
3,7,4'-Trimethylquercetin	9
Quercetagetin	6
Dihydroquercetin	3
Kaempferol	3
Luteolin	3
Caffeic acid	3

[a] Partially purified enzyme preparation (Section C.3.1.) desalted on Sephadex G-25 and assayed as described in Section C.6.1.

[b] Total methylating activity of quercetin (control = 100%) amounted to 13,000 dpm/mg protein.

contaminant, kaempferol and dihydroquercetin were not accepted for methylation by the OMT system of Chrysosplenium, whereas rhamnetin and myricetin were poorly accepted (25 and 5% of quercetin activity in Table 4).

Phenylpropanoids such as caffeic or ferulic acids, and flavones such as luteolin or its 7-glucoside were not accepted as substrates (Table 4) indicating the specificity of this enzyme system towards flavonols.

D.3. The OMTs of C. americanum

D.3.1. Separation of three distinct flavonol OMTs

Preliminary experiments on the purification of the enzyme system of C. americanum were described in sections C.3.1. to C.3.4., except that 20 g of leaves were used in the extraction procedure, Sephadex G-100 was used instead of Sephacryl S-200 and there was no hydroxyapatite step.

Chromatography of the ammonium sulphate pellet on Sephadex G-100 (Fig. 9A) resulted in the recovery of OMT activity against the five different substrates (Fig. 6). All activity was confined to a discrete peak whose molecular weight was larger than ovalbumin and which coincided with the molecular weight of BSA (65,000).

Further purification of the enzyme protein was achieved by chromatofocusing on Polybuffer ion exchanger and resulted in three peaks with OMT activity (Fig. 9B). Activity peak I catalysed the methylation of quercetin and gave one product which was identified as 3-methylquercetin (Fig. 9C). The second peak (II) catalysed further O-methylation of 3,7-dimethylquercetin and 3,7-dimethylquercetagetin

C. americanum OMT
(Seph. G-100)
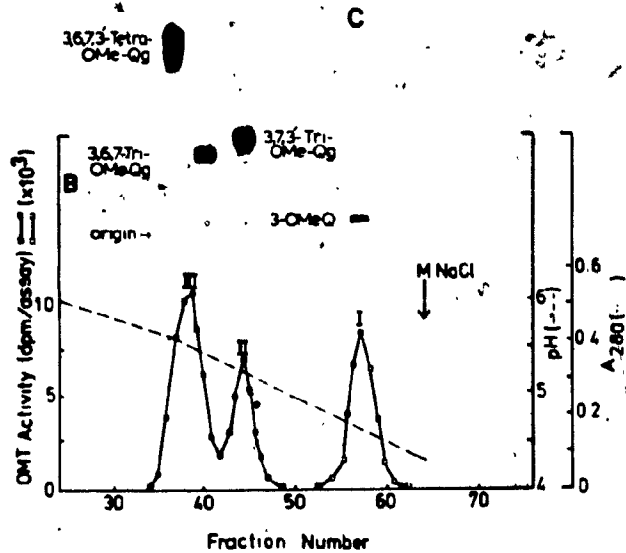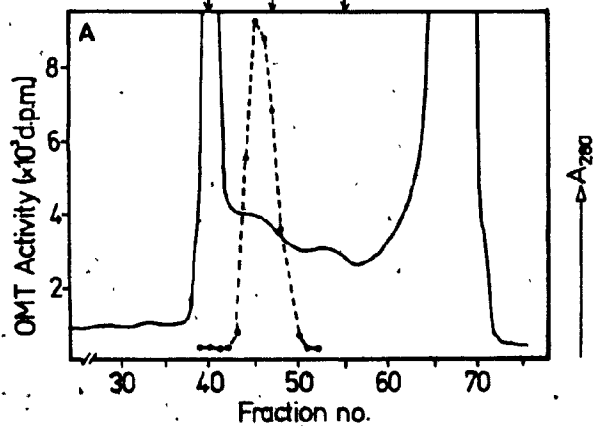
Figure 9.   Characterization of the 3-, 6- and 4'-OMTs from

C. americanum

A.   Sephadex G-100 chromatography (1.5 x 70 cm) of

partially purified OMTs from a resuspended

30-70% $(NH_4)_2$ SO_4 pellet (1 ml). The sample

was applied and 2-ml fractions were collected

at a rate of 9 ml/hr. OMT activity was assayed

using 3,7-dimethylquercetagetin (···●···) and

quercetin as methyl acceptors. The quercetin

methylating activity co-eluted with the 6- and

4'-OMTs and was 50% as active as the former

activities.

B.   Chromatography on Polybuffer ion exchanger

(1 x 28 cm) of active fractions (10 ml) from

Sephadex G-100, as described in section C.3.4.

OMT activity was assayed using quercetin (-□-)

and 3,7-dimethylquercetagetin (-■-) as

substrates.

C.   Continued on next page

Figure 9.　　Continued

　　　C.　　Photograph of an autoradiogram of the

chromatographed reaction products of the 3-, 6-

and 4'-OMT peaks. Peak I was only active

against quercetin and transformed this

substrate into 3-methylquercetin (3-methyl Q)

whereas peak II accepted 3,7-dimethyl-

quercetagetin for O-methylation at the 4'

position of the A-ring. In addition, Peak III

transformed 3,7,3'-trimethylquercetagetin to

the 6-substituted tetramethylated derivative.

The reaction products were identified by

co-chromatography with reference compounds as

described in Fig. 8.

to their respective 4'-methyl derivatives. Peak III O-methylated
3,7-dimethylquercetagetin and 3,7,3'-trimethylquercetagetin to their
respective 3,6,7-tri- and 3,6,7,3'-tetramethyl ethers (Fig. 9B, C).
Attempts to detect the 7-OMT activity on chromatofocused fractions
(Fig. 9B) were unsuccessful although partially purified enzyme
preparations (Fig. 9A) were active against 3-methylquercetin.

D.3.2. Purification of individual OMTs from C. americanum

In order to purify larger amounts of flavonoid OMTs for kinetic
studies, the previous procedure (D.3.) was modified as described in
section C.3. The elution profiles of enzyme activities from
Sephacryl S-200, hydroxyapatite and chromatofocusing on Polybuffer ion
exchanger are shown in Figures 10, 11 and 12 respectively.

Sephacryl S-200 chromatography (Fig. 10) resulted in the elution
of all OMTs as a single discrete peak similar to that obtained with
Sephadex G-100 (Fig. 9A). However, unlike Sephadex G-100, this column
permitted the complete removal of chlorophyll-containing protein.
Protein fractions with OMT activity were pooled and chromatographed on
a hydroxyapatite column as described in section C.3.3.

Fractions from hydroxyapatite were assayed against the five
different substrates resulting in the separation of two major peaks of
activity (Fig. 11). Peak 1 was variably active against all five
substrates (Fig. 11B, C) whereas peak 2 was only active against
quercetin, 3,7-dimethylquercetin and 3,7-dimethylquercetagetin. This
purification step permitted the separation of the 6- and 7-OMTs from
the majority of the 3- and 4'-enzymes. Each hydroxyapatite peak was
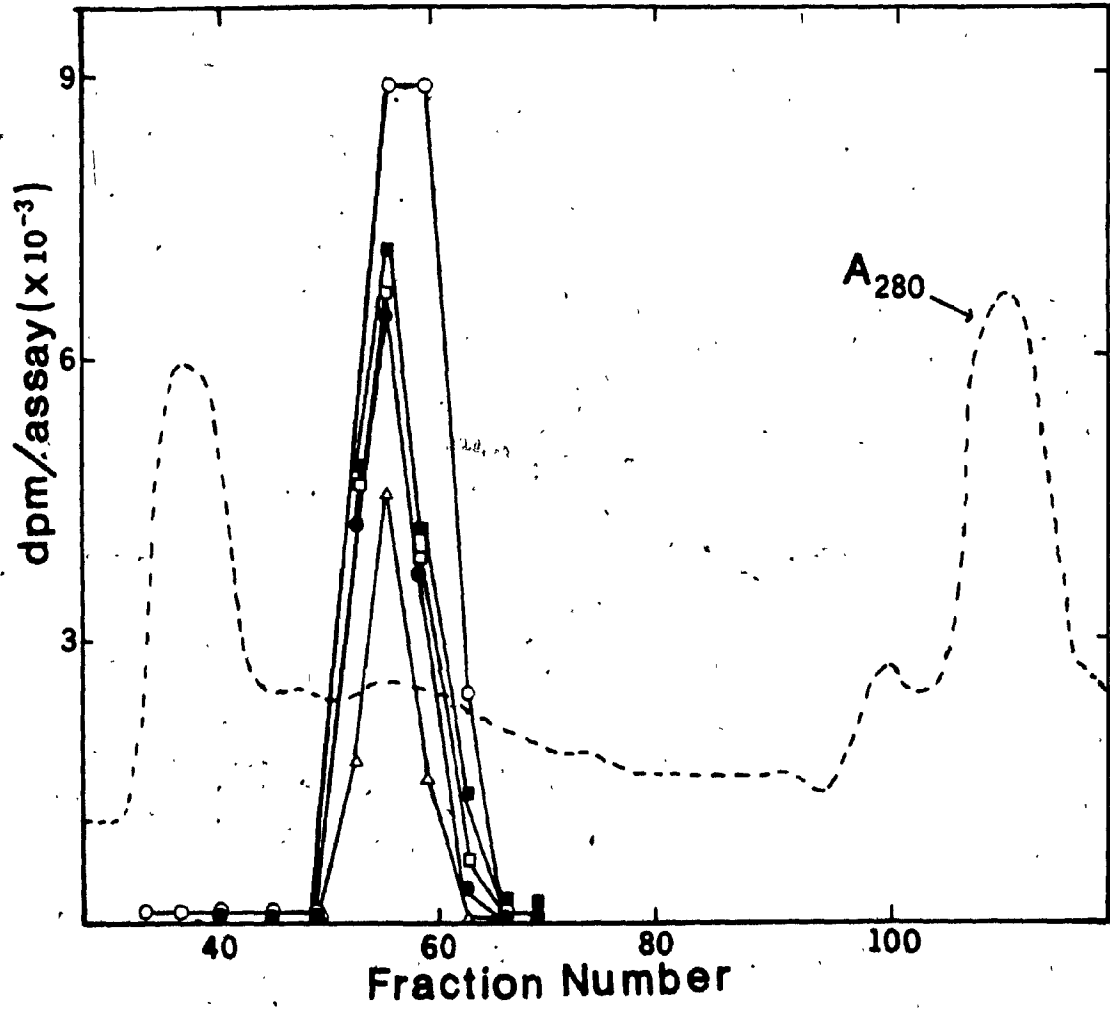
Figure 10.   Sephacryl S-200 (2.5 x 118cm) chromatography of
              of partially purified OMTs.

              As described in section C.3.2., OMT activity
              was assayed using quercetin (─●─), 3-methyl-
              quercetin (─△─), 3,7-dimethylquercetin (─■─)
              3,7-dimethylquercetagetin (─○─) and 3,7,3'-
              trimethylquercetagetin (─□─) as substrates
              (Fig. 6).
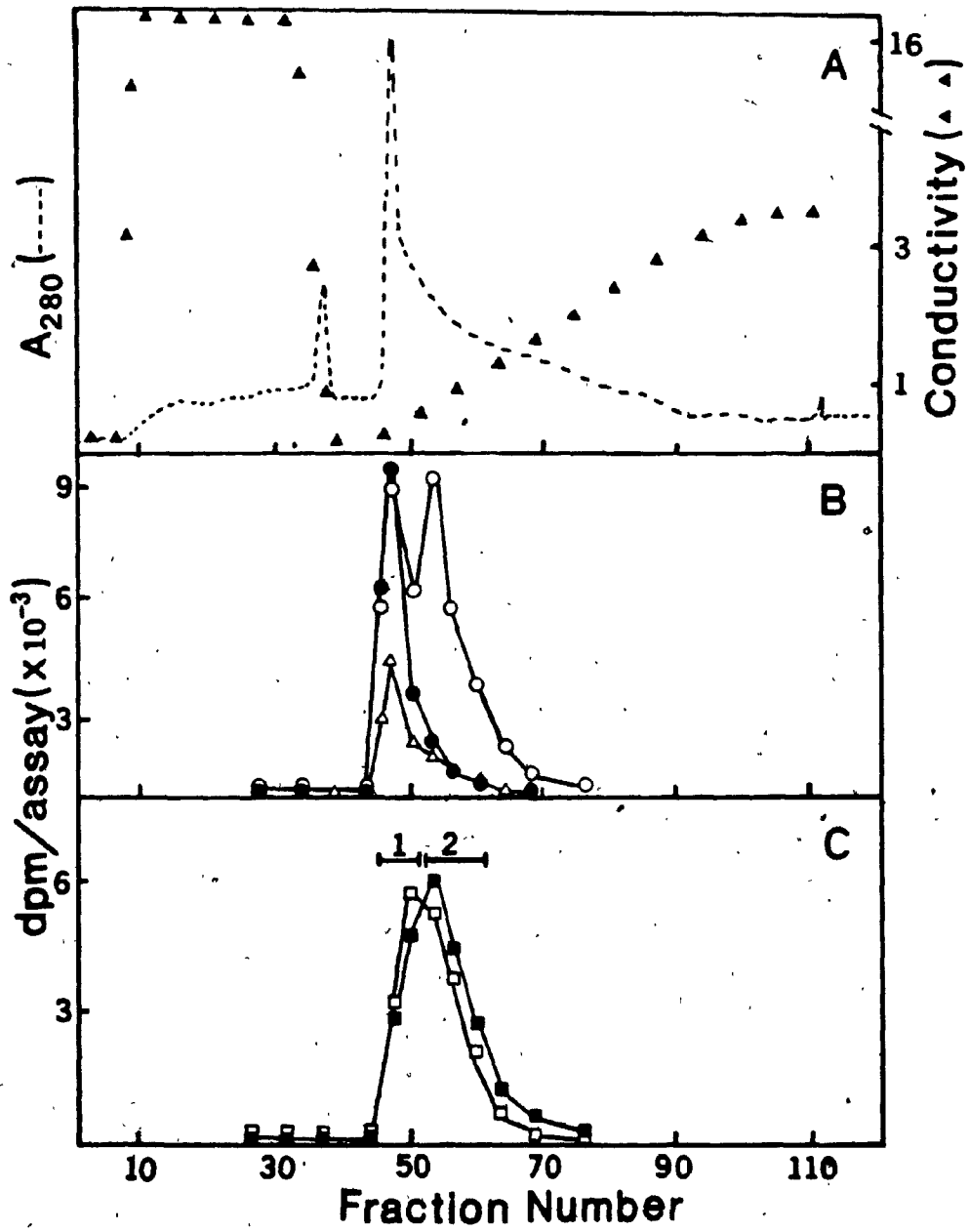
Figure 11. Hydroxyapatite chromatography of partially
purified OMT from Sephacryl S-200.

The procedure was as described in section
C.3.3. Note that the high conductivity (▲)
was initially due to 0.5 M NaCl present in the
sample being applied onto the column. OMTs
were assayed using quercetin (─□─),
3-methylquercetin (─▲─), 3,7-dimethyl-
quercetin (─■─), 3,7-dimethyl- quercetagetin
(─○─) and 3,7,3'-trimethyl-quercetagetin
(─●─) as substrates (Fig. 6).

Figure C labels: 374Q, 3673Qg, 374Qg, 37Q, 3Q, Q, 3Q, 37Q, 37Qg, 37Qg

Axis labels:
pH (▲ ▲) — vertical left axis
dpm/assay (×10^{-3}) — vertical right axis
Fraction Number — horizontal axis
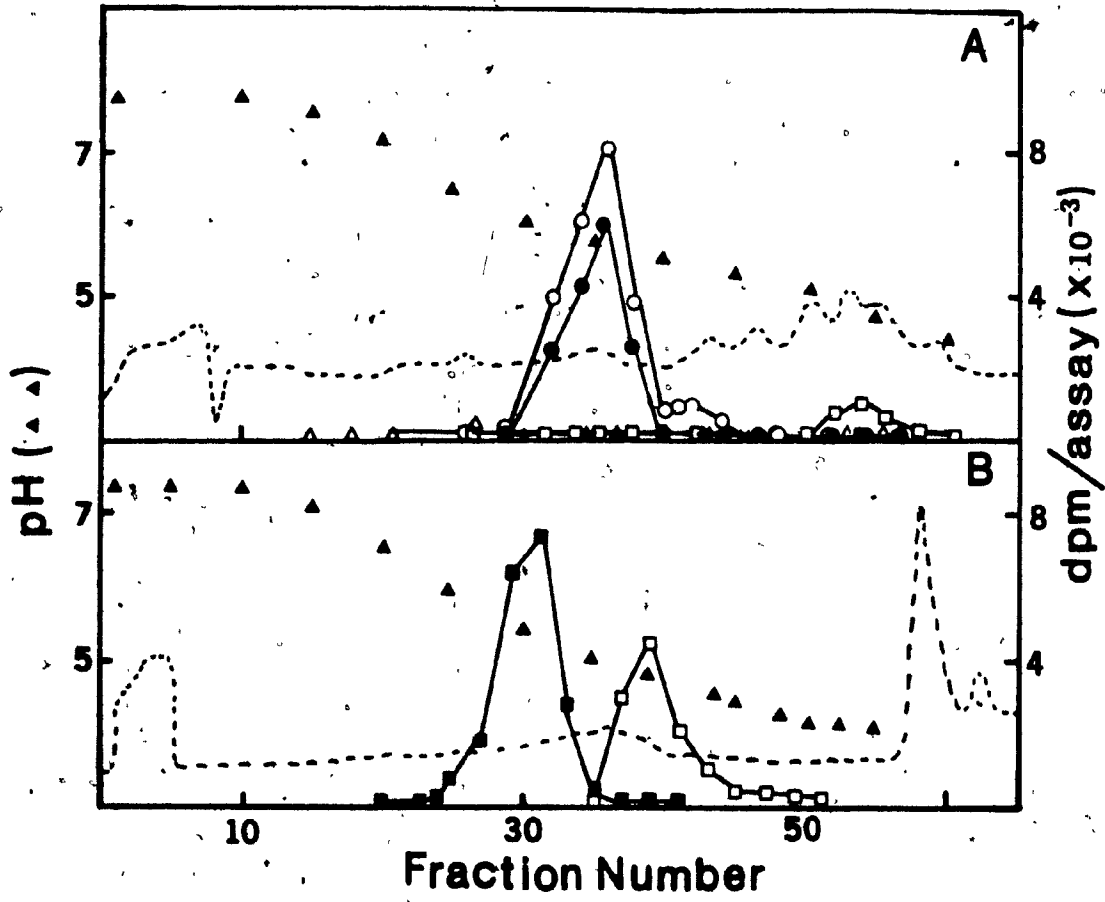
Panels A, B, C
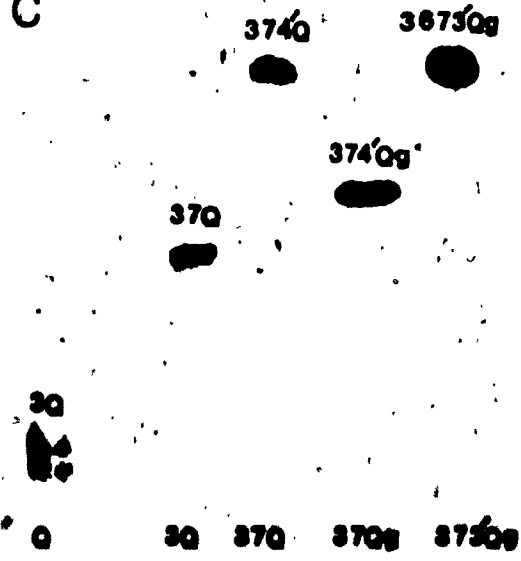
Figure 12.   Chromatography on Polybuffer ion exchanger
             (1 x 28 cm) of peaks 1 and 2 from hydroxy-
             apatite.

A.   Chromatofocusing of 6- and 7-OMTs on Polybuffer
     ion exchanger. Fractions 41-50 from the
     hydroxyapatite column were applied to and
     eluted from this column as described in section
     C.3.4.

B.   Chromatofocusing of 3- and 4'-OMTs on
     Polybuffer ion exchanger. Fractions 51-63 from
     the hydroxyapatite column were applied to and
     eluted from this column as described in section
     C.3.4. OMTs were assayed using quercetin
     (—□—), 3-methylquercetin (—△—),
     3,7-dimethyl- quercetin (—■—), 3,7-dimethyl-
     quercetágatin (—○—) and 3,7,3'-trimethyl-
     quercetagetin (—●—) as substrates.

C.   Continued on next page

Figure 12.   Continued

C.   Photograph of an autoradiogram of the

chromatographed reaction products of purified.

3-, 6- and 4'-OMTs. The 3-OMT activity

transformed quercetin (Q) to 3-methyl-Q (3Q);

the 7-OMT from hydroxyapatite (Fig. 11, peak 1)

accepted 3Q to give 3,7-dimethylquercetin

(3,7Q); the 4'-OMT methylated either 3,7Q or

3,7-dimethylquercetagetin (3,7Qg) to their

respective trimethylated derivatives (3,7,4'Q

and 3,7,4'Qg); the 6-OMT transformed

3,7,3'-trimethylquercetagetin to the 6

methylated derivative (3,6,7,3'-Qg). The

reaction products were identified by

co-chromatography with reference compounds as

described in Fig. 8.

subsequently submitted to chromatofocusing on Polybuffer ion exchanger using Polybuffer PB-74. The 6-OMT was eluted at its apparent isoelectric point (pH 5.77) free from contaminating 3- or 4'-activities (Fig. 12A). The 7-OMT activity was lost during chromatofocusing, possibly due to instability of the enzyme in the purified form, or to the loss of some factor required for its activity. The 3- and 4'-OMTs were further separated on a second Polybuffer ion exchanger and eluted at pH 4.8 and 5.4, respectively (Fig. 12B). This procedure assured that the 6- and 4'-OMTs whose apparent pI's differed only by 0.3 pH unit were not cross-contaminated.

The combined purification steps described above resulted in an increase in specific activity of the three focused enzymes of 85-, 92- and 164-fold for the 3-, 6- and 4'-OMTs, respectively, as compared with those of the crude enzyme preparation (Table 5). The 7-OMT which co-purified with the 6-OMT on hydroxyapatite showed a 6-fold increase in specific activity.

Examination of enzyme preparations after SDS PAGE revealed that the purified enzymes were contaminated with several bands of protein (Appendix I, Fig. 3A). Furthermore, partially purified enzyme preparations (Sephadex G 100, Fig. 9A) were active when extracted from nondenaturing gels (Appendix I, Fig. 3B), whereas purified preparations (after chromatofocusing) were inactivated by this procedure. OMT activity against quercetin, 3,7-dimethylquercetin and 3,7-dimethylquercetagetin could be localized in the same region of the gel, indicating that the 3-,6- and 4'-OMTs behaved similarly under these electrophoretic conditions.

Table 5. Purification of <u>Chrysosplenium</u> O-methyltransferases[a]

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Total activity (pkat)	Purification (-fold)	Recovery %
3-OMT					
Crude extract[b]	103	0.62	64		100
Amm. sulphate[c]	80	0.55	44	0.9	69
Sephacryl S-200	20	1.5	31	2.4	48
Hydroxyapatite[d]	5	5.9	29	9.5	45
PBE-94[e]	0.25	53	13.3	85	21
6-OMT					
Crude extract[b]	103	0.42	50		100
Amm. sulphate[c]	80	0.55	44	1.1	88
Sephacryl S-200	20	1.66	33	3.4	66
Hydroxyapatite[f]	5	3.1	15.5	6.4	31
PBE-94[g]	0.25	44	11.1	92	22
4'-OMT					
Crude extract[b]	103	0.84	84		100
Amm. sulphate[c]	80	0.94	74	1.2	88
Sephacryl S-200	20	1.06	53	1.3	63
Hydroxyapatite[d]	5	6.1	31	7.6	37
PBE-94[e]	0.25	120	30	164	38

See following page for footnotes a-g.

Footnotes for Table 5.

[a] The purification process was performed with 86 g fresh material and the substrates used for the 3-, 6- and 4'-OMTs were quercetin, 3,7,3'-trimethylquercetagetin and 3,7-dimethylquercetin, respectively. The 7-OMT which co-eluted with 6-OMT until the hydroxyapatite step was purified 6-fold.

[b] After treatment with Dowex 1X2 and desalting on Sephadex G-25.

[c] 30-70% salt saturation, then desalted on Sephadex G-25.

[d] Peak 2 from hydroxyapatite column.

[e] Chromatofocusing column #2.

[f] Peak 1 from hydroxyapatite column.

[g] Chromatofocusing column #1.

D.3.3. Substrate specificity of purified OMTs

The 3-OMT, which focused at pH 4.8, exhibited expressed specificity towards the 3-position of flavonols with 3',4'- (or 4',5'-) hydroxylation pattern such as quercetin, but not quercetagetin (Table 6). Furthermore, the latter did not act as a substrate inhibitor when added at concentrations of up to 80 μM to enzyme assays (Fig. 13). 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 4'-position (as in tamarixetin) resulted in complete loss of activity. Furthermore, the latter compounds were good product inhibitors with inhibition constants of the same order of magnitude as for 3-methylquercetin (Appendix I, Fig. 4). When myricetin was used as a possible substrate, enzyme activity dropped by 90% (Table 6). Such strict position specificity indicates that quercetin is the best substrate for this enzyme and that 3-methylation may be the first step in the sequential methylation of these flavonols.

The 7-OMT, which was studied in the hydroxyapatite fraction, accepted 3-methylquercetin for further methylation at the 7-position (Table 6). 3-Methylquercetagetin was not available as substrate, however, the enzyme was unable to methylate any of the mono-, di- or trimethyl quercetins tested. The expressed specificity of this enzyme towards the 7-position of quercetin suggests that 7-methylation is the next step in the sequential methyl transfers of Chrysosplenium flavonoids.
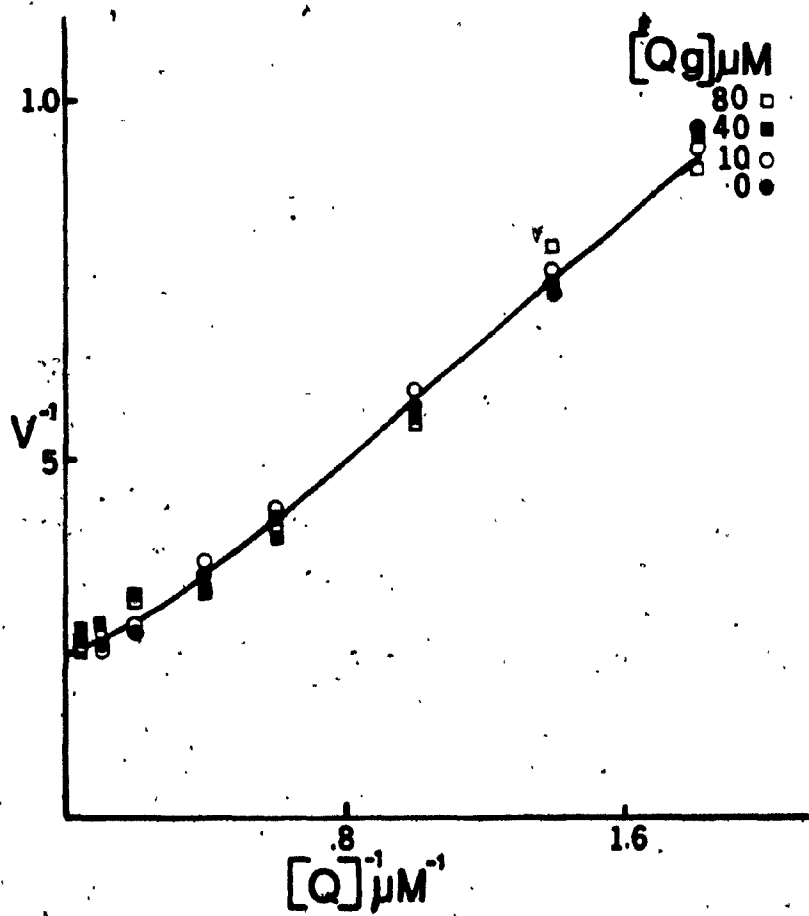
Figure 13. Inhibition of 3-OMT by alternate substrate.

1/v versus 1/Q (quercetin) at various fixed
concentrations of quercetagetin (Qg). Each
incubation mixture contained 6 g/ml enzyme
protein, 10 mM $MgCl_2$, 10mM 2-mercaptoethanol
and 10% DMSO (in 100 mM phosphate buffer, pH 8)
and the reaction was carried out at 30°C for 30
min. Velocities were expressed as μM/s/mg
protein.

Table 6. Substrate specificity of Chrysosplenium O-methyltransferases[a,b]

Substrate	Relative activity (%)[c]			
	3-OMT	6-OMT	7-OMT[d]	4'-OMT
Quercetin (Q)	100	0	0	0
Rhamnetin (7-Methyl-Q)	30	0	0	0
3-Methyl-Q	0	0	100	0
8-Hydroxykaemferol	0	25	0	0
Quercetagetin (Qg)	0	5	0	0
3,7-Dimethyl-Q	0	0	0	100
3,7-Dimethyl-Qg	0	100	0	95
3,7,3'-Trimethyl-Qg	0	40	0	0

[a] Enzyme preparations were used after chromatofocusing (for the 3-, 6- and 4'-OMTs) or after hydroxyapatite (for 7-OMT).

[b] The standard enzyme assay was used (Section C.6.1.) using 0.1 - 1.0 µM of the indicated substrates.

[c] The methylating activities of these enzymes (100%) amounted to 6500, 14000, 4500 and 9000 dpm/assay for the 3-, 6-, 7- and 4'-OMTs, respectively.

[d] The substrate specificity for the 7-OMT was generated considering that this preparation was contaminated with 6-OMT; therefore, the activities against 3,7-dimethyl- and 3,7,3'-trimethylquercetagetin were deducted accordingly.

The two other protein fractions which focused at pH 5.4 and 5.8 accepted partially methylated flavonol intermediates for further O-methylation at the 4'- and 6-positions, respectively. The 4'-OMT converted both 3,7-dimethylquercetin and its 6-hydroxy analog equally well to their respective 4'-methyl derivatives, whereas the 6-OMT accepted 3,7-dimethylquercetagetin and 3,7,3'-trimethylquercetagetin producing their respective 6-methyl derivatives at a ratio of 2:1 (Table 6). The fact that the 4'-OMT did not react with trimethyl substrates suggests that 4'-methylation may precede that at position 6; thus establishing the third and fourth steps in the sequential methylation of these flavonols.

None of the four enzymes described here exhibited any activity with phenylpropanoids, flavones, dihydroflavonols or any of their glucosides thus indicating their specificity towards hydroxylated and partially methylated flavonols (Table 6).

D.3.4. Other enzyme properties

D.3.4.a. pH optima

The O-methylating activity of each enzyme was measured against its best substrate in the presence of histidine-HCl, imidazole-HCl, Tris-HCl and glycine-NaOH, buffers over a pH range of 6.0-9.5. The curves obtained (Fig. 14) show the relative activities of the four OMTs as well as their pH optima which ranged between 7.8 and 9.0. However, there was a gradual shift in optimal activity, from lower to higher pH, with increasing methylation of the substrate used. Therefore, 3- and 7-OMTs which utilised quercetin and 3'-methyl-quercetin, respectively exhibited their optimal activities at
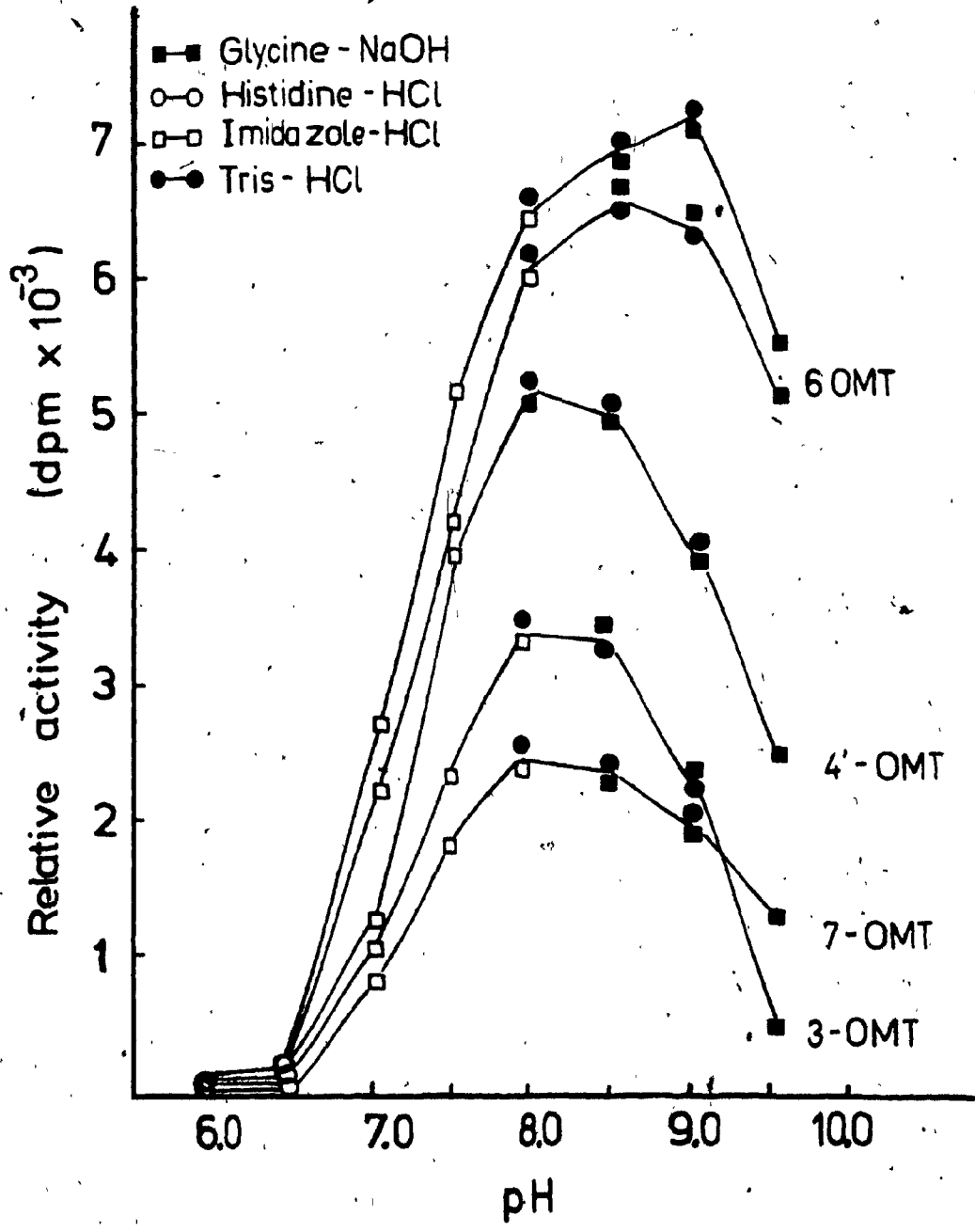
Figure 14. pH optima of purified OMTs after

chromatofocusing, except for the 7-OMT where

the partially purified extract from

hydroxyapatite (Fig. 11, peak 1) was used. The

substrates used were quercetin for the 3-OMT;

3-methylquercetin for the 7-OMT; 3,7-dimethyl-

quercetin for the 4'-OMT; and both 3,7-

dimethylquercetagetin and 3,7,3'-trimethyl-

quercetagetin for the 6-OMT.

pH 7.8-8.2; whereas those of the 6- and 4'-OMTs, which accepted di- or trimethyl substrates, were between pH 8.8 and 9.0 (Fig. 14).

D.3.4.b. Requirement for cofactors

Of the three purified enzymes studied, the 6-OMT showed absolute requirement for Mg^{2+} (Table 7). The latter could not be replaced by other cations tested. Further investigation of the effect of Mg^{2+} indicated that this activation was saturable (Fig. 15A). Lineweaver-Burke plots (Fig. 15A insert) were linear with $30 \mu M Mg^{2+}$ required for half maximal saturation. When the effect of EDTA was investigated (Table 5, Fig. 15B), it was found to inhibit the 6-OMT, whereas it had no effect on the activity of the 3- and 4'-OMTs.

The addition of 14 mM 2-mercaptoethanol in the assay mixture did not increase any of the four enzyme activities, as compared with those of the controls. However, SH-group inhibitors such as PCMB or NEM, were potent inhibitors of enzyme activity (Table 7). The former was particularly effective at 1 mM in inhibiting the three OMTs, whereas the addition of 14 mM 2-mercaptoethanol partially prevented this inhibition.

D.3.4.c. Molecular weight

Partially purified enzyme preparations (C.3.1.) or individual OMTs (C.3.5.) were chromatographed on Sephacryl S-200 which had been calibrated with standard proteins. Both partially purified enzymes and purified OMTs had the same elution volume which corresponded to an approximate molecular weight of 57,000 (Fig. 16).

Table 7. Effect of inorganic ions and other reagents on OMT activity[a]

Additions	Concentration (mM)	Relative activity (%).		
		3-OMT	6-OMT	4'-OMT
Mg^{2+} (control)	1	100	100	100
Mg^{2+}	0	95	5	140
Mn^{2+}	1	110	15	150
Ca^{2+}	1	100	5	150
K^+	1.	115	5	140
EDTA	1	100	110	150
EDTA	10	100	24	150
2-Mercaptoethanol	14	105	100	100
PCMB (minus ME)	1	33	5	15
PCMB 14 mM ME	1	90	60	75
N-Ethylmaleimide[b]	1	48	80	45
Iodoacetamide[b]	1	105	100	100

[a] The standard enzyme assay was used (Section C.6.1.) using 0.5 µM of quercetin, 3,7-dimethylquercetagetin and 3,7-dimethylquercetin as substrates for the 3-, 6- and 4'-OMTs, respectively.

[b] In absence of 2-mercaptoethanol.
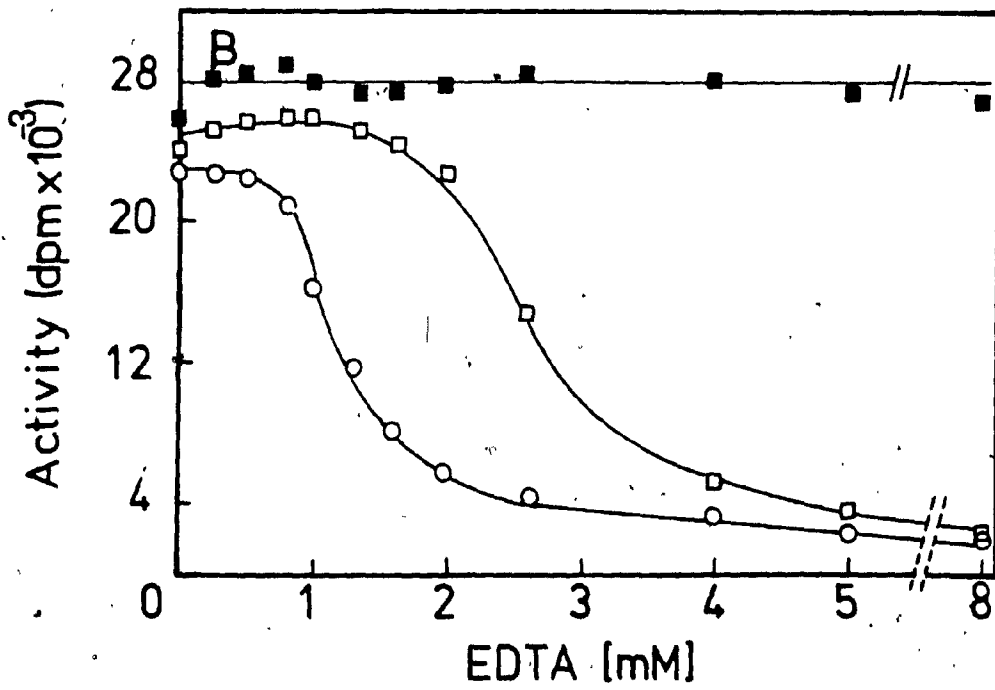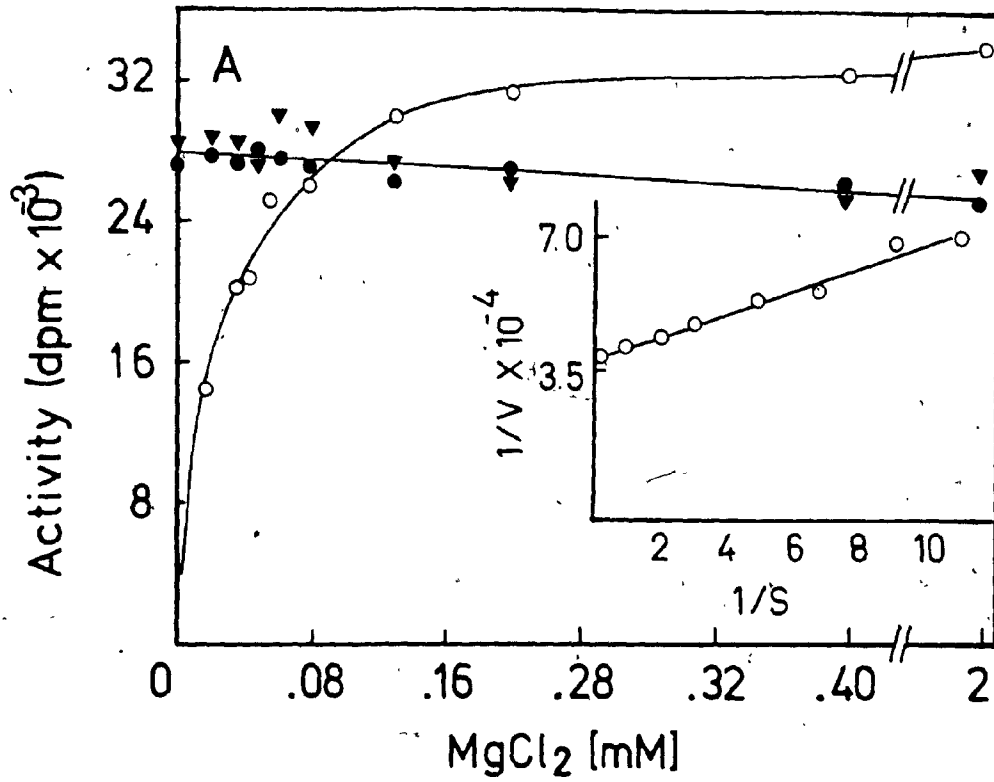
Figure 15.   Effect of Mg^{2+} and the metal chelator EDTA on
              OMT activity.

A. Mg^{2+} saturation curves for the 3- (—●—) 4'-
      (—▲—) and 6- (—O—) OMT activities. A
      Lineweaver-Burke plot of the latter (inset)
      gave an activation constant of 30 μM.

B.   Effect of EDTA on the 6-OMT activity in the
      presence of 0.38 (—O—), 0.76 (—□—) and 3.8
      (—■—) mM of $Mg\ Cl_2$.

log molecular weight
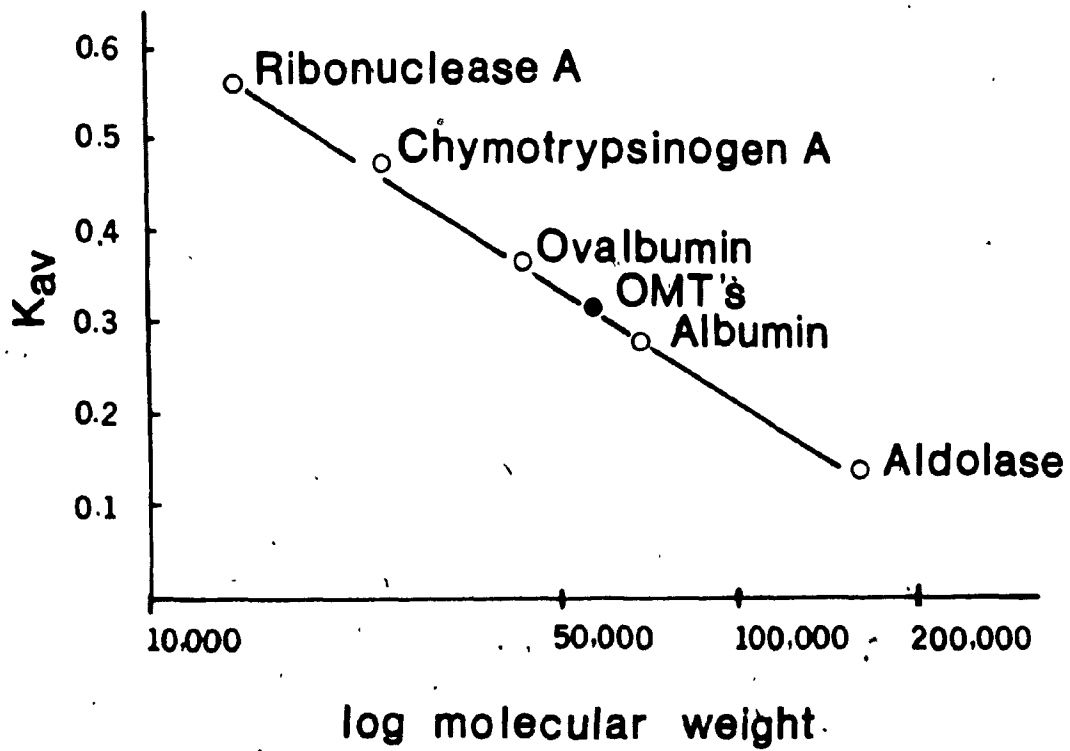
Figure 16.   Determination of Molecular Weight of OMTs

Using a calibrated Sephacryl S-200 column
(2.5 x 118 cm)

D.3.4.d. Stability of 3-, 6-, and 4'-OMTs

Except for the partially purified (C.3.1.) enzyme which lost 50%
of its activity after 15 days (Fig. 17A), the chromatofocused
fractions were quite stable upon storage in 20% glycerol, 5 mM DTT and
0.2M phosphate, pH 8 at -20°C for several months (Fig. 17B).

D.3.5. Localisation studies: OMTs and Glucosyltransferases (GTs)
from Chrysosplenium

Preliminary studies on the localisation of the flavonoid specific
OMTs and GT were performed as described in section C.9. These
experiments demonstrated that all of the OMT activity could be found
on top of the gradient indicating their presence in the cytosol
(Fig. 18). However, it should be noted that in this system, we cannot
differentiate between cytoplasmic and vacuolar constituents. In
addition, there seemed to be two GT activities, one cytoplasmic and
the other bound to some unidentified membrane fraction. The
cytoplasmic activity was present only if a suitable flavonoid
substrate was added to the assay mixture, whereas the membrane-bound
activity could be detected even without the addition of flavonoid
substrate, indicating the glucosylation of some endogenous extractable
acceptor. Furthermore, when radioactive products from assays of the
respective fractions were submitted to TLC on Polyamide, followed by
autoradiography, the cytoplasmic fraction gave products corresponding
to the glucosylated flavonoid substrate, whereas the membrane-bound
activity produced a single spot which chromatographed with the solvent
front (Table 8).
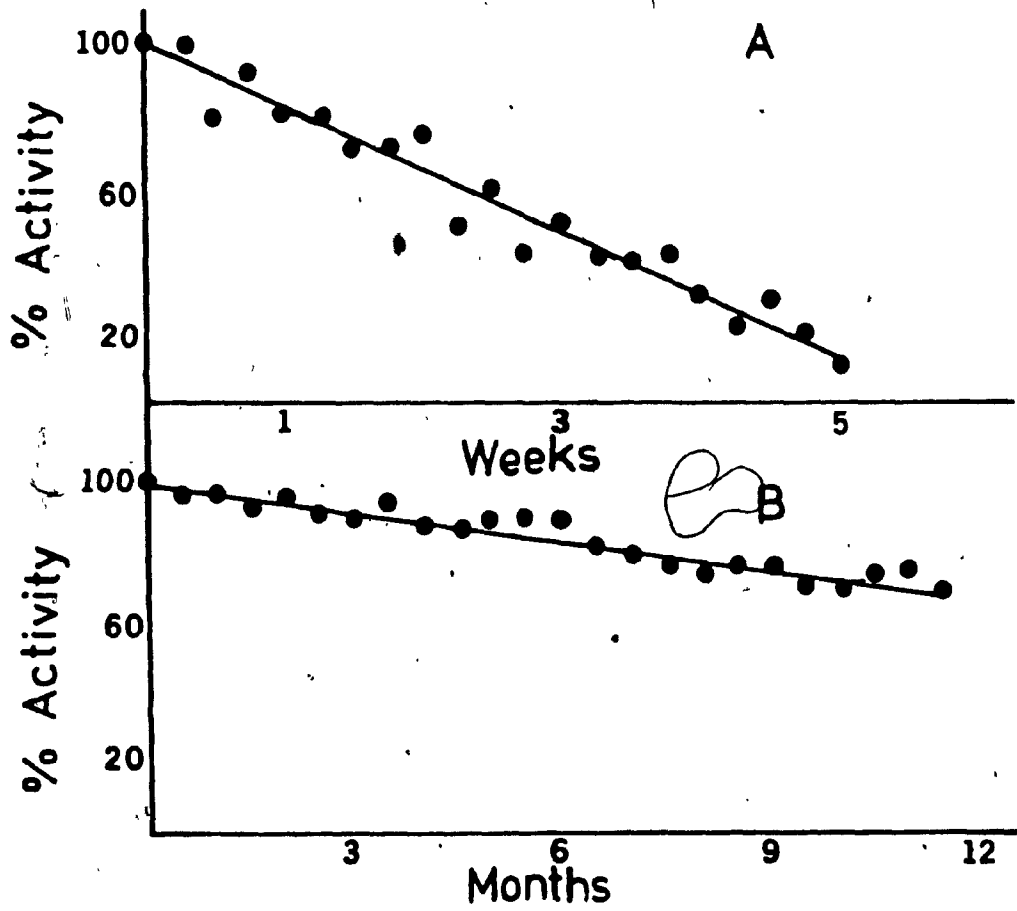
Figure 17.   Time course of 3-, 6- and 4'-OMT inactivation

      A.   Partially purified OMT preparations (section

         C.3.1.) were stored at 4°C and assayed several

         times over the course of 5 weeks.

      B.   Purified 3-, 6- and 4'-OMTs were stored at

         -20°C and assayed over the course of several

         months. This represents the time course of

         inactivation for the 3-OMT, and is

         representative of the 6- and 4'-OMTs as well.
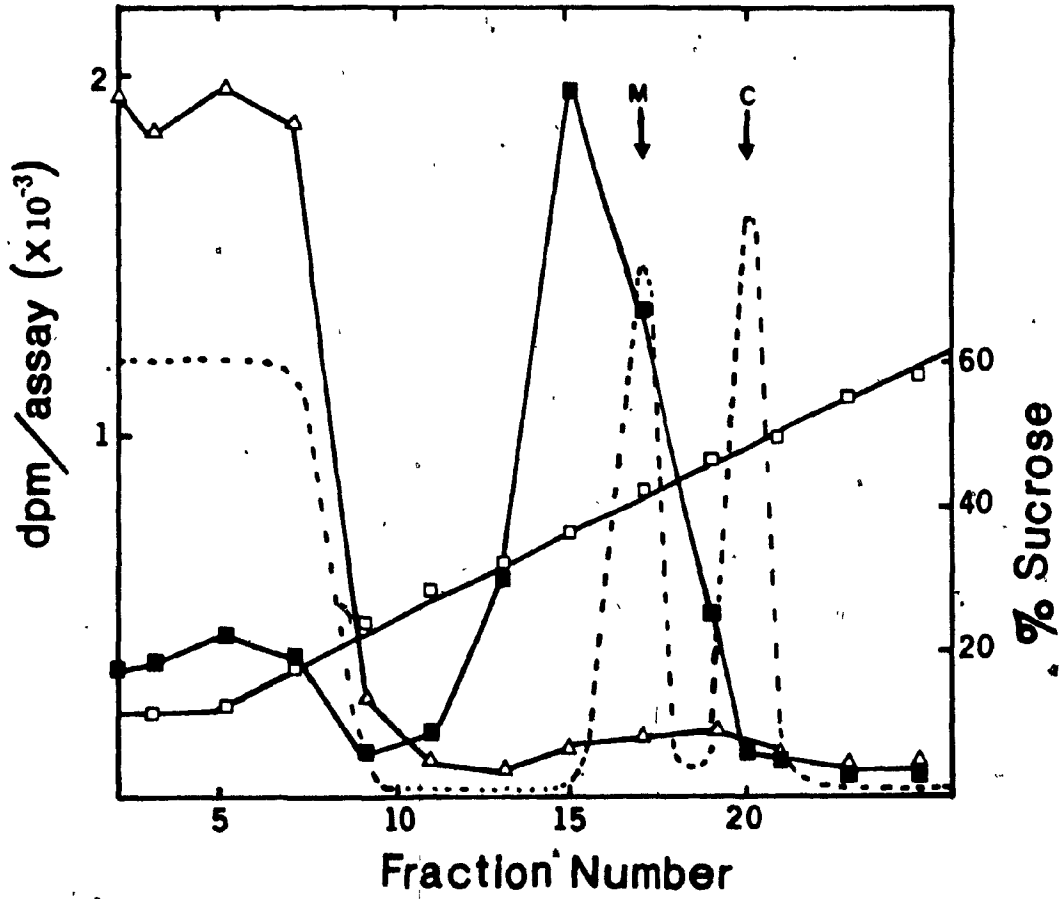
Figure 18.   Frationation of organelles from C. americanum

leaf cells.

Sucrose concentrations (—□—) are expressed on

a % w/w basis and (----) is absorbance at

280. The (M) represents the usual location of

mitochondria and, in this case, broken

chloroplasts as well. The (C) represents

intact chloroplast.s. Glucosyltransferase

activity (—■—) was assayed using 2'-hydroxy-

3,7,4'-trimethylquercetin as substrate. OMTs

(—△—) were assayed with 3,7-dimethyl-

quercetagetin, a substrate for the 6- and

4'-OMTs; 3,7-dimethylquercetin was 60% as

active (specific for the 4' OMT) and quercetin

specific for the 3-OMT was 25% as active. No

OMT activity could be found beyond 20% w/w

sucrose.

Table 8.   Identification of reaction products of <u>Chrysosplenium</u>
            O-glucosyltransferases isolated on sucrose gradients.

Fraction number[a]	Substrate	Identification of products[c]	Relative amount (%)	R_f^d
(1-10)	+	2'	50	0.55
		5'	50	0.45
	−	no products		
(13-18)	+	unknown	100	0.95
	−	unknown	100	0.95

[a] Fractions 1-10 and 13-18 were pooled (Fig. 8) and glucosyltransferase
   activity was assayed as described by Bajaj (103).

[b] 2'-hydroxy-3,7,4'-trimethylquercetin (#1, Fig. 3) was used as
   substrate and the products formed were 2'- and 5'-O-glucosylated
   derivatives.

[c] The reaction products were identified by co-chromatography with
   reference compounds, visualization in UV light and autoradiography.

[d] On Polyamide 6-MN TLC plates developed in toluene-ethyl formate-
   ethanol-water (60:20:19:1).

It is clear from these preliminary studies thàt neither the OMTs
nor the GT appeared to be associated with either the chloroplasts or
mitochondria of this tissue. However, more detailed studies should be
carried out in order to confirm these results as well as to exclude
other possible microcompartments (e.g. endoplasmic reticulum).

D.4. The OMTs of Lotus corniculatus

D.4.1. Comparative OMT activity of different organs of Lotus

The O-methylating activity of partially purified extracts
(C.4.1.) of different organs of the plant was compared using three
flavonol substrates: quercetin, 8-hydroxyquercetin and 8-hydroxy-
kaempferol. and one phenylpropanoid substrate, caffeic acid. The
results, shown in Table 9, clearly indicate that the flowering buds
exhibited the highest methylating activity towards flavonol substrates
and the lowest towards caffeic acid. Both apical buds and mature
leaves showed a similar degree of flavonoid O-methylation which was
50-80% lower than that of flowering buds, whereas activity towards
caffeic acid was greater in mature leaves than apical buds. Three
week-old seedlings, on the other hand, exhibited the lowest degree of
flavonoid methylation which amounted to 15% of that of flower buds.

It is interesting to note that the pattern of methylated products
formed from each flavonoid substrate was similar for the different
plant organs used (Table 10). Both 8-hydroxykaempferol and
8-hydroxyquercetin were methylated either at the 3- or 8- positions at
a ratio of 2:1. Quercetin on the other hand, was equally methylated
at positions 3 and 3', with trace amount of label found in

Table 9. O-Methylating activity of different organs of Lotus[a]

Plant organ	8-Hydroxy-kaempferol	8-Hydroxy-quercetin	Quercetin	Caffeic acid
	DPM / g fresh weight / min[b]			
Three-wk-old seedlings	2350	2150	2150	1100
Apical buds	4200	5760	8750	2800
Mature leaves	4050	6020	6750	4900
Young flower buds	19750	15600	14290	1000

[a] Buffer extracts of the indicated organs desalted on Sephadex G-25.

[b] The standard enzyme assay was used with the indicated substrates as was described in Section C.6.1. See Table 10 for identification of reaction products.

Table 10. Identification of the reaction products of <u>Lotus</u> OMTs[a]

| Substrate | Reaction products[b] | | |
	Methyl derivative	Relative amount (%)[c]	R_f value[d]
8-Hydroxy-	8-	30	0.65
kaempferol	3-	70	0.50
8-Hydroxy-	8-	30	0.40
quercetin	3-	70	0.30
Quercetin	3-	50	0.40
	3'-	45	0.55
	Dimethyl[e]	5	0.70
Caffeic acid	3-	90	0.65

[a] Plant organs were used as source of enzyme as described in Table 9.

[b] Identified by co-chromatography with reference compounds and auto-radiography.

[c] As % of total activity shown in Table 9.

[d] On Polyamid-6 using benzene-methyl ethyl ketone-methanol (70:15:15) as solvent system.

[e] 3,3'- or 3,7-Dimethylquercetin.

dimethylated products (Table 10). These results clearly indicate the presence of 8- and 3'-OMT activities in the flower buds of Lotus, together with another enzyme activity which attacks position 3 of quercetin, 8-hydroxyquercetin or 8-hydroxykaempferol.

D.4.2. OMT activities in Lotus flower buds

Further characterization of OMT activity was carried out with flower buds at different stages of development (C.4.2) (Table 11). Enzyme activities which catalysed the methylation of positions 3, 8 and 3' of flavonols were assayed with 8-hydroxykaempferol and quercetin whereas caffeic OMT activity was assayed with caffeic acid. The results indicated that 8-OMT activity steadily increased until the third stage of development and then decreased, whereas 3- and/or 3'-OMT activity was already high at stage one and decreased rapidly after stage two (Table 11). On the other hand, caffeic OMT remained uniformly low throughout development, which corresponds to the lack of lignification in flower tissues.

D.4.3. Separation of 8- and 3'-OMTs in Lotus flower buds

OMTs from young flower buds were extracted and purified as described in sections C.4.2.-C.4.4. Chromatography on Sephadex G-100 resulted in a single peak of activity (Fig. 19A) which catalysed the methylation of positions 8, 3 or 3' of different flavonoid substrates. Further purification of the latter by chromatofocusing on Polybuffer ion exchanger resulted in two peaks of OMT activity which focused at pH 5.5 and 5.1 (Fig. 19B).

Table 11. OMT activities of <u>Lotus</u> flower buds at different stages
of development[a]

Substrate	OMT activity $(\mu M / mg / min)$[b]				
8-OH-kaempferol	1.88	1.61	2.15	1.73	0.80
Quercetin	1.81	1.18	0.60	0.52	0.36
Caffeic acid	0.26	0.14	0.18	0.34	0.38

	OMT activity $(\mu M / flower / min)$				
8-OH-kaempferol	0.04	0.08	0.14	0.03	0.01
Quercetin	0.04	0.06	0.04	0.01	0.005
Caffeic acid	0.005	0.01	0.01	0.005	0.005

[a] Flower buds at different stages of development were extracted as
described in Section C.4.2. without fractionation with ammonium
sulphate. Extracts were desalted on Sephadex G-25 and used as
the enzyme source.

[b] Enzyme assays were carried out as described in Section C.6.1.
Values represent total activity in reaction products.
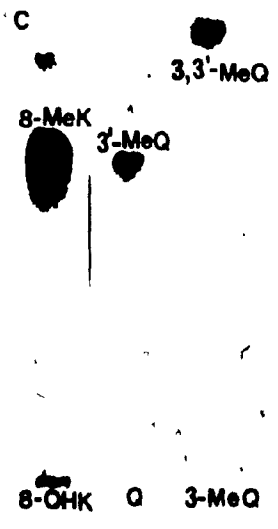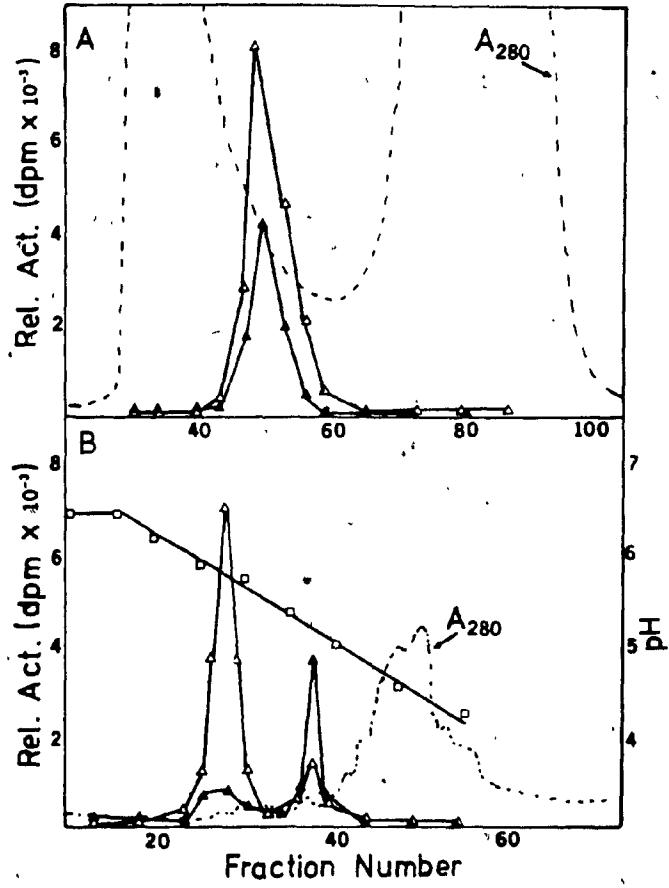
Figure 19.   The OMTs of <u>Lotus</u> flower buds

OMT activity was assayed using 8-hydroxy-
kaempferol (—△—) and quercetin (—▲—) as
substrates.

A.   Chromatography on Sephadex G-100 (2 x 100 cm)
of partially purified OMTs as described in
sections C.4.2. - C.4.3.

B.   Chromatofocusing on Polybuffer ion exchanger
(1 x 28 cm) as described in section C.4.4.

C.   Photograph of an autoradiograph of the
chromatographed reaction products of the 8- and
3'-OMTs peaks from chromatofocusing. The 8-OMT
was assayed againt 8-hydroxykaempferol (8-OHK)
and gave 8-methoxykaempferol·(8-MeK) as a major
reaction product. The 3'-OMT was assayed
against Quercetin (Q) and 3-methylquercetin
(3-MeQ) and gave 3'-methylquercetin (3'-MeQ)
and 3,3'-MeQ as the major reaction products.
The reaction products were identified by
co- chromatography on Polyamide 6-MN TLC plates
with reference compounds, 8-MeK, 3'-MeQ and
3,3'-MeQ. The solvent system used was benzene-
methyl ethyl ketone-methanol (70:15:15).

Whereas the Sephadex G-100 fraction catalysed the methylation of a number of flavonoid substrates, the chromatofocused peak 1 exhibited preferential affinity for 8-hydroxyflavonols and gave rise to their 8-methyl derivatives (Table 12). On the other hand, peak 2 methylated 8-hydroxyflavonols as well as luteolin, quercetin and 3-methyl-quercetin. Examination of the autoradiographed reaction products (Fig. 19C) clearly indicated that peak 1 contained predominantly an 8-OMT activity; whereas peak 2 consisted mainly of 3- and 3'-OMTs.

D.4.4. Separation of OMT activities in Lotus shoots

The OMT profiles of shoot tips were studied using the methods described in sections C.4.2.-C.4.4. A single OMT peak was obtained from Sephadex G-100 (Fig. 20A) similar to that of flower buds (D.4.3.). However, chromatofocusing of active fractions from Sephadex G-100 resolved two peaks of OMT activity at pH 6.1 and 5.2 (Fig. 20B) instead of 5.5 and 5.1 (Fig. 19B). Furthermore, peak 1 (pH 6.1) was very active against quercetin but not 8-hydroxykaempferol,whereas peak 2 (pH 5.2) was more active against 8-methylquercetin than either 8-hydroxykaempferol or quercetin. These results indicated that the flavonoid OMTs present in shoots represent a different group of enzymes from those present in flower buds.

D.4.5. Purification of 8-OMT from Lotus flower buds

The purification procedure described in section C.5.-C.5.3. was carried out in order to purify enough enzyme for kinetic studies. Sephadex G-100 chromatography (Fig. 21A) resulted in a single peak of

Table 12. Substrate specificity of Lotus OMTs at different stages of purification[a]

Substrate[c]	Relative activity (%)[b]		
	Sephadex G-100	Peak 1	Peak 2
8-OH-kaempferol	100	100	88
8-OH-quercetin	80	84	100
Quercetin	45	30	57
8-MeO-quercetin	10	7	23
Luteolin	32	10	56
3-Me-quercetin	19	7	52
7-Me-quercetin	16	6	26
Quercetagetin	3	0	0

[a] Peaks 1 and 2 were recovered after chromatofocusing on Polybuffer ion exchanger (PBE-94).

[b] Control activities (100%) amounted to 17500, 5500 and 2400 dpm/assay for the Sephadex G-100, peak 1 and peak 2, respectively.

[c] The following substrates were assayed and found to be poor methyl acceptors (<5% activity): 3'-; 4'-; 3',4'-; 3,7-; 7,4'- and 3,3',4'- methylquercetin, as well as 3,7- and 3,7,3'-methyquercetagetin; kaempferol.
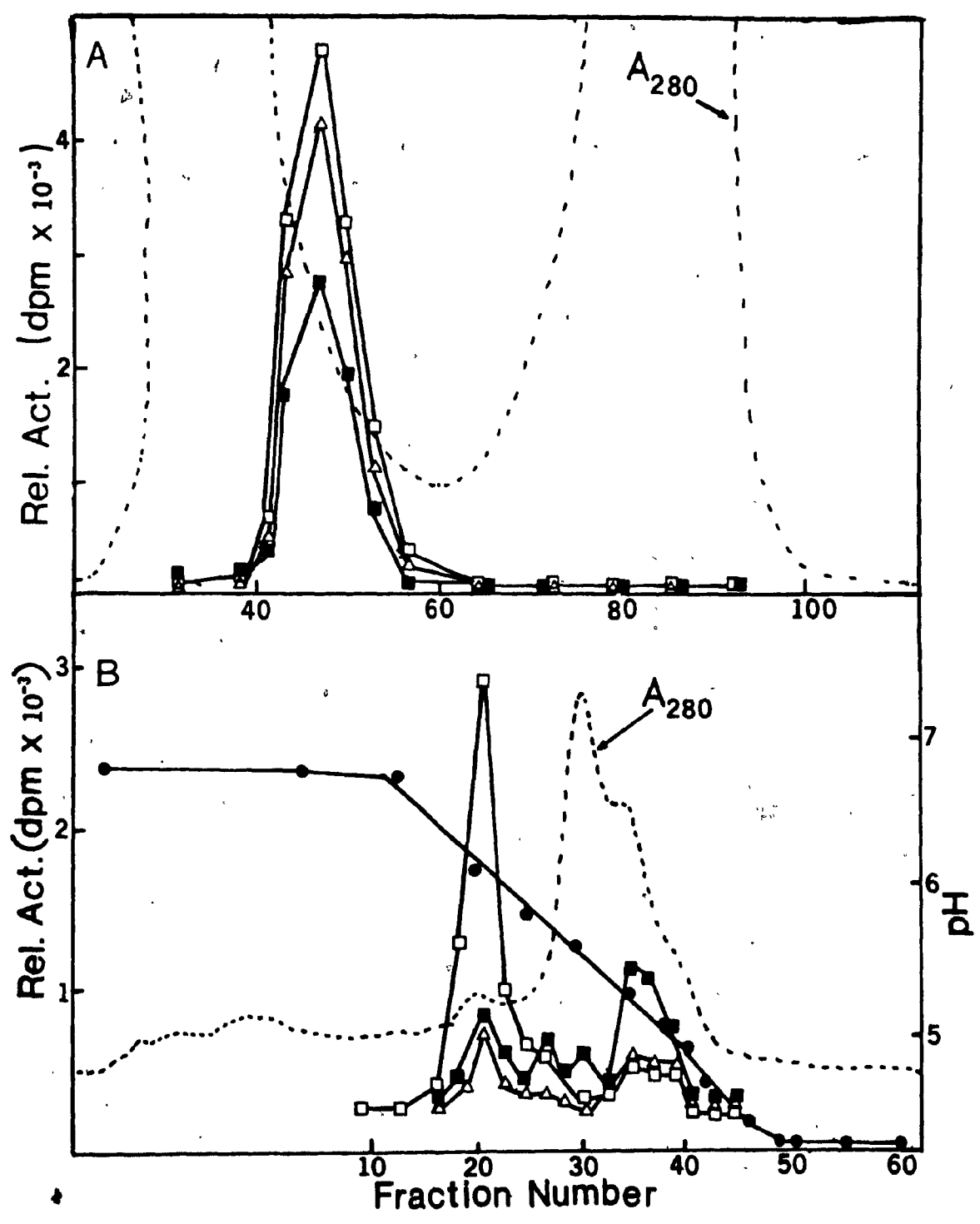
Figure 20.   The OMTs of _Lotus_ shoots

OMT activity was assayed using 8-hydroxy-
kaempferol (▬■▬), quercetin (▬□▬) and
3-methylquercetin (▬△▬) as substrates.

A.   Chromatography on Sephadex G-100 (2 x 100 cm)
of partially purified OMTs as described in
sections C.4.2. - C.4.3.

B.   Chromatofocusing on Polybuffer i-on exchanger
(1 x 28 cm) as described in section C.4.4.

activity similar to the previous profile (Fig. 18A). Active fractions
were applied to SAH-Agarose (Fig. 21B) which resulted in partial
separation of quercetin methylating activity. from the 8-OMT. I The
enzyme was concentrated and further purified by chromatography on
hydroxyapatite (Fig. 21C) where the activities against quercetin and
8-hydroxykaempferol co-eluted. Final separation of these two
activities was, achieved by chromatofocusing on Polybuffer ion
exchanger (Fig. 21D) where the 8-OMT activity was eluted at pH 5.6.
This purification procedure, described in Table 13, resulted in ca
1200-fold purification and a 4% yield of the total 8-OMT in the crude
enzyme preparation.

D.4.6. Properties of the purified 8-OMT

D.4.6.a. Substrate specificity

The extensively purified 8-OMT was shown to possess strict
position specificity for the 8-position of 8-hydroxykaempferol and
8-hydroxyquercetin and did not accept quercetin as substrate (Table
14). Furthermore, TLC of radioactive products on Polyamide (as
described in Table 7) followed by autoradiography indicated that
8-methoxykaempferol was the only reaction product formed when
8-hydroxykaempferol was used as substrate. It is interesting to note
that quercetagetin, the 6-hydroxy derivative of quercetin was not
accepted for methylation at position 6, whereas the corresponding
flavone, 6-hydroxyapigenin was a poor methyl acceptor. This is in
contrast with the 6 OMT found in Chrysosplenium, where 8-hydroxy-
kaempferol was a good methyl acceptor (Table 6).
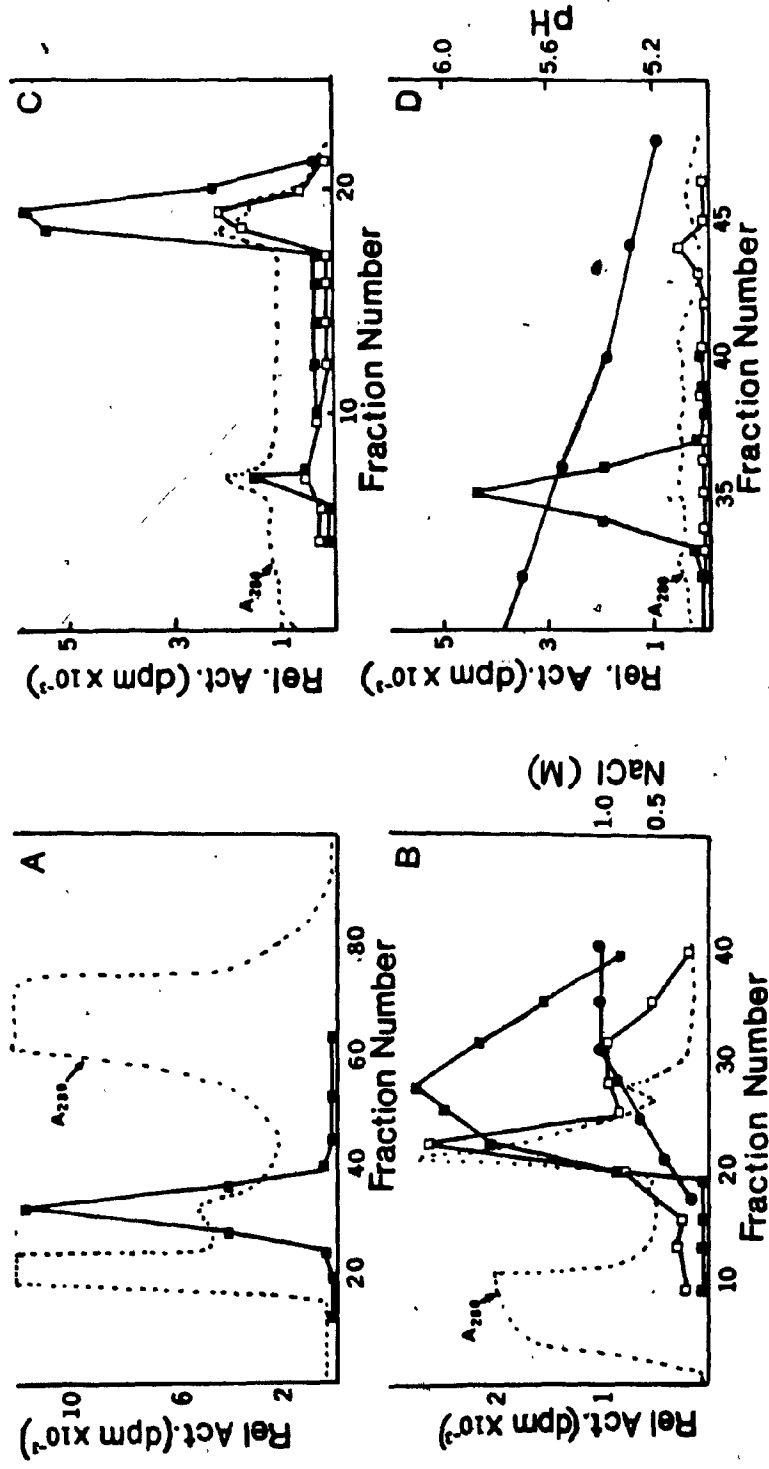
Figure 21. Purification of 8-OMT from Lotus flower buds

OMT activity was assayed using 8-hydroxy-
kaempferol (—■—), and quercetin (—□—) as
substrates.

A.   Chromatography on Sephadex G-100 (2 x 100 cm)
as described in section C.5.

B.   Chromatography on SAH-Agarose (1 x 5 cm) as B.
described in section C.5.1.

C.   Chromatography on hydroxyapatite as described
in section C.5.2.

D.   Chromatofocusing on Polybuffer ion exchanger
as described in section C.5.3.

Table 13. Purification of _Lotus_ 8-0-methyltransferase[a]

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Total activity (pkat)	Purification (-fold)	Recovery (%)
Dowex 1X2	98	0.27	26.46		100
Amm. sulphate[b]	52	0.42	21.85	1.5	82
Sephadex G-100	19	1.21	23	4.5	83
SAH-Agarose	0.17	43.98	7.48	163	28
Hydroxyapatite	0.007	331.9	2.32	1230	9
PBE-94[c]	-	-	1.03	-	4

[a] The purification procedure was performed with 20 g fresh material and the substrate used with enzyme assays was 8-OH-kaempferol.

[b] 30-70% salt saturation, after desalting on Sephadex G-25.

[c] Protein content of the chromatofocused fraction was too low to be measured with the Bio-Rad method.

Table 14. Substrate specificity of purified <u>Lotus</u> 8-OMT[a,b]

Substrate	Relative activity (%)[c]
8-Hydroxyquercetin	100
8-Hydroxykaempferol	·61
6-Hydroxyapigenin	13
Luteolin	7
3,7-Dimethylquercetagetin	6
8-Methoxyquercetin	5
3;7-Dimethylquercetin	4
Quercetin	4
3-Methylquercetin	4
3,7,3'-Trimethylquercetagetin	2
Quercetagetin	2

[a] The enzyme preparation used was that recovered after the chromato-focusing step (Table 13).

[b] The standard enzyme assay was used (Section C.6.1.) with 1.5 μM of the indicated substrates.

[c] The total methylating activity with 8-OH-quercetin (control = 100%) amounted to 11000 dpm/assay.

D.4.6.b. pH optimum

The 8-OMT activity was measured against 8-hydroxykaempferol in the presence of 0.1M phosphate buffer over a pH range of 6.1-8.4. The curve obtained (Fig. 22A) showed an optimum between pH 7-8.4 with maximal activity at pH 7.9.

D.4.6.c. Requirements for cofactors

The activity of the 8-OMT was increased 4-fold upon the addition of $MgCl_2$ to the reaction mixture (Fig. 22B). However, upon the addition of 1 mM EDTA to the assay mixture, the enzyme activity was completely abolished in the absence of Mg^{2+} ions (Fig. 22B).

D.4.7. Enzyme kinetics of the C. americanum OMTs

Preliminary experiments (Appendix I, Fig. 5) established that the reaction was linear with time at all concentrations of SAM and flavonoid substrate used.

D.4.7.a. Inhibition by flavonoid substrate

When the flavonoid substrate was varied at a constant SAM concentration, Michaelis-Menten kinetics were observed below K_m, whereas higher flavonoid concentrations resulted in substrate inhibition (Fig. 23). Above 10 µM flavonoid, substrate inhibition was most notable with the 6-OMT whereas the other two enzymes were only slightly inhibited. Such inhibitions may have resulted from the combination of the substrate with the wrong enzyme form and/or from decreased solubility of the flavonoid substrate at higher concentrations. The binding of substrate with the wrong enzyme form
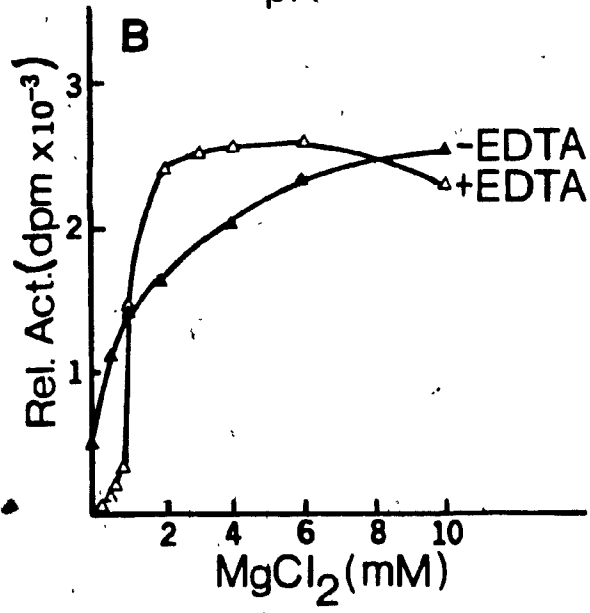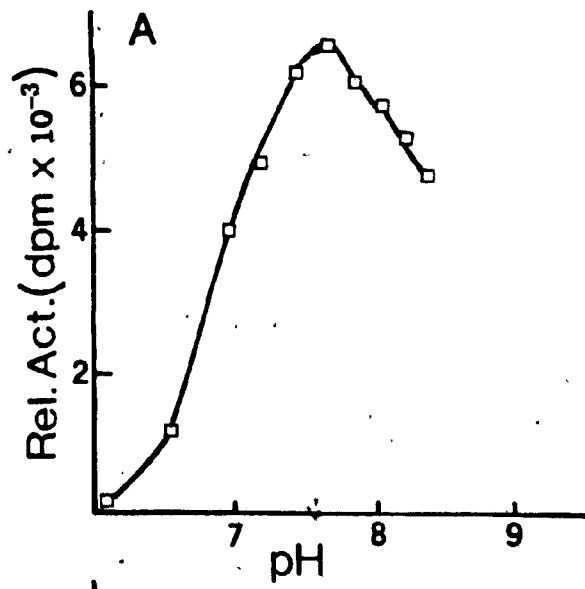
Figure 22.   pH optima and Mg^{2+} requirement for 8-OMT of

Lotus

    A.   pH optima of 8-OMT with 200 mM phosphate

buffer.

    B. Mg^{2+} saturation curve for the 8-OMT in the

presence and absence of 1 mM EDTA.
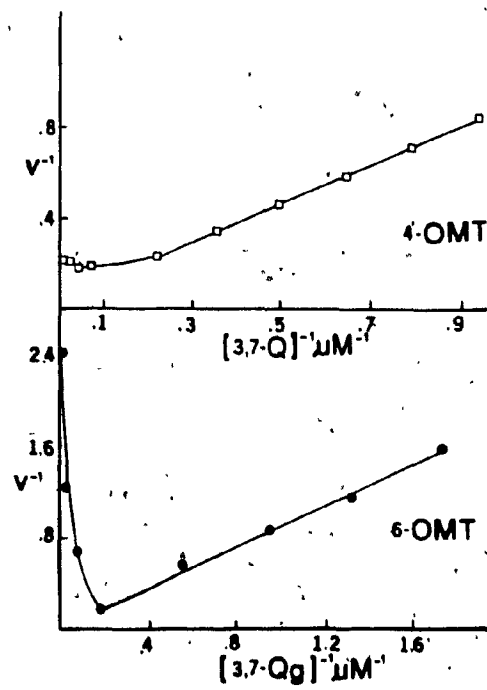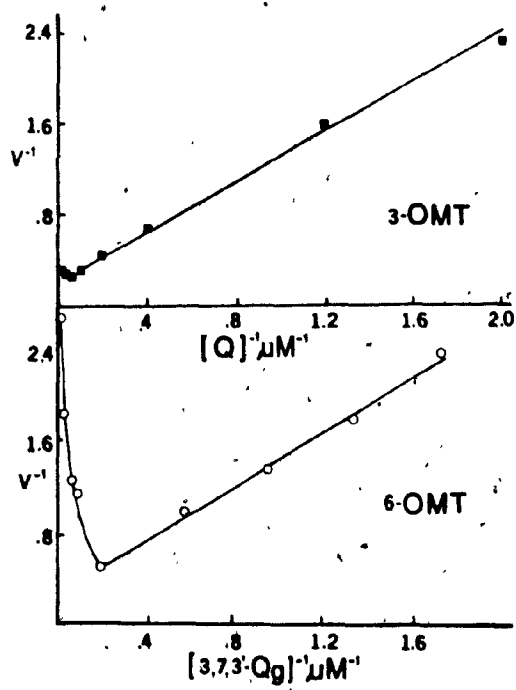
Figure 23.   Substrate inhibition by flavonol substrate

1/v versus 1/Flavonol at constant SAM
concentrations (76.6 µM, of which 16.6 µM was
$[^{14}CH_3]$-SAM containing 220,000 dpm). Each
incubation mixture contained 6 µg/ml of the
3-,4'- or 6-OMTs, 10 mM $MgCl_2$, 14 mM
2-mercaptoethanol, 5% DMSO in 100 mM phosphate
buffer pH 8. The amount of $^{14}CH_3$ labelled
flavonol formed was determined after 30 min.
incubation at 30°C. Velocities are expressed
as µM/s/mg protein. Q; 3,7-Q; 3,7,3'Qg; and
3,7-Qg represent quercetin, 3,7-dimethyl-
quercetagetin and 3,7-dimethylquercetagetin
respectively.

usually occurs at high substrate concentrations and/or when the
reaction is studied in the non-physiological direction (117).
However, the substrate inhibition observed in Fig. 23 occurred neither
at high substrate concentrations nor was it in the non-physiological
direction. When SAM was the variable substrate the reaction was
saturable without apparent substrate inhibition.

D.4.7.b. Substrate interaction kinetics

Since the 3-, 6- and 4'-OMTs showed Michaelis-Menten kinetics and
substrate inhibition was observed with flavonol, therefore, substrate
interaction kinetics were performed at flavonol concentrations below
10 μM.

Estimates of the parameters derived from nonlinear regression
analysis of the substrate interaction kinetic data are shown in Table
15. The variance ratio test indicated that for the 6-OMT, the K_{ia} K_b
term was significant for the interaction between SAM and
3,7,3'-trimethylquercetagetin, i.e. the lines of the double reciprocal
plot intersect (Fig. 24C and 25C). For the interaction between SAM
and quercetin (3-OMT) (Fig. 24A and 25A) or 3,7-dimethylquercetin
(Fig. 24B and 25B) equation one did not give a statistically better
fit to the data. However, when the data was fitted by a line
intercept computer programme (method of least squares), it was
apparent that the reciprocal plots (Fig. 24 A-C and 25 A-C) were
converging and not parallel.

In a sequential mechanism, represented by equation I (section
C.10.) where K_{ia} is the dissociation constant of enzyme and A, double
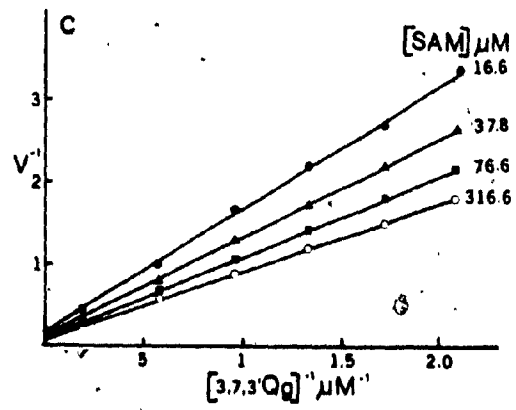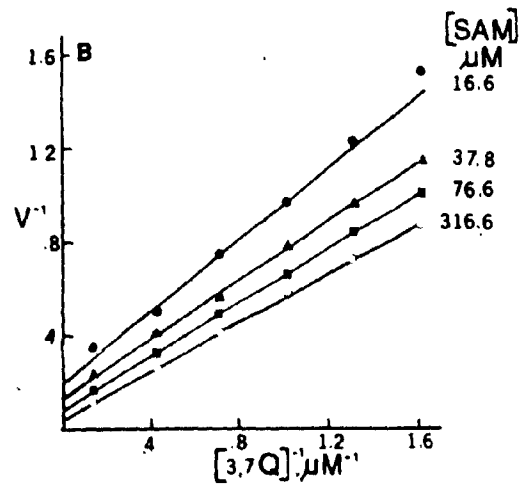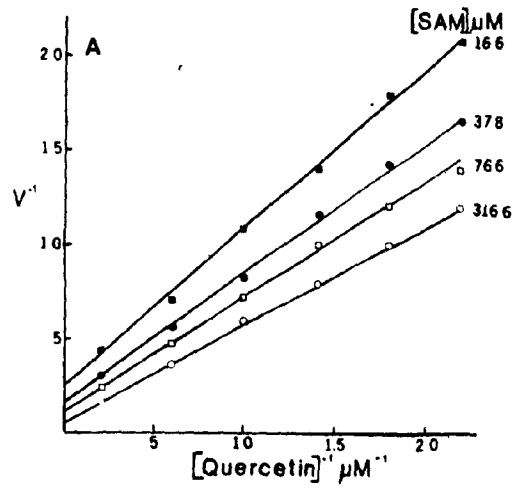
Figure 24. Initial velocity - 1/v versus 1/Flavonol at

various fixed concentrations of SAM for the

3-, 6-, and 4'-OMTs.

A. 1/v versus 1/Q (quercetin) at various fixed

concentrations of SAM. Each incubation mixture

contained 6 μg/ml of 3-OMT. OTher conditions

are as in Fig. 23.

B. 1/v versus 1/3,7Q (3,7-dimethylquercetin) at

various fixed concentrations of SAM. Each

incubation mixture contained 6 μg/ml of

4'-OMT. Other conditions were as in Fig. 23.

C. 1/v versus 1/3,7,3'Qg (3,7,3'-trimethyl-

quercetagetin at various fixed concentrations

of SAM. Each incubation mixture contained

6 μg/ml of 6-OMT. Other conditions were as in

Fig. 23.
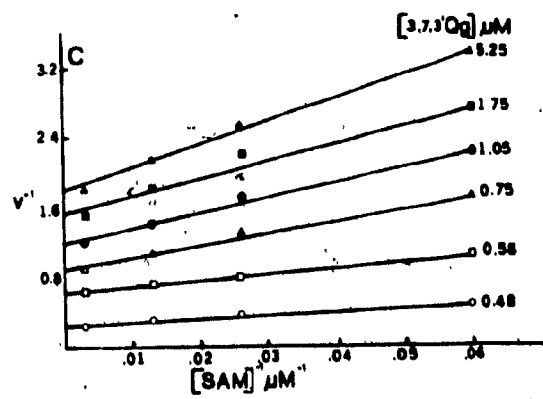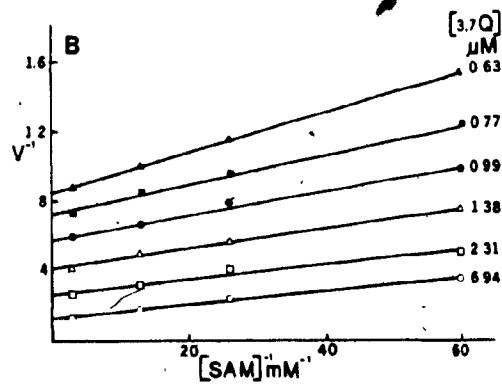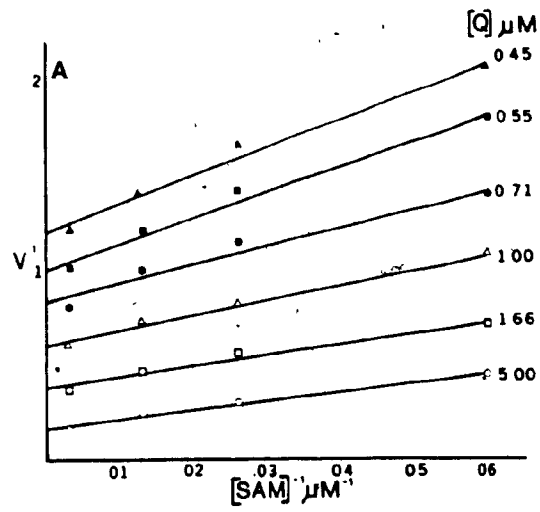
Figure 25.   Initial velocity - 1/v versus 1/SAM at various

fixed concentrations of SAM for the 3-, 6-, and

4'-OMTs.

A.   1/v versus 1/SAM at various fixed

concentrations of quercetin (Q). The data are

the same as those shown in Fig. 24A.

B.   1/v versus 1/SAM at various fixed

concentrations of 3,7-dimethylquercetin (3,7Q).

The data are the same as those shown in Fig.

24B.

C.   1/v versus 1/SAM at various fixed

concentrations of 3,7,3'-trimethyl-

quercetagetin (3,7,3'Qg). The data are the

same as those shown in Fig. 24C.

reciprocal plots will yield a family of lines which intersect at a point dependent on the ratio of K_{ia}/K_a. As K_{ia}/K_a decreases, the intersection point becomes more negative, with minus infinity corresponding to parallel lines (equation II). The possibility was, therefore, considered that the mechanisms of the 3- and 4'- OMTs were sequential and that failure of the computer programme to distinguish between the two equations velocity was due to a small value for K_{ia}/K_a which was not resolved by this programme.

In order to determine the value of the K_{ia} to K_a ratio, K_{ia} was calculated from the kinetic constants given in Table 15. If SAM was assumed to be the first substrate to bind to the enzyme, then the value of K_{ia}, the dissociation constant for the Enzyme-SAM complex was 5, 7, and 12 μM for the 3-, 4'- and 6-OMTs, respectively. On the other hand, the values of K_a were 114, 130 and 50 μM for the 3-, 4'- and 6-OMTs. Therefore, K_{ia} was 23, 18 and 4 times smaller than K_a for the 3-, 4'- and 6-OMTs, respectively and could explain the tendency of the former two enzymes to give parallel double reciprocal plots. Another factor which favored a sequential binding mechanism for the three enzymes was the fact that product inhibition patterns (see section D.4.7.c.) did not conform to those expected for a ping pong mechanism. Therefore, the kinetic constants given in Table 15 for the substrate interaction kinetics of the 3- and 4'-OMTs were generated from equation I.

The following equation expresses the initial rate for the three enzymes, assuming that SAM is the first substrate to bind, K_{ia} has a

Table 15. Estimates of parameters of substrate interaction kinetics

for Chrysosplenium 3-, 6- and 4'-O-methyltransferases[a]

Enzyme	Substrate		Parameter value			Kinetic
	A	B	Term	μM	SD[*]	pattern
3-OMT	SAM	Quercetin	K_a	114	33	Intersecting
			K_{ia}	5	-	
			K_a/K_{ia}	23	-	
			K_b	12	3.3	
			$K_{ia}K_b$	85	34	
			V	24	4.6	
6-OMT	SAM	3,7,3'-Tri-methylquercet-agetin	K_a	51	28	Intersecting
			K_{ia}	12	-	
			K_a/K_{ia}	4	-	
			K_b	18	6	
			$K_{ia}K_b$	214	55	
			V	20	6	
4'-OMT	SAM	3,7-Dimethyl-quercetin	K_a	130	25	Intersecting
			K_{ia}	7	-	
			K_a/K_{ia}	19	-	
			K_b	15	2.6	
			$K_{ia}K_b$	73	33	
			V	29	4	

See following page for foornote

Footnote for Table 15

[a] The parameter values presented for the 6-OMT were those for the kinetic equation, as chosen by the variance ratio test; whereas the values for the 3- and 4'-OMTs were chosen because the postulated sequential mechanism better fits the product inhibition data.,

[*] S.D. is standard deviation

non zero value and the presence of more than one binding site for the
flavonol substrate.

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + [A][B]^2/K_{ib}} \qquad VIII$$

D.4.7.c. Product inhibition kinetics

Previous substrate interaction kinetics established that the 3-,
6- and 4'-OMTs followed a sequential rather than ping pong mechanism.
Further information on the order of substrate binding was obtained
from product inhibition studies. The rate equations derived for a
bi bi sequential mechanism (117, 118) predict that the last product
released is a competitive inhibitor with respect to the first
substrate since both bind to the same enzyme form, whereas
non-competitive inhibition is expected with respect to the other
substrate.

The results obtained for the 3-OMT (Fig. 26 A-D) are in agreement
with a modified version of the theoretical predictions mentioned
above. SAH was a competitive inhibitor with respect to SAM (Fig. 26A)
and uncompetitive with respect to quercetin (Fig. 26B).
3-Methylquercetin was a noncompetitive inhibitor with respect to both
SAM (Fig. 26C) and quercetin (Fig. 26D). The kinetic patterns
obtained (Table 16-18) exclude random addition of substrates and/or
release of products, in which case four competitive inhibition
patterns would be expected. Three (Fig. 26 A, C, D) of the four
(Fig. 26B) kinetic patterns obtained are consistent with an ordered

Figure 26.   Product inhibition kinetics of the 3-OMT from

Chrysosplenium americanum

A.   Product inhibition by S-adenosyl-L-

homocysteine: 1/v versus 1/SAM (16.6 μ M of

$[^{14}CH_3]$-SAM, 0.1 μCi in addition to varying

amounts of unlabelled SAM) at different fixed

concentrations of SAH and at a constant

concentration of quercetin (4 μM). Each

incubation mixture contained 6 μg/ml of the

3-OMT, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 5%

DMSO in 100 mM phosphate buffer, pH 8. The

amount of $^{14}CH_3$ labelled flavonol formed was

determined after 30 min incubation at 30°C.

Velocities were expressed as μM/s/mg protein.

B.   Product inhibition by S-adenosyl-L-

homocysteine: 1/v versus 1/Q

(quercetin) at different fixed concentrations

of SAH and at constant concentration of SAM

(8.3 μM of $[^{14}CH_3]$-SAM, 0.05 μCi). Other

conditions were as in (A).

C & D.   Continued on next page

Figure 26. Continued

C. Product inhibition by 3-methylquercetin (3Q):
1/v versus 1/SAM at different fixed concentra-
trations of 3Q and at constant concentrations
of quercetin (4 uM). Other conditions were as
in (A), except that DMSO was 10%.

D. Product inhibition by 3-methylquercetin (3Q):
1/v versus 1/Q (quercetin) at different fixed
concentrations of 3Q and at constant
concentration of SAM (8.3 uM of $[^{14}CH_3]$-SAM,
0.05 uCi). Other conditions were as in (A),
except that DMSO was 10%.

Table 16. Estimates of parameters of product inhibition kinetics for 3-0-methyltransferase[a]

Substrate	Product	Parameter value			Kinetic pattern
		Term	μM	SD	
SAM	SAH	K'_a	25	5.7	Competitive
		K_{is}	4.5	0.9	
SAM	3-Methylquercetin	K'_a	44	4.5	Noncompetitive
		K_{is}	29	5.3	
		K_{ii}	65	9.0	
Quercetin	SAH	K'_a	1.3	0.1	Uncompetitive
		K_{ii}	8.4	0.6	
Quercetin	3-Methylquercetin	K'_a	4.5	0.3	Noncompetitive
		K_{is}	27	2.8	
		K_{ii}	128	36	

[a] The parameter values were those chosen for the kinetic equation by a variance ratio test.

bi bi mechanism where SAM and SAH were leading partners in the reaction.

The results of the product inhibition studies for the 4'-OMT (Fig. 27 A-D) were similar to those of the 3-OMT. When SAM was the variable substrate, SAH (Fig. 27A) was a competitive inhibitor and 3,7,4'-trimethylquercetin (Fig. 27B) a noncompetitive inhibitor. The inhibition by both SAH (Fig. 27C) and 3,7,4'-trimethylquercetin (Fig. 27D) was uncompetitive and noncompetitive, respectively when 3,7-dimethylquercetin was the variable substrate. The presence of SAH at higher concentrations (Fig. 27C inset) enhanced the substrate inhibition observed with 3,7-dimethylquercetin. The kinetic patterns obtained were identical to those for the 3-OMT, indicating that this enzyme also binds substrates and releases products in a given order.

The results obtained for the 6-OMT (Fig. 28 A-D) agree partly with the patterns observed for the 3-.and 4'-OMTs. Figure 28A shows that SAH was again a competitive inhibitor when SAM was the variable substrate, whereas 3,6,7,3'-tetramethylquercetagetin (Fig. 28B) was a noncompetitive inhibitor. However, figures 28C and 28D show that both SAH and 3,6,7,3'-tetramethylquercetagetin, respectively, were uncompetitive inhibitors of 3,7,3'-trimethylquercetagetin. However, considering the poor solubility of the tetramethylated product, the results obtained should be interpreted with caution. In contrast with the 4'-OMT, which required higher SAH concentrations to observe substrate inhibition (Fig. 27C inset), the 6-OMT did so at lower concentrations of this inhibitor (Fig. 28D).
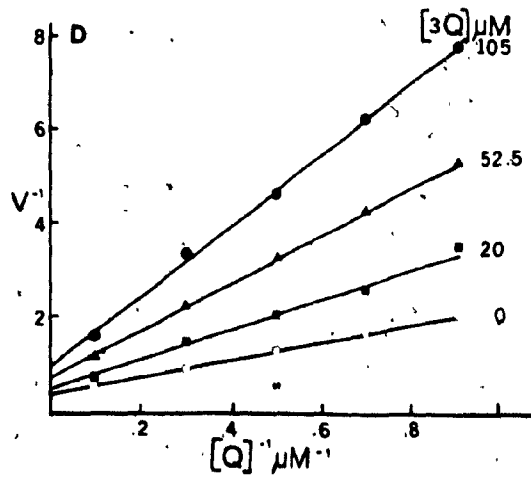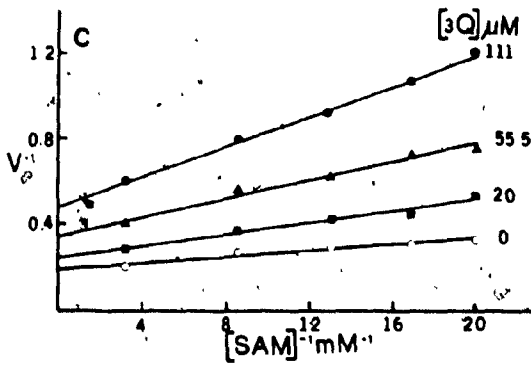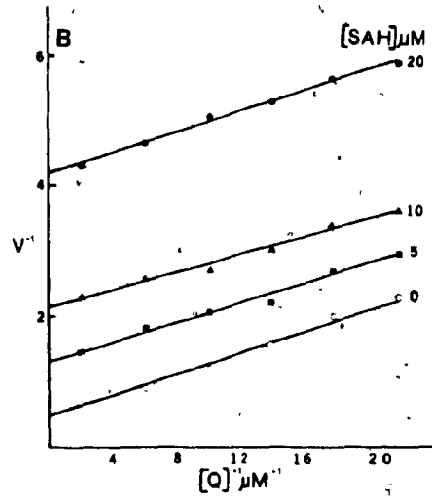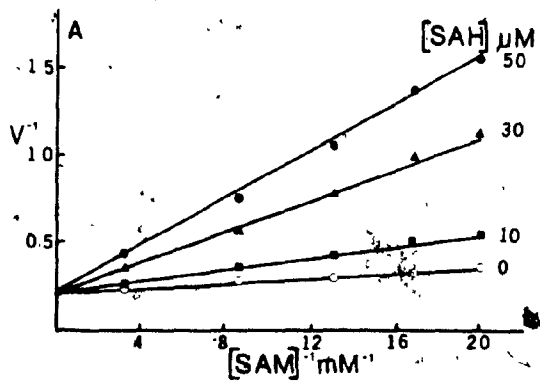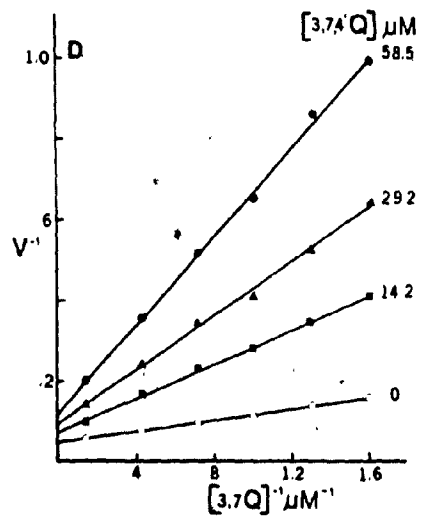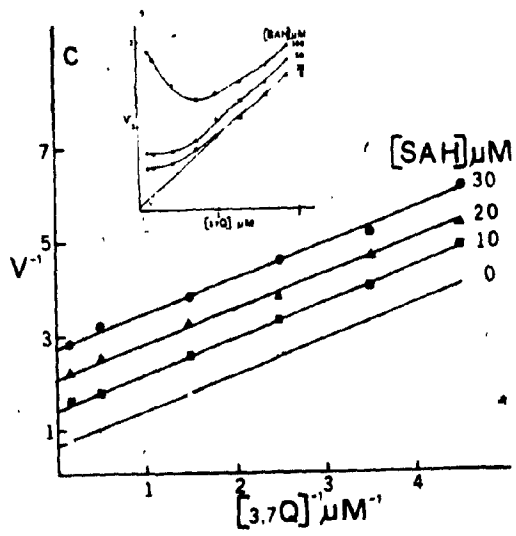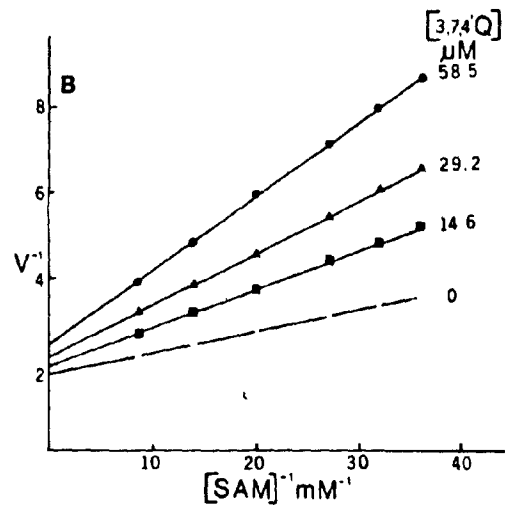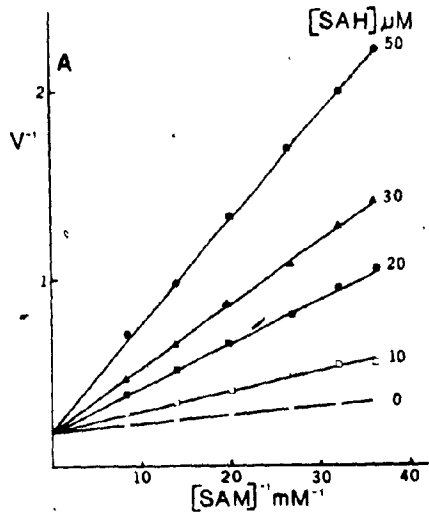
Figure 27.   Product inhibition kinetics of the 4'-OMT from

Chrysosplenium americanum

A.   Product inhibition by S-adenosyl-L-homo-

cysteine: 1/v versus 1/SAM at different fixed

concentrations of SAH and at a constant

concentration of 3,7-dimethylquercetin (3,7Q) ·

(7 µM). Other conditions were as Fig. 26A ·

except that 6 µg/ml of the 4'-OMT as used for

assays.

B.   Product inhibition by 3,7,4'-trimethylquercetin

(3,7,4'Q): 1/v versus 1/SAM at different

concentrations of 3,7,4'Q and at constant

concentration of 3,7-dimethylquercetin (7µ M)

Other conditions were as in (A) except that the

final DMSO concentration was 10%.

C & D.   Continued on next page

Figure 27.   Continued

C.   Product inhibition by S-adenosyl-L-homo-

cysteine: 1/v versus 1/3,7Q (3,7-dimethyl-

quercetin) at different fixed concentra-

tions of SAH and at constant concentration of

SAM (8.3 μM of [^{14}CH$_3$]-SAM, 0.05 μCi). Other

conditions were as in Fig. 26A. Inset, effect

of S-adenosyl-L-homocysteine on substrate

inhibition by 3,7Q: 1/v versus 1/3,7Q at

different fixed concentrations of SAH and at

constant concentration of SAM (8.3 μM of

[^{14}CH$_3$]-SAM, 0.05 μCi). Other conditions were

as in Fig. 26A.

D.   Product inhibition by 3,7,4'-trimethylquercetin

(3,7,4'Q): 1/v versus 1/3,7Q (3,7-dimethyl-

quercetin) at different fixed concentrations of

3,7,4'Q and at constant concentrations of SAM

(8.3 μM of [^{14}CH$_3$]-SAM, 0.5 μCi). Other

conditions were as in Fig. 26A, except that the

final DMSO concentrations was 10%.
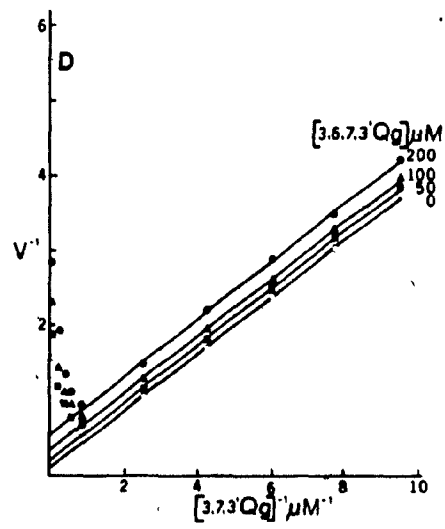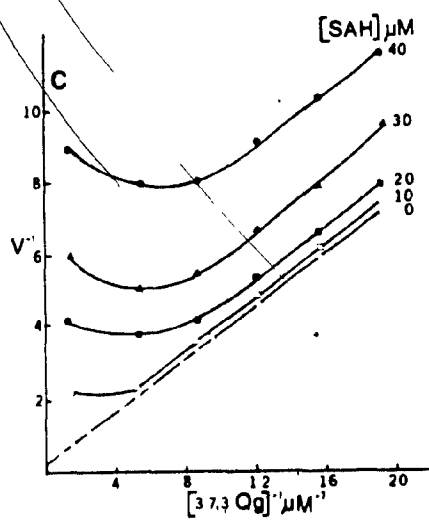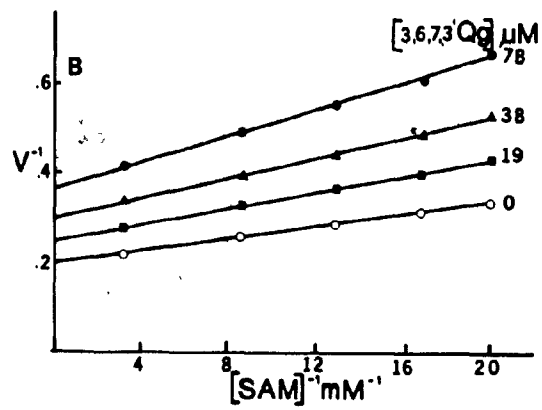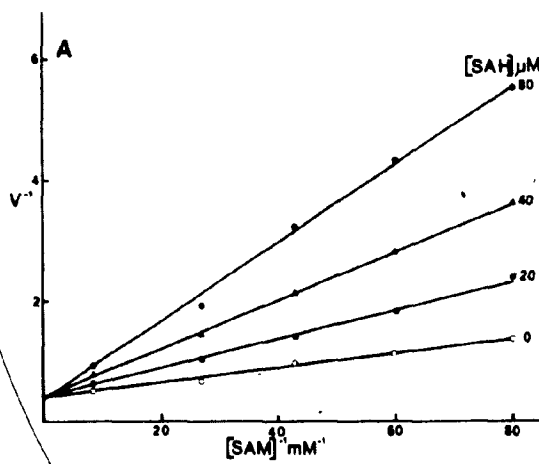
Figure 28.   Product inhibition kinetics of the 6'-OMT from

Chrysosplenium americanum

A.   Product inhibition by S-adenosyl-L-homo-

cysteine: 1/v versus 1/SAM at different fixed

concentrations of SAH and at a constant

concentration of 3,7,3'trimethyl-

quercetagetin (3,7,3'Qg) (5.8 μM). Other

conditions were as Fig. 26A except that 6 μg/ml

of the 6-OMT was used for assays.

B.   Product inhibition of the 6-OMT by 3,6,7,3'-

trimethylquercetagetin: (3,6,7,3'Qg): 1/v

versus 1/SAM at different concentrations of

3,6,7,3'Qg and at constant concentration of

3,7,3'-trimethylquercetagetin (5.6 μM). Other

conditions were as in Fig. 28A,.except that the

final DMSO concentration was 10%.

C & D.   Continued on next page

Figure 28.   Continued

C.   Product inhibition of 6-OMT by S-adenosyl-L-

homocysteine: 1/v versus 1/3,7,3'Qg

(3,7,3'-triimethylquercetagetin) at different

fixed concentrations of SAH and at constant

concentration of SAM (8.3 μM of [^{14}CH$_3$]-SAM,

0.05 μCi). Other conditions were as in Fig.

26A.

D.   Product inhibition of 6-OMT by 3,6,7,3' tetra-

methylquercetagetin (3,6,7,3'Qg): 1/v versus

1/3,7,3'Qg (3,7,3'-trimethyl-quercetagetin) at

different fixed concentrations of 3,6,7,3'Qg

and at constant concentration of SAM (8.3 μM of

[^{14}CH$_3$]-SAM, 0.5 μCi). Other conditions were

as in Fig. 26A, except that the final DMSO

concentration was 10%.

Table 17. Estimates of parameters of product inhibition kinetics
for 6-O-methyltransferase[a]

| Substrate[b] | Product | Parameter value | | | Kinetic |
		Term	µM	SD	pattern
SAM	SAH	K'_a	26	2.3	Competitive
		K_{is}	16	1.3	
SAM	3,6,7,3'-Tetra-Me-Qg	K'_a	36	2.2	Noncompetitive
		K_{is}	74	14	
		K_{ii}	81	6	
3,7,3'-Tri-Me-Qg	SAH		n.d.	n.d.	Uncompetitive
3,7,3'-Tri-Me-Qg	3,6,7,3'-Tetra-Me-Qg	K'_a	10	0.6	Uncompetitive
		K_{ii}	167	16	

[a] The parameter values were those chosen for the kinetic equation by a variance ratio test.

[b] Qg, quercetagetin

n.d., not determined

Table 18. Estimates of parameters of product inhibition kinetics for 4'-O-methyltransferase[a]

Substrate[b]	Product	Parameter value			Kinetic pattern
		Term	µM	SD	
SAM	SAH	K'_a	21	2.8	Competitive
		K_{is}	4.4	0.4	
SAM	3,7,4'-Tri-Me-Q	K'_a	27	1.2	Noncompetitive
		K_{is}	23	0.8	
		K_{ii}	140	32	
3,7-Di-Me-Q	SAH	K'_a	1.1	0.24	Uncompetitive
		K_{ii}	10	1.95	
3,7-Di-Me-Q	3,7,4'-Tri-Me-Q	K'_a	1.4	0.88	Noncompetitive
		K_{is}	3	0.33	
		K_{ii}	10	0.09	

[a] The parameter values were those chosen for the kinetic equation by a variance ratio test.

[b] Q, quercetin

D.5. Enzyme kinetics of <u>Lotus</u> 8-OMT

D.5.1. Saturation with substrate

Preliminary studies on the 8-OMT of <u>Lotus</u> established that saturation with either 8-hydroxykaempferol or SAM gave Michaelis-Menten kinetics (Fig. 29A, B), with no apparent substrate inhibition by either substrate.

D.5.2. Substrate interaction kinetics

Estimates of the parameters derived from non-linear regression analysis of the substrate interaction kinetic data are shown in Table 19. The variance ratio test indicated that the K_{ia}/K_b term was significant for the interaction between SAM and 8-hydroxykaempferol, implying that the lines of the double reciprocal plots intersect (Fig. 30A, B).

The initial velocity results are consistent with a sequential binding mechanism where both substrates must bind prior to any product release, thus excluding a ping pong mechanism. However product inhibition studies are required in order to distinguish between an ordered and a random mechanism.

D.5.3. Product inhibition kinetics

Further information on the order of substrate binding was obtained from product inhibition studies. The results obtained for the 8-OMT (Fig. 31 A-D, Table 20) indicate an ordered binding of substrates and release of products. SAH was a noncompetitive inhibitor with respect to SAM (Fig. 31A) and noncompetitive with respect to 8-hydroxykaempferol (Fig. 31B). 8-Methoxykaempferol was a
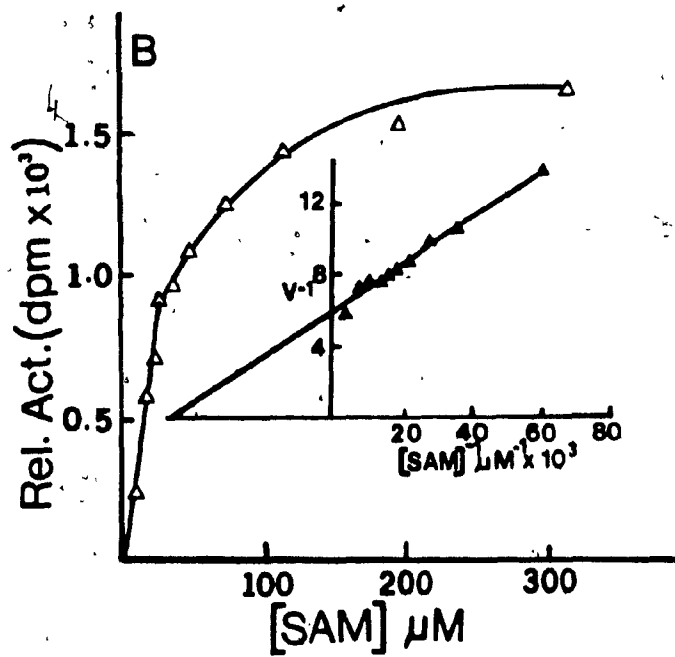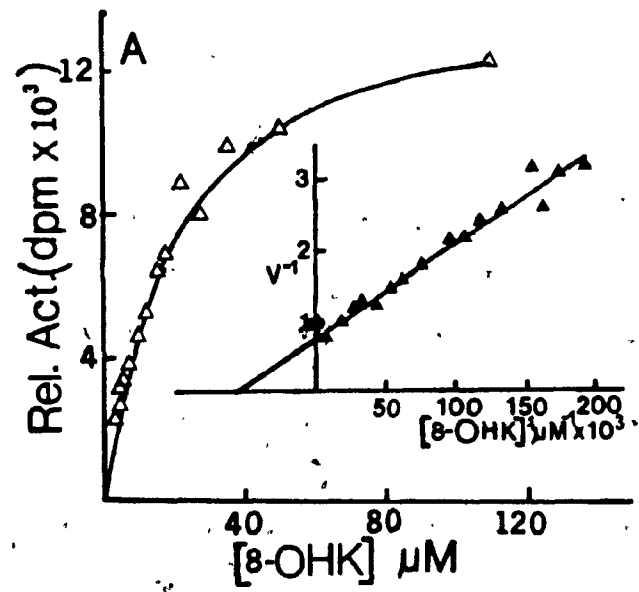
Figure 29.   Saturation with 8-hydroxykaempferol and SAM as

substrate for the 8-OMT of Lotus corniculatus.

A.   Effect of 8-hydroxykaempferol (8-OHK)

concentration on the reaction rate of 8-OMT.

The concentration of SAM was 66 µM (containing

10 µM of $[^{14}CH_3]$-SAM, 0.061 µCi).   Inset.

double reciprocal plot of 1/v versus 1/8-OHK.

Each incubation mixture contained .05 µg/ml of

8-OMT, 10 mM $MgCl_2$, 14 mM 2-mercaptoethanol and

5% DMSO in 100 mM phosphate, pH 8.

B.   Effect of SAM concentration on the reaction

rate of 8-OMT.   (16.6 µM of $[^{14}CH_3]$-SAM,

0.1 µCi, in addition to varying amounts of

unlabelled SAM).   The concentration of 8-OHK

was 10 µM.   Inset: double reciprocal plot of

1/v versus 1/SAM.   Other conditions were as in

(A).
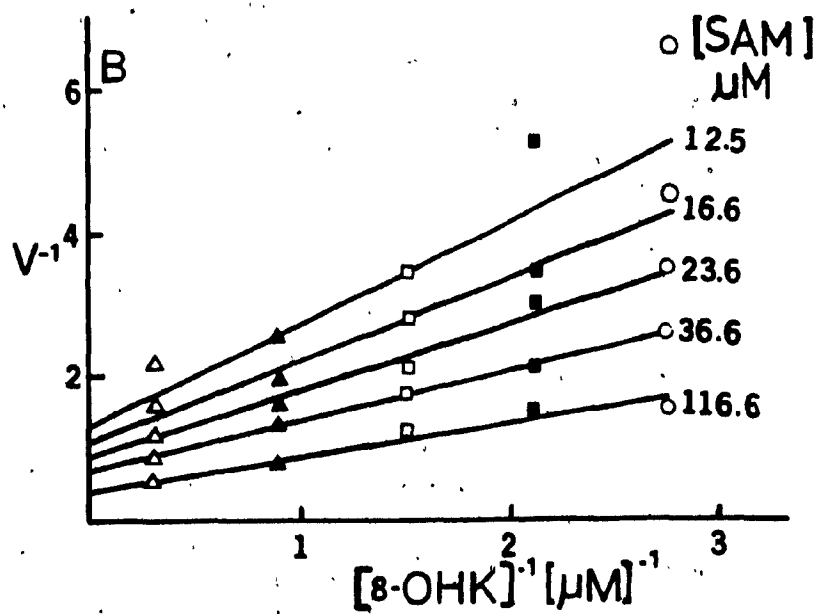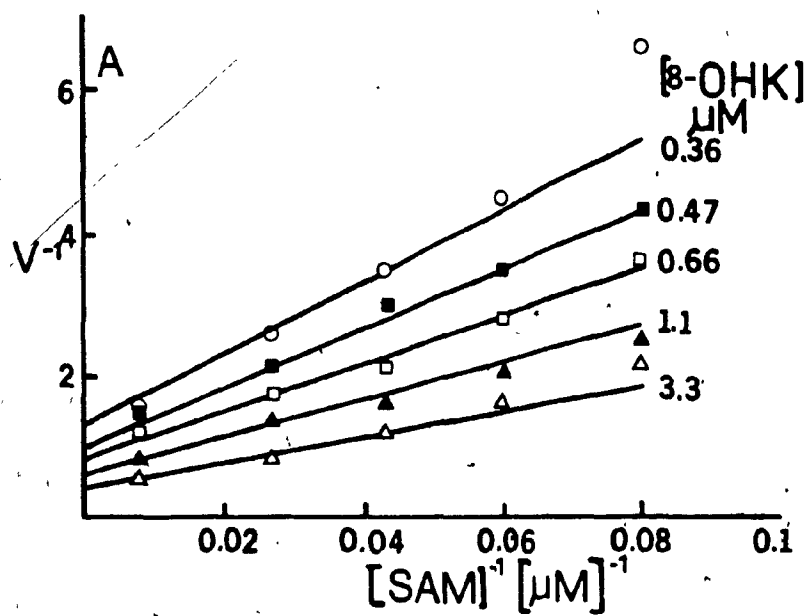
Figure 30.   Substrate interaction kinetics for the 8-OMT of

Lotus.

A.   Initial velocity. 1/v versus 1/8-hydroxy-

kaempferol (8-OHK) at various fixed

concentrations of SAM (16.6 µM of [$^{14}CH_3$]-SAM,

0.1 µCi), in addition to varying amounts of

unlabelled SAM). Each incubation mixture

contained .05 µg/ml of 8-OMT, 10 mM $MgCl_2$, 14

mM 2-mercaptoethanol   and 5% DMSO in 100 mM

phosphate buffer, pH 8. The amount of $^{14}CH_3$

labelled flavonol formed was determined after

30 min incubation at 30°C. Velocities are

expressed as µM/s/mg protein.

B.   1/v versus 1/8-OHK fixed concentrations of

SAM. The data are the same as those shown in

Fig. 29A.

Table 19. Estimates of parameters of substrate interaction kinetics
for <u>Lotus</u> 8-O-methyltransferase[a]

Substrate		Parameter value			Kinetic
A	B.	Term	μM	SD	pattern
SAM	8-Hydroxykaempferol	K_a	53	10.5	Intersecting
		K_{ia}	35	-	
		K_a/K_{ia}	1.5	-	
		K_b	1.3	0.3	
		$K_{ia}K_a$	45	6.6	
		V	3.6	0.4	

[a] The parameter values obtained were those for the kinetic equation
as chosen by the variance ratio test.

non-competitive inhibitor with respect to SAM (Fig. 31C) and competitive with respect to 8-hydroxkaempferol (Fig. 31D). Therefore, the kinetic patterns obtained (Table 20) exclude random addition of substrates and/or release of products.
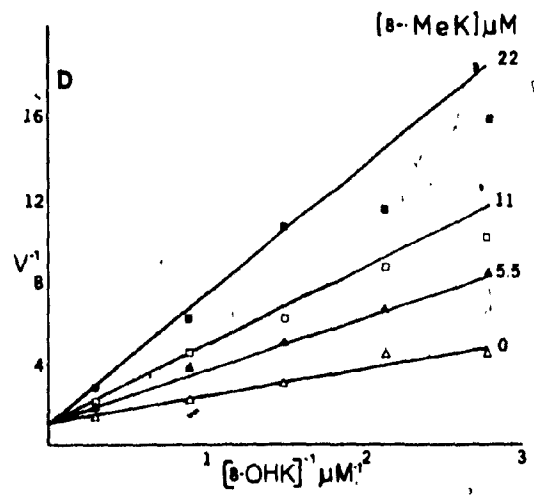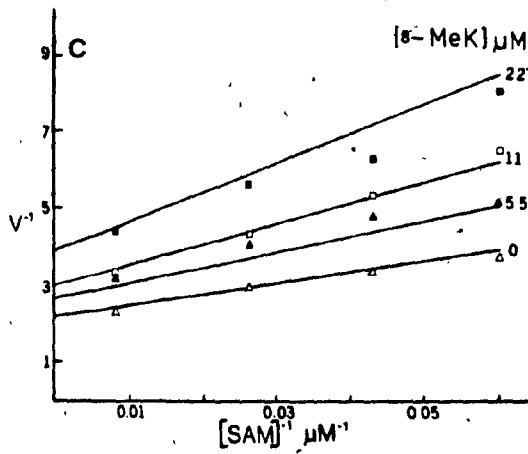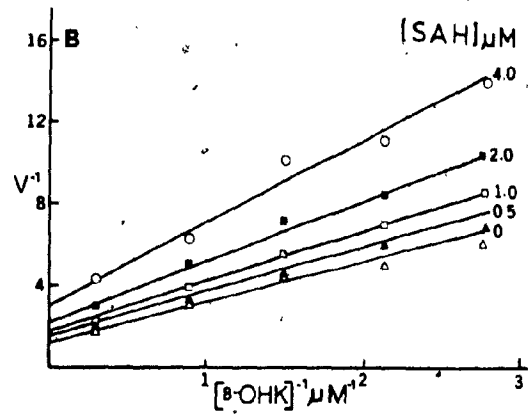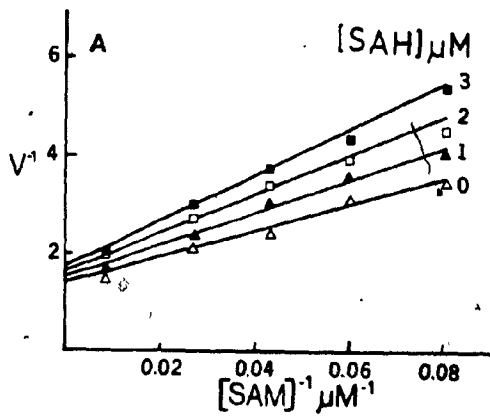
Figure 31. Product inhibition kinetics of the 8-OMT from

Lotus corniculatus.

A. Product inhibition by S-adenosyl-L-homo-
cysteine: 1/v versus 1/SAM (16.6 μM of
$[^{14}CH_3]$-SAM, 0.1 μCi, in addition to varying
amounts of unlabelled SAM) at different fixed
concentrations of SAH and at a constant
concentration of 8-hydroxykaempferol (8-OHK)
(0.66 μM). Each incubation mixture contained
.05 μg/ml of 8-OMT, 10 mM MgCl$_2$, 14 mM
2-mercaptoethanol,   5% DMSO in 100 μM
phosphate buffer, pH 8. The amount of $^{14}CH_3$
labelled·labelled flavonol formed was
determined·after 30 min incubation at 30°C.
Velocities are expressed as μM/s/mg protein.

B. Product inhibition by S-adenosyl-L-
homocysteine: 1/v versus 1/8-OHK (8-hydroxy-
kaempferol) at different fixed concentrations
of SAH and at constant concentrations of SAM
(8.3 μM of $[^{14}CH_3]$-SAM, 0.05 μCi). Other
conditions were as in (A).

C & D. Continued on next page

Figure 31.   Continued

C.   Product inhibition by 8-methoxykaempferol

(8-MeK): 1/v versus 1/SAM at different fixed

concentrations of 8-MeK and at constant

concentrations of 8-hydroxykaempferol

(0.66 μM). Other conditions were as in (A)

except for DMSO which was 10%.

D.   Product inhibition of 8-methoxykaempferol

(8-MeK): 1/v versus 1/8-OHK (8-hydroxy-

kaempferol) at different fixed concentrations

of 8-MeK and at constant concentrations of SAM

(8.3 μM, 0.05 μCi). Other conditions were as

in (A) except for DMSO which was 10%

Table 20. Estimates of parameters of product inhibition kinetics
for 8-O-methyltransferase[a]

| Substrate | Product | | Parameter value | | Kinetic |
		Term	µM	SD	pattern
SAM	SAH	K'_a	20	2.2	Noncompetitive
		K_{is}	4	1.1	
		K_{ii}	15	6.2	
SAM	8-Methoxy-kaempferol	K'_a	14	2.8	Noncompetitive
		K_{is}	14	6	
		K_{ii}	28	7.6	
8-Hydroxy-kaempferol	SAH	K'_a	1.7	0.14	Noncompetitive
		K_{is}	3.8	0.8	
		K_{ii}	3.7	0.4	
8-Hydroxy-kaempferol	8-Methoxy-kaempferol	K'_a	1.3	0.13	Competitive
		K_{is}	5.9	0.5	

[a] The parameter values were those chosen for the kinetic equation by
a variance ratio test.

E. DISCUSSION

The results presented here clearly demonstrate the existence in
C. americanum of four, novel OMTs which exhibited strict position
specificity for the 3-, 6-, 7- and 4'-hydroxyl groups of flavonols.
In addition, L. corniculatus was shown to contain a novel 8-OMT. The
fact that none of these enzymes accepted any of the phenylpropanoid
compounds tested, or other intermediates of flavonol biosynthesis
clearly indicates their expressed specificity for hydroxylated
flavonols and their partially methylated derivatives. In view of the
differential specificity of the five OMTs for their best substrates,
we wish to propose the following systematic names: S-adenosyl-
L-methionine:quercetin-3-O-methyltransferase; S-adenosyl-L-methionine:
3-methylquercetin 7-O-methyltransferase; S-adenosyl-L-methionine:
3,7,3'-trimethyl-quercetagetin 6-O-methyltransferase; S-adenosyl-
L-methionine:8-hydroxykaempferol/quercetin 8-O-methyltransferase and
S-adenosyl-L-methionine:3,7-dimethylquercetin 4'-O-methyltransferase.
Therefore, except for the 5-, 2'- and 5'-positions, the O-methylation
of all other hydroxyls on the flavonoid ring system has been
demonstrated with these two plant tissues.

Chrysosplenium OMTs are quite distinct from the much studied
flavonoid enzymes which exhibited a wide range of specificity towards
the 3'-position of flavones/flavonols or their glucosides (38-40).
The enzymes reported here mediated position-oriented methylations
which followed an orderly sequence depicted in Figure 32, thus clearly
establishing that O-methylation of flavonoids occurs at the C_{15} level
in Chrysosplenium as well as with the Lotus 8-OMT. However recent

QUERCETAGETIN (Qg)

QUERCETIN (Q)

3-MeQg

3-MeQ

3,7-MeQg

3,7-MeQ

3,7,4'-Me Qg

3,7,4'-MeQ

3,6,7,4'-Me Qg
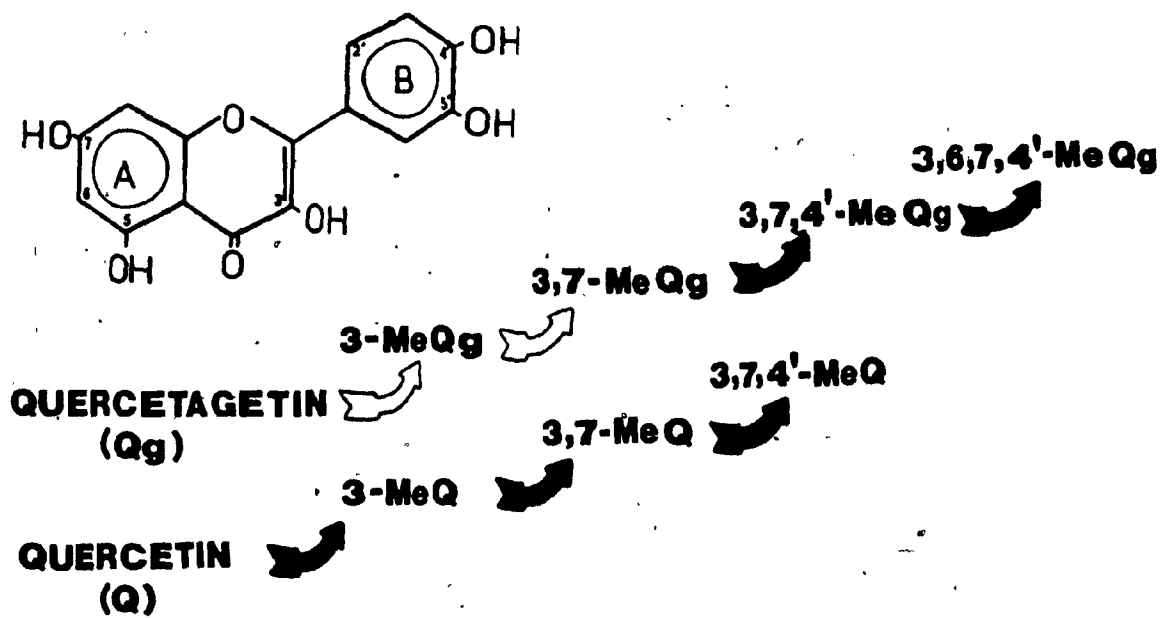
Figure 32.   Proposed pathway for the sequential

O-methylation of flavonoids in Chrysosplenium

americanum.

Q and Qg are quercetin and quercetagetin

respectively, and the numbers preceeding Q and

Qg indicate their methylated positions.

The black arrows demonstrate reactions which

have been shown in Chrysosplenium, whereas

the white arrows demonstrate potential enzyme

reactions which have not been demonstrated.

work with the anthocyanin 3'-OMT of Petunia hybrida (119) and the vitexin-2"-θ-rahmnoside 7-OMT of Avena sativa (120) demonstrated that, unlike Chrysosplenium and Lotus OMTs, these enzymes accepted only glycosylated substrates. This suggests that the order of methylation and glucosylation does not necessarily follow a set pattern, but is determined by the specific tissue involved.

The fact that feruloyl-CoA (3-methyl-4-hydroxycinnamoyl CoA) was not converted with any significant efficiency by the chalcone synthase of Chrysosplenium to the corresponding chalcone or flavanone (D. Barron, unpublished) suggests that methylation takes place at a later stage during flavonoid biosynthesis. It is not unexpected, therefore, that a polyhydroxyflavonol of common occurrence, such as quercetin, would be the natural methyl acceptor in the multiple sequence of methyl transfers in Chrysosplenium. This is supported by the fact that 3-OMT exhibited its highest affinity and strict position specificity for this substrate. These results are in agreement with the findings of Brunet and Ibrahim (48) who demonstrated that a number of 3-hydroxyflavones displayed a high methyl acceptor ability which correlated with the highest nuclear electron density for the 3-hydroxyl group of flavonols.

Quercetagetin, the 6-hydroxy derivative of quercetin, was neither accepted for O-methylation nor did it act as a substrate inhibitor. This raises the question as to how do methylated derivatives of quercetagetin (Fig. 13) arise in Chrysosplenium. Two possible routes may be postulated. The first is that quercetin is methylated at position 3 with subsequent divergence of the pathway into

polymethylated derivatives of quercetin or quercetagetin. Such a
hypothesis implies the introduction of a hydroxyl group at position 6
of 3-methylquercetin, or of a later methylated intermediate. The
second possibility is that two specific 3-OMTs may be involved, one
for quercetin and the other for quercetagetin. However, the latter
enzyme has yet to be found in this tissue.

It is interesting to note that neither quercetagetin nor its
3,7-dimethyl- or 3,7,3'-trimethyl derivatives were accepted as
substrates by Lotus 8-OMT (Tables 12, 14) whereas the 6-OMT of
Chrysosplenium did methylate the 8-position (being meta to the side
chain) of 8-hydroxykaempferol. The fact that quercetagetin was not
accepted for O-methylation by the 3-, 6- or 8-OMTs, indicates that
hydroxylation of position 6 may well take place after quercetin has
undergone partial methylation, contrary to earlier proposals (14,
121). Furthermore, the same hypothesis may be true for the
hydroxylation at the 2'-position of Chrysosplenium flavonoids
(Fig. 3), since any change in the substitution of ring B of quercetin
resulted in almost complete loss of activity (Tables 4 & 6). The
validity of the latter hypothesis could be tested if a sample of
2'-hydroxyquercetin was available for use as a substrate.

Different OMTs were shown to occur in Lotus flowers (Fig. 19B) as
compared with shoot tips (Fig. 20B). 8-OMT activity was found
primarily in the flowering stage which coincided with the presence of
8-methoxyquercetin, 8-methoxykaempferol and 8-methoxyisorhamnetin in
this tissue (106). Furthermore, the vegetative parts of Lotus
contained only the methylated product isorhamnetin (106) and displayed

low 8-OMT activity as compared with the quercetin methylating activities (3- and 3'-OMTs) (Fig. 20B). These results indicate a tissue-specific control of OMT expression in Lotus as a function of morphological differentiation. Therefore, the 8-OMT is expressed during flowering for the production of 8-0-methylated flavonols that are responsible for the intense yellow color of flower petals in this species (106).

The five OMTs reported here had pH optima similar to those reported for other flavonoid-specific enzymes (38-44, 119, 120), whereas their molecular weights were intermediate between those of the flavone/flavonol (38-40) and the isoflavone OMTs (41). The 3-, and 4'-OMTs displayed no Mg^{2+} requirement and were not inhibited by EDTA. Similar results were reported for the isoflavone 4'-OMT of Cicer arietinum (41) and the vitexin-2"-O-rahmnoside 7-OMT of Avena (120). On the other hand, the 6- and 8-OMTs showed absolute requirement for Mg^{2+} and their activity was inhibited by EDTA. Similar results were reported for the 3'-OMT of parsley cell suspension cultures (38). Very recently, two distinct N-methyltransferases involved in the stepwise methylation of phosphatidylethanolamine to phosphatidyl-choline were isolated from rat liver microsomes. The first enzyme had an absolute requirement for Mg^{2+} ions and was shown to be limiting the rate of synthesis of these phospholipids (49). It is not certain whether the absolute requirement for Mg^{2+} by the 6- and 8-OMTs has any significance in the regulation of O-methylation in Lotus and Chrysosplenium.

Preliminary studies on the subcellular localisation of OMTs and GT in Chrysosplenium (Fig. 18) seem to indicate that none of these

enzymes could be localised within any of the cell organelles.

Instead, these reactions seem to take place in the cytosol. Similarly,
it was postulated that the enzymes of the latter part of the pathway
of flavonol synthesis (70, 71) as well as methionine synthase and
methionine adenosyltransferase (65), the enzymes catalysing the
synthesis of SAM, were localised in the cytoplasm. These results are
in sharp contrast with those of Charrière-Ladreix et al. (72) who
claimed that the stepwise methylation of flavonols takes place on the
chloroplast membrane. The cytoplasmic localisation of the anthocyanin
3'-OMT from the flowers of Petunia hybrida (122) and the demonstration
that ultracentrifugation caused non-specific adhesion of this OMT to
membranes, confirm that flavonol OMTs are probably not associated with
organelles such as mitochondria or chloroplasts.

A cytoplasmic localisation of OMTs and GT raises the question as
to the assembly and accumulation of the final products in
C. americanum. Many of the partially methylated intermediates and end
products of this pathway are mostly lipophilic and thus, sparingly
soluble in the aqueous environment of the cytoplasm. Therefore, both
intermediates and end-products could be compartmented within a loose
protein aggregate where these intermediates could be channeled rapidly
through the biosynthetic sequence (123), without coming in contact
with the cytoplasmic pool. Furthermore, it may be suggested that the
enzymes of flavonol synthesis are associated with a membrane system
where the final hydrophobic products could be sequestered and/or
shuttled towards the site of accumulation. The fact that
polymethylated flavonol glucosides of Chrysosplenium were found on top

of sucrose gradients (Fig. 18), which may contain cytoplasmic as well as vacuolar contents, seems to indicate that the plant vacuole could be the site of accumulation of these compounds. This hypothesis is supported by the fact that a number of secondary products of plant metabolism accumulate in the central vacuole of the plant cell (122, 124, 125). However, a more rigorous approach is required to provide conclusive evidence for the localisation of these enzymes and their methylated end-products in this tissue.

The purification procedures utilized for the OMTs of both Chrysosplenium and Lotus were successful in separating a number of flavonoid-specific OMTs. The isolation of these OMTs would not have been achieved without the use of chromatofocusing on Polybuffer ion exchanger (Fig. 14 & 25C). Furthermore, the availability of specific flavonoid substrates made it possible to purify these enzymes and study their substrate specificity. These facts may explain why such enzymes could not be studied previously.

It should be noted that purified Chrysosplenium OMT preparations were extensively contaminated with other proteins (Appendix 1, Fig. 3A). This problem is related to the fact that, unlike the enzymes of primary metabolism, those of secondary metabolism are low in abundance. Furthermore, the amount of protein found in plants is extremely low as compared with that in animal tissues. Gulliver and Tipton (126) isolated 55 mg of pure catechol OMT (with a 25% yield) from 1 Kg of porcine liver containing 700 g of protein. Assuming that the abundance of Chrysosplenium OMTs was similar to that of the porcine liver, then 72 Kg of plant material would be required to

isolate similar amounts of flavonoid OMTs from Chrysosplenium! This comparison illustrates one of the many difficulties involved in obtaining purified enzymes from plant materials.

The 3-, 6-, and 4'-OMTs of C. americanum exhibited similar steady state kinetic characteristics. One noticeable difference with respect to substrate inhibition was observed, namely that the 6-OMT was severely inhibited by substrate concentrations close to. K_m whereas the other two enzymes, though inhibited, were less affected by their respective flavonoid substrates. In view of the substrate inhibition observed with these three enzymes, substrate interaction kinetics were carried out at flavonoid substrate concentrations below K_m.

Substrate interaction kinetics were performed with the 3-, 4'- and 6-OMTs. For the 6-OMT, both the computer programme (Table 15) and the plotting of data by the method of least squares (Fig. 24C) indicated converging Lineweaver-Burke plots, suggesting a sequential reaction mechanism. For the 3- and 4'-OMTs, the computer programme indicated that the equation for a sequential mechanism (equation I) did not give a significantly better fit than the equation for a ping pong mechanism (equation II) (Appendix II, Table VI), whereas the data plotted by the method of least squares gave clearly converging lines (Fig. 24A, B). Therefore, the data suggest either a ping pong mechanism giving a series of parallel lines or a sequential mechanism giving a series of quasi-parallel lines (79). As stated in the results, the degree of convergence will depend on the value of K_{ia}, the dissociation constants for the first substrate to bind. Assuming

that SAM was the first substrate to bind, the apparent dissociation

constants (K_{ia}) for the enzyme-SAM complex were calculated from the

values of $K_{ia} K_b$ and K_b given in Table 15. It is remarkable that the

values so obtained were similar for each OMT, and might be identical

given that K_{ia} was derived from kinetic constants with standard

deviations close to 20%. Assuming an average K_{ia} value of

8 µM for all three OMTs (as calculated from Table 15), this value

would be 14, 16 and 6 times smaller than K_a for the 3-, 4'- and

6-OMTs, respectively. The fact that the K_{ia} values were small

relative to K_a may explain why the computer programme could not better

fit the data for the 3- and 4'-OMTs to equation I than II.

Since the substrate interaction kinetics provided equivocal

results for two of the three Chrysosplenium OMTs, more information was

obtained from product inhibition studies. In a ping pong mechanism,

SAM must bind first and SAH be released prior to binding of flavonol

substrate. Therefore, a methylated enzyme intermediate is produced in

which case SAM and SAH will bind to different forms of the enzyme that

are reversibly connected. Slope and intercept effects would be

expected resulting in noncompetitive inhibition between SAM and SAH.

However, the inhibitions observed for the 3-, 6- and 4'-OMTs were

competitive (Fig. 26A, 27A & 28A). Similarly, the inhibition by the

flavonol product with SAM as variable substrate should be competitive

for a ping pong mechanism, whereas the inhibition observed for the

three enzymes was noncompetitive (Fig. 26C, 27B & 28B). Furthermore,

the fact that three out of four inhibition patterns could not be

fitted to those expected for a ping pong mechanism suggests that the

3-, 6- and 4'-OMTs followed a sequential binding mechanism where both substrates had to bind prior to any product release.

Assuming that the mechanism for the 3-, 6- and 4'- OMTs of C. americanum is sequential, the binding of substrates and the release of products could be random or ordered. In a random mechanism four competitive product inhibition patterns would be expected (79); except when dead-end inhibition occurs, alternative patterns are possible. Coward et al. (34), studying rat liver catechol-OMT, proposed that this reaction proceeds by random binding of substrates and products with the formation of a dead-end complex between SAM and the methylated products, metanephrine or paranephrine. This mechanism was proposed because these two products were noncompetitive inhibitors against SAM as the variable substrate, rather than giving the expected competitive inhibition. However, SAH was a competitive inhibitor against either SAM or epinephrine (methyl acceptor) as the variable substrate and suggested that this inhibitor was competing with both substrates for the same enzyme form. The results obtained from our studies with the 3-, 6- and 4'-OMTs indicated that competitive inhibition was observed only between SAM and SAH (Fig. 26A, 27A and 28A), whereas uncompetitive inhibition was observed between the flavonoid substrate and SAH (Fig. 26B, 27C & 28C). Therefore, most product inhibition patterns obtained for Chrysosplenium OMTs could not be fitted to those expected for a random mechanism.

In an ordered mechanism one competitive and three noncompetitive product inhibition patterns would be expected (79). In the case of the 3- and 4'-OMTs of Chrysosplenium, three out of four kinetic

patterns obtained fit this mechanism whereas in the case of the 6-OMT, two out of four patterns are consistent with ordered binding of substrates and release of products. The 'simplest' mechanism that fits the kinetic data for substrate interaction and product inhibition of C. americanum OMTs is that shown in Figure 33. In this reaction sequence, A represents SAM; B, the flavonoid substrate; P, the flavonoid product and Q, SAH. All three enzymes exhibited the common kinetic behavior that SAH was a competitive inhibitor of SAM. This observation is consistent with the hypothesis that SAM and SAH are leading reaction partners in the catalytic cycle, with the flavonoid substrate or product binding only when the SAM-SAH site is occupied. Similarly, the other data would fit this reaction mechanism if the effect of substrate inhibition was taken into account.

At high concentrations, substrates will often act as dead-end inhibitors particularly when the reaction is being studied in the non-physiological direction (117, 118). We found that each of these enzymes was inhibited by its respective flavonoid substrate at concentrations near K_m values and occurring in the physiological direction (Fig. 23). Furthermore, inhibition by the flavonoid substrate at concentrations below K_m was observed when SAH was added to enzyme assays (Fig. 27C inset and Fig. 28C). Thus, in two cases (6- and 4'-OMTs) the abortive complex EQB could be distinguished kinetically and might explain why SAH was an uncompetitive inhibitor when flavonoid was the variable substrate (Fig. 26B, 27C &28C). Uncompetitive substrate inhibition is characteristic of ordered systems and occurs when B reacts in dead-end fashion with EQ (Fig. 33) (79, page 824).
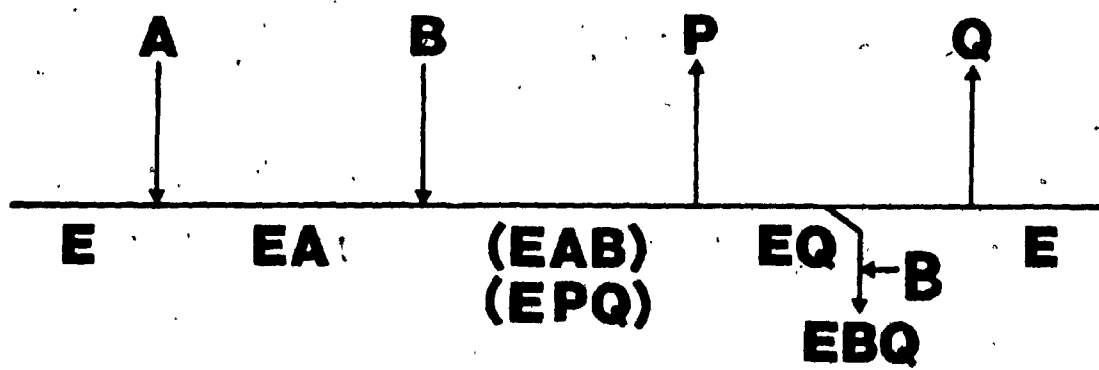
Figure 33.   Proposed kinetic mechanism for the 3-, 6- and

4'-OMTs of Chrysosplenium americanum: A,

S-adenosyl-L-methionine; B, flavonol substrate;

P, methylated flavonol product; Q, S-adenosyl-

L-homocysteine.

A dead-end inhibitor will affect the slope of the double reciprocal plot if (a) it and the varied substrate combine with the same enzyme form or (b) it combines before the varied substrate with an enzyme form that is reversibly connected to the form combining with the varied substrate (79). A dead-end inhibitor affects the intercept of a reciprocal plot if it and the varied substrate combine with different enzyme forms (79). In the case of Chrysosplenium OMTs, the flavonol (B, Fig. 33) would act both as a substrate and as a dead-end inhibitor; the substrate combines with E-SAM (EA) and the dead-end inhibitor combines with E-SAH (EQ). Furthermore, the reversible connection between these two enzyme forms is broken since there is a product release step (P) and free E is removed to form E-SAH-Flavonol. Therefore, inhibition by SAH against flavonol as variable substrate gives only the intercept effects seen in Figures 26B, 27C and 28C. All other inhibitions were noncompetitive (Figures 26C, D; 27B, D; 28B) except for the 6-OMT where the flavonol product was uncompetitive with the flavonol substrate (28D). The latter inhibition is difficult to explain in terms of an ordered binding mechanism, since an abortive EAP or EP complex would give noncompetitive inhibition patterns. However, as stated before, the problems encountered with the solubility of the tetramethylated product may limit the value of the latter results (Fig. 28D). Given the overall similarity in inhibition patterns for the three enzymes, it is reasonable to suggest that they follow the same reaction sequence (Fig. 33).

Similar substrate interaction and product inhibition kinetics
were observed for the five charge isozymes of rabbit adrenal glands
(76, 77) which catalyze the N-methylation of norepinephrine. Several
isozymes gave quasi-parallel double reciprocal plots when the
interaction of substrates was investigated, and in the case of isozyme
3, SAH was an uncompetitive inhibitor of norepinephrine. The only
characteristic that was common to all isozymes was the competitive
inhibition observed between SAM and SAH. Therefore, a model similar
to that shown in Figure 33 was proposed which also included a number
of abortive complexes.

Substrate inhibition which does not normally occur at
physiological concentrations may be of interest in that it may set
upper limits to the in vivo levels of these flavonoid substrates. For
example, the different sensitivities of muscle and heart isozymes of
lactate dehydrogenase to substrate inhibition by pyruvate reflect the
different pyruvate levels in these two organs (127, 128). Similarly,
the five norepinephrine N-methyltransferase isozymes of rabbit adrenal
glands had different sensitivities to substrate inhibition and were
postulated to appear at various stages of growth of the animal
depending on the requirements for epinephrine (76, 77). The fact that
Chrysosplenium OMTs were inhibited by concentrations above 10 µM of
the flavonoid substrate indicates that their in vivo levels may be
substantially lower than this value. Furthermore, the low levels of
SAH effective in enhancing inhibition of flavonoid substrate might be
physiologically significant. Alternatively, these data may indicate a
very tight control of the levels of all substrates and products which
would serve to regulate the methylation sequence in this plant.

The kinetic parameters (Table 15) showed that the three enzymes displayed similar affinities for their respective flavonoid substrates as indicated by their apparent K_b values. However, the affinity for SAM (K_a) was similar for the 3- and 4'-OMTs whereas it was at least 2 times greater for the 6-OMT. The numerical value of K_m is of interest (79, page 34) since this may establish a measure for the intracellular level of the substrate. For example, if the intracellular concentration of SAM was much smaller than K_m, then the reaction rate would be very sensitive to changes in total SAM concentration, but most of the catalytic potential of the enzyme would be wasted since v would be much smaller than V_{max}. Previous studies with plant tissues (55, 56) have established the levels of SAM at 16 µM whereas in animal tissues (53) this level was 40 µM. Assuming that Chrysosplenium contained levels of SAM corresponding to 16 µM, that V_{max} was similar for all three enzymes and that the levels of other substrates were saturating, then the in vivo velocity for the 3-, 4' and 6-OMTs would be 12, 11 and 24% of V_{max}, respectively. These calculations reflect the higher affinity of the 6-OMT for SAM and suggest that these enzymes would only be operating at a fraction of their maximal efficiencies under such conditions while being very sensitive to changes in SAM concentration. Such predictions, however, may not take into account other known or unknown variables which may affect the rates of these enzyme-catalysed reactions. It would be of interest to determine the actual concentrations of substrates and products which occur in Chrysosplenium in order to better evaluate the former predictions.

Whereas the 3-, 6- and 4'-OMTs of Chrysosplenium showed similar types of product inhibition, there were marked differences in the magnitude of their apparent inhibition constants (Table 16-18). These observations could provide further support for the view that the reactants, both substrates and intermediate products, of the OMT sequence play an important role in the regulation of each enzyme and thus, the whole pathway.

Poulton et al. (54) suggested that transmethylation reactions in plants might be controlled by the intracellular SAM/SAH ratio and Cantoni (53) found that some methyltransferases were more sensitive to this ratio than others. It can be seen in Table 21 that when the K_m for SAM is high relative to the apparent K_i for SAH, as demonstrated for the 3- and 4'-OMTs of Chrysosplenium, the percent relative activity decreases. However, when the K_m for SAM approaches the apparent K_i for SAH (6-OMT) the percent relative activity increases. Therefore, the 3- and 4'-OMTs were more sensitive to inhibition than the 6-OMT. These characteristics suggest that OMTs earlier in the sequence of O-methylation may regulate the rate of O-methylation in Chrysosplenium. It is interesting to note that the isoflavone 4'-OMT from Cicer (41), the two anthocyanin 3'/5'-OMTs from Petunia (129) and the caffeic OMT from soyabean (130), which catalyse final methylation steps, showed a behavior similar to that of the 6-OMT rather than the 3- or 4'-OMTs (Table 21). Whether these findings will correlate with the behavior of these enzymes in vivo remains to be established. However, we believe that the Chrysosplenium system demonstrates how the SAM/SAH ratio could serve to regulate the overall pathway of methylation in this tissue.

Table 21. Effect of SAM/SAH ratio on O-methyltransferase activity

Enzyme[c]	Source	Kinetic pattern[d]	K_m SAM (µM)	K_i SAH	K_m/K_i	% Relative activity	
						4[a]	1.5[b]
3-OMT	C. americanum	C[e]	114	4.5	25.3	24.6	18.6
4'-OMT	C. americanum	C	130	4.4	29.5	23.1	17.7
6-OMT	C. americanum	C	51	16	3.2	65	51.7
4'-OMT	Cicer arietinum	C	160	30	5.3	65.2	58.5
3'/5'-OMT	Petunia hybrida	C	50	70	0.7	89	82
3'/5'-OMT	Petunia hybrida	C	95	70	1.35	85	79
Caffeic OMT	Glycine max	C	15	6.9	2.2	68	49
8-OMT[f]	L. corniculatus	NC[g]	53	4	13.2	13	13
7-OMT	Avena sativa	NC	1.6	2.5	0.64	8.6	8.6

[a] SAM 42.4 µM, SAH 10.6 µM (55)

[b] SAM 16 µM, SAH 10.6 µM (55)

[c] The enzymes from C. americanum were: quercetin 3-OMT, 3,7-dimethyl quercetin 4'-OMT, 3,7,3'-trimethylquercetagetin 6-OMT; C. arietinum, isoflavone 4'-OMT (41); P. hybrida, anthocyanin 3'/5'-OMT (129);

See following page for footnotes b-g

Footnotes to Table 21 (contd.)

c contd. <u>G</u>. <u>max</u>, caffeic acid 3-OMT (130); <u>L</u>. <u>corniculatus</u>, 8-hydroxy-
kaempferol/8-hydroxyquercetin 8-OMT; <u>A</u>. <u>sativa</u>, vitexin-2"-0-rhamnoside
7-OMT (120).

d C, competitive; NC, noncompetitive

e When the kinetic pattern for product inhibition was competitive the
following equation was used to determine % relative activity :

$$\% \text{ Inhibition} = \frac{[I]}{[I] + K_i(1+\frac{[S]}{K_m})} \times 100$$

f Assuming that SAM and SAH are leading reaction partners (Fig. 34b).

g When the kinetic pattern for product inhibition was noncompetitive,
the following equation was used to determine the percentage
relative activity:

$$\% \text{ Inhibition} = \frac{[I]}{K_i + [I]}$$

In both e and g the % rel. activity was obtained from 100% minus
% inhibition.

Also, the effects of other products has not been considered in this
determination.

Our studies with Chrysosplenium OMTs indicate that flavonoid products were good inhibitors of the reaction (Table 16-18). In general, flavonoid product concentrations in the same range of K_m for flavonol were required to inhibit the reaction (Fig. 26D, 27D & 28D). It is interesting to note that the Michaelis constant for the glucosylation of the flavonoid substrate (104), the last step in the biosynthesis of polymethylated flavonols, was in the same concentration range (10 μM) as those found for the OMTs studied here. On the other hand, the apparent inhibition constant for the glucosylated product was in the mM range (104) indicating that glucosylation was not inhibited by high concentrations of products formed. The fact that mono- and dimethylated flavonol intermediates do not accumulate in vivo (102), suggest that only catalytic amounts of each intermediate would be synthesized for its utilization by the next enzyme in the biosynthetic sequence, until final products are formed. Therefore, inhibition by flavonol product may not be important in regulation of methylation if methylated intermediates do not accumulate.

The product inhibition patterns which were observed for the 8-OMT from L. corniculatus could be expected for a steady-state ordered bi bi mechanism with 8-hydroxykaempferol binding before SAM, followed by the release of SAH and 8-methoxykaempferol (Fig. 34a). An alternative mechanism which may also fit the data is the mono-iso Theorell-Chance mechanism with the inverse binding sequence proposed above and an isomerization of the free enzyme (92) (Fig. 34b). The latter hypothesis is attractive in that SAM and SAH would be leading reaction
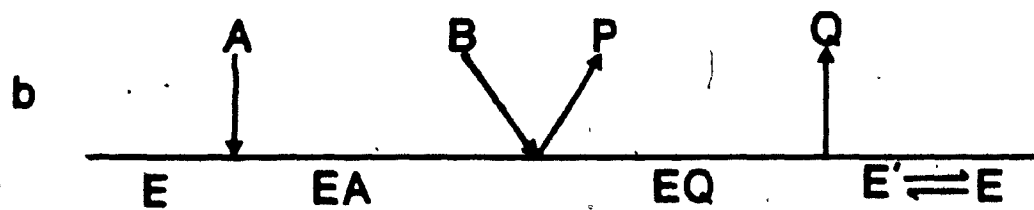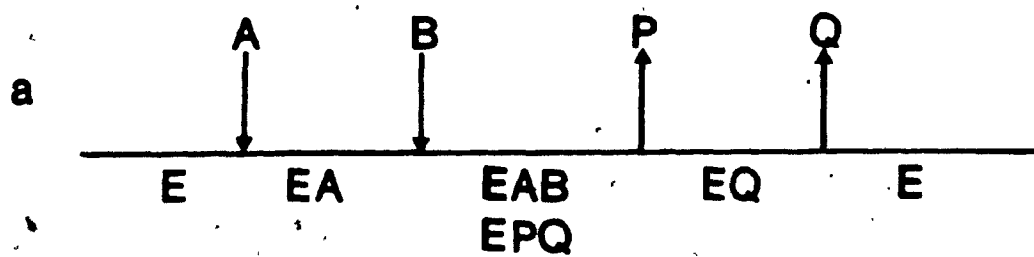
Figure 34.   Proposed kinetic mechanisms for the 8-OMT of
             Lotus _corniculatus_

A.   A, 8-hydroxykaempferol; B, S-adenosyl-L-
     methionine; P, S-adenosyl-L-homocysteine; Q,
     8-methoxykaempferol.

B.   Order of substrate binding and release of
     products is the inverse of (A).

partners, as was found in most other methyltransferase systems studied (41, 51, 77, 78). However, a distinction between both mechanisms can only be obtained by binding studies.

Very recently, a vitexin 2"-O-rhamnoside 7-OMT from Avena sativa (oat) was purified and characterized kinetically (120). The kinetic patterns generated for substrate interaction and product inhibition were identical to those of the Lotus 8-OMT. The authors favored the mechanism shown in Figure 34b, based on the efficient binding of the Avena enzyme to SAH-Sepharose. Unlike the 7-OMT from oat which was eluted from SAH-Sepharose only in the presence of SAM, the Lotus 8-OMT did not bind as tightly to SAH-Agarose (Fig. 21B) and was eluted from this column with 0-1M NaCl gradient. Furthermore, both enzymes were inhibited noncompetitively by SAH when SAM was the variable substrate and competitively by the flavonoid product when the flavonoid was the variable substrate. Therefore, these two enzymes differ from those of Chrysosplenium as well as other OMTs (Table 21) since the inhibition between SAM and SAH for the latter was competitive.

It is interesting to note that the degree of inhibition caused by a competitive inhibitor depends on the concentration of the substrate, that of the inhibitor, the K_m and the K_i. However, in the case of a noncompetitive inhibitor the degree of inhibition depends only upon the concentration of inhibitor and the K_i. Therefore, in the latter case, when the concentration of inhibitor is equal to K_i, 50% inhibition will be observed at all substrate concentrations. Thus, the 7-OMT of Avena and the 8-OMT of Lotus require that the level of SAH be kept very low for the continuation of these reactions (Table

21). On the other hand, the 3-, 6- and 4'-OMTs of Chrysosplenium were variably active at SAH concentrations similar in magnitude to those of SAM. These examples illustrate how the mechanism of the reaction could determine the requirements for regulation of different methyltransferases. Therefore, the regulation of methyltransferase activity may not always be controlled by the intracellular SAM/SAH ratio, but by the concentration of SAH only.

The kinetic data obtained for the four OMTs reported here cannot, however exclude other uncommon reaction mechanisms. For example, a covalent enzyme-methylated intermediate cannot be totally excluded. If the methyl group from SAM was transferred to the enzyme, but SAH could not dissociate before the binding of flavonoid substrate, sequential kinetics are obtained (131).

The steric course of the one-carbon transfer to nucleophilic O, N, S or C atoms of different acceptor molecules has been investigated for several SAM-dependent methyltransferases. All transfer reactions examined so far resulted in an inversion of configuration of the transferred methyl group. Therefore, a direct transfer of the methyl group from SAM to the acceptor substrate via an SN_2-type mechanism appears to be involved (81-86). Based on the results from binding studies with different affinity gels, the ordered mechanism was also postulated for three OMTs from cell cultures of Ruta graveolens; two different furanocoumarin OMTs and a caffeic OMT, respectively (51).

It is tempting to postulate that the synthesis of polymethylated flavonols in Chrysosplenium may occur on the surface of a protein aggregate (123). It is evident from our work that if such an

aggregate exists _in vivo_, the various components appear to be loosely associated, unlike the membrane-bound pathway of cyanogenic glucoside biosynthesis (132) or the multifunctional protein of the arom complex (133). One of the problems associated with the latter systems (132, 133) was the difficulty in studying the individual enzyme reactions involved. In contrast, we have not been able to isolate a protein aggregate catalysing the sequence of methylation in this tissue. Whereas the results reported here do not establish the existence of an OMT aggregate in _Chrysosplenium_, however, the following lines of evidence tend to support this concept: (a) incorporation of ^{14}C-cinnamate into end-products after 5-10 min pulse, without labelling of low methylated intermediates, (b) sequential multistep methylation of quercetin at positions 3, 7 and 4' by cell free extracts, (c) absence of mono- and dimethylated intermediates among the products which accumulate _in vivo_, (d) similarity of the kinetic mechanism of the enzymes studied, (e) regulation of all enzymes studied by a very specific range of substrate and product concentrations as shown by kinetic constants, (f) the presence of inhibition by flavonoid substrate in the three OMTs studied. This indirect evidence, however, does not unequivocally demonstrate the existence of a protein aggregate, and presents a major challenge to be investigated in future research on the OMT system of _Chrysosplenium_.

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G. APPENDIX

G.1. Appendix I: Miscellaneous figures.

G.2. Appendix II: Statistical Tables.

G.3. Appendix III: Perspectives for future work.
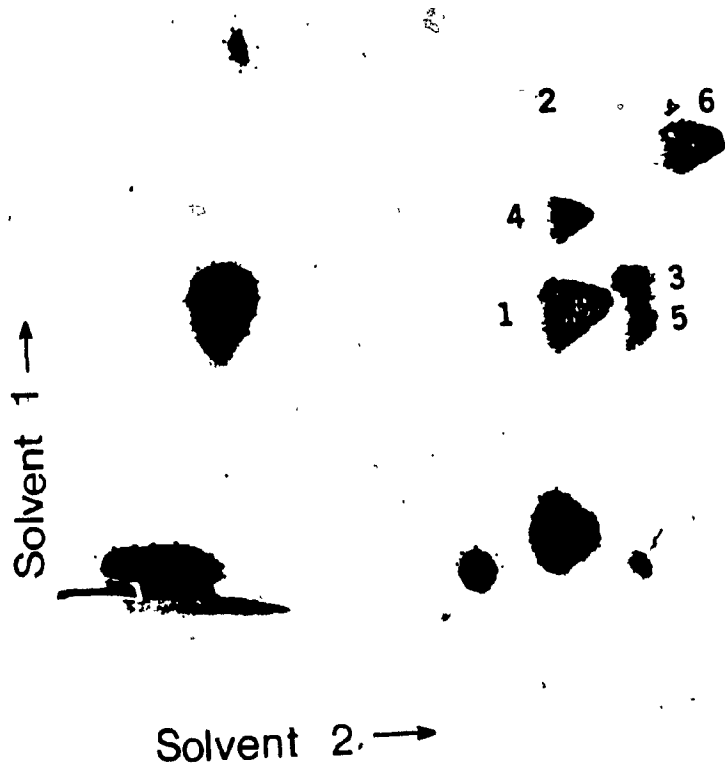
Appendix I.

Figure 1.   Incorporation of 2-^{14}C- cinnamic acid into

methylated flavonol glucosides of <u>Chrysosplenium</u>.

The labelled products are numbered as in figure 3.

It should be noted that except for number 2, all

the flavonols which accumulate in this tissue

were readily labelled. The other major spots

of radioactivity do not correspond to flavonoids

· and remain to be identified.

The reaction products were chromatographed on

Polyamide 6-MN TLC. plates in two directions:

1. Toluene-ehtyl formate-ethanol-water (60:20:19:1)

2. Water-n-butanol-acetone-dioxane (70:15:10:5)

(102) followed by autoradiography.

M   Q   R
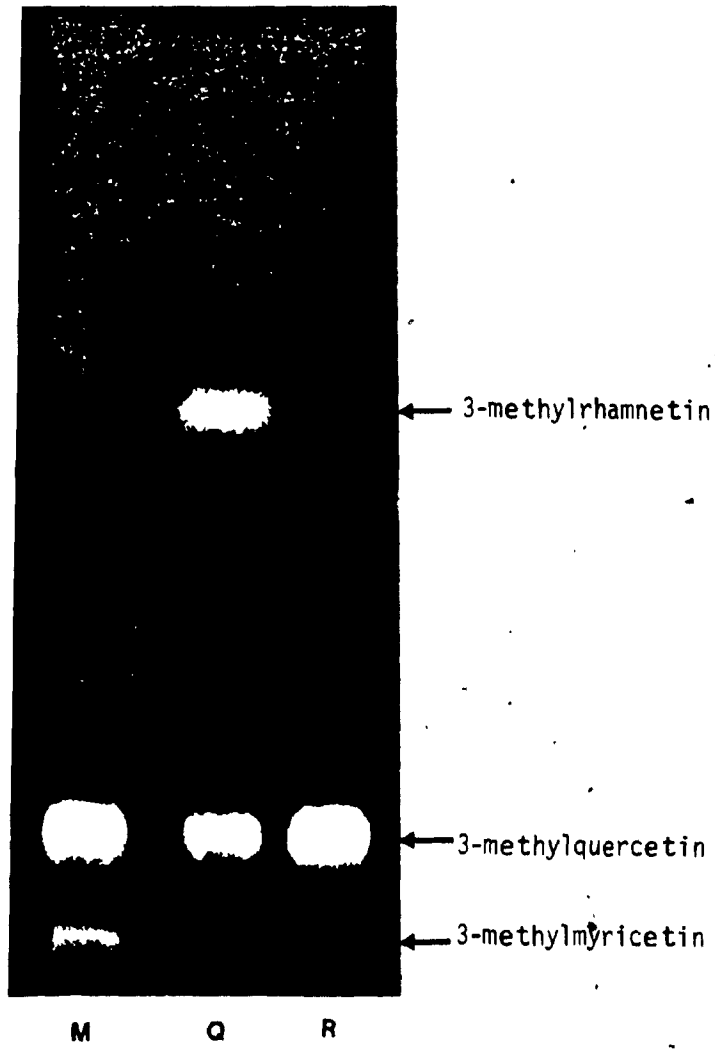
Appendix I.

Figure 2.   Effect of impure substrates in the study of
              substrate specificity for the 3-OMT.

              Myricetin (M) (5'-hydroxyquercetin) and rhamnetin
              (R) (7-methylquercetin) both contained quercetin
              (Q) impurities which accounts for the major
              incorporation of radioactivity into 3-methyl-
              quercetin.
              Products were chromatographed with reference
              compounds on Polyamide 6-MN TLC plates in
              solvent A (Table 3) followed by autoradiography.
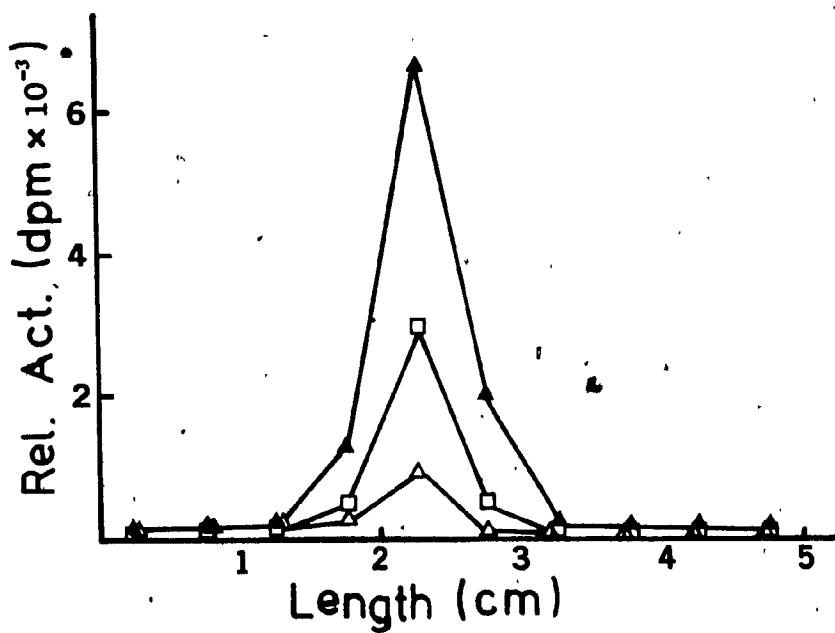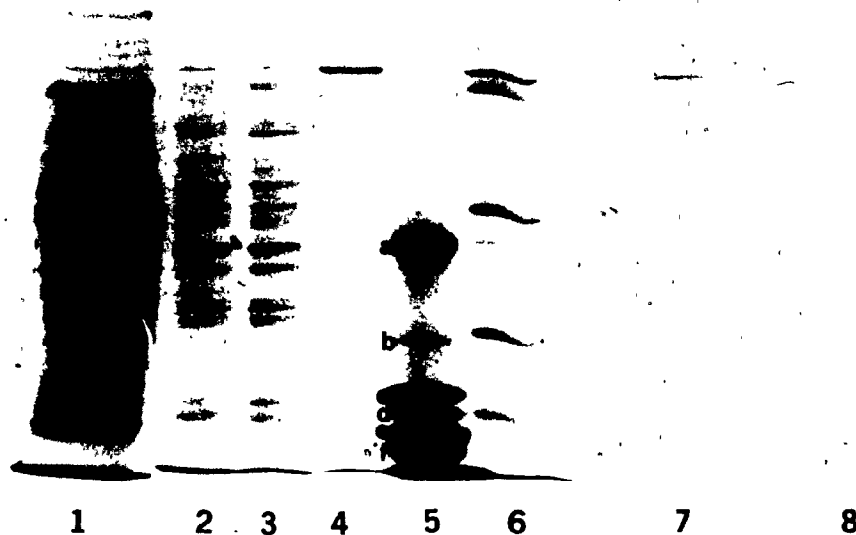
Appendix I.

Figure 3A.   Photograph of Coomasie Blue stained proteins
              submitted to SDS-PAGE as described in section
              C.3.7.1.

              Lane 1 is desalted $(NH_4)_2SO_4$ pellet (120 µg);
              lane 2 is after Sephacryl S-200 (24 µg) (Fig. 10);
              lane 3 is hydroxyapatite peak 1 (30 µg) (Fig. 11);
              lane 4 is hydroxyapatite peak 2 (20 µg) (Fig. 11);
              lane 5 is protein standards: bovine serum albumin(a)
              (MW, 66000), ovalbumin(b) (MW, 45000), pepsin(c)
              (MW, 34700), trypsinogen(d) (MW, 24000), ß-lacto-
              globulin(e) (MW, 8400) and lysozyme(f) (MW, 14300);
              lane 6 is 6-OMT from Polybuffer ion exchanger (28 µg)
              (Fig. 12A); lanes 7 and 8 are 4'- and 3-OMTs,
              respectively, from Polybuffer ion exchanger (10 µg)
              (Fig. 12B).

Figure 3B.   Activity profile of OMTs isolated from native
              polyacrylamide gels (section C.7.).

              OMT activity was assayed using quercetin (—x—),
              3,7-dimethylquercetin (—□—) and 3,7-dimethyl-
              quercetagetin (—▲—) for the 3- 4'- and 6-OMTs,
              respectively.
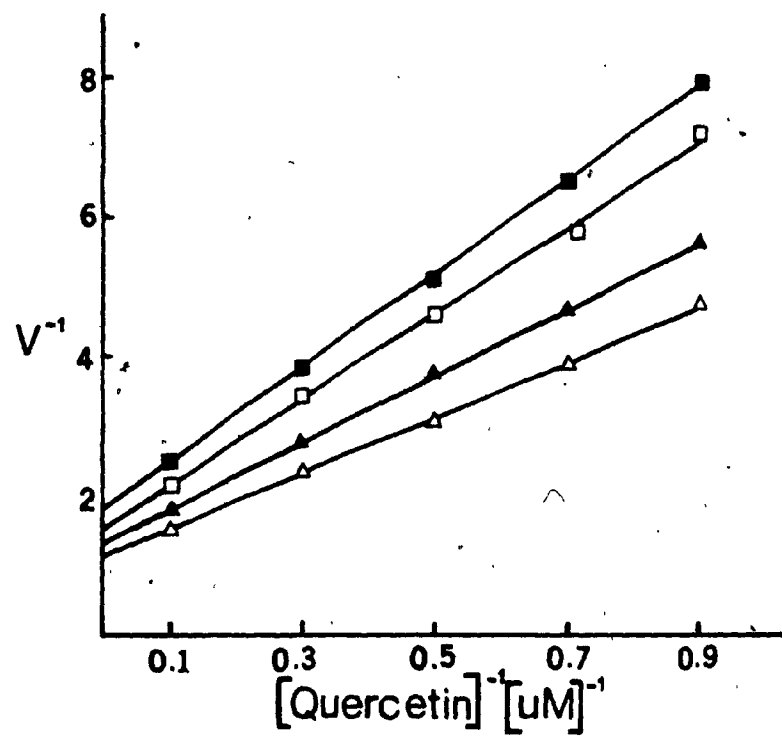
Appendix I.

Figure 4.   Product inhibition by 20 µM 3-methylquercetin
(—■—), 3'-methylquercetin (—□—) or 4'-methyl-
quercetin (—▲—) of 3-OMT activity. (—△—) is
the control without flavonol product inhibitor.
1/v versus 1/Quercetin with assay conditions
as described in figure 26D.
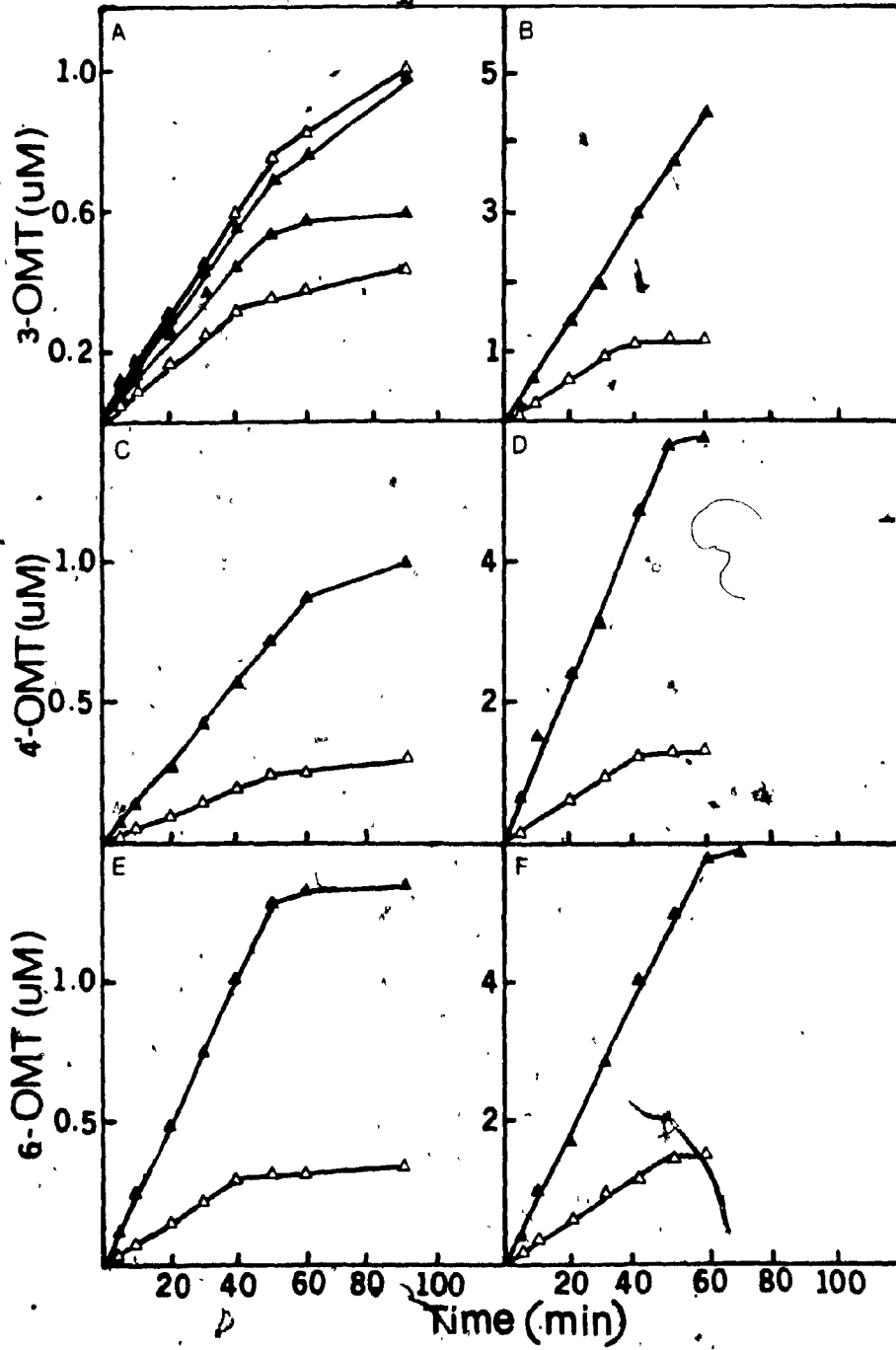
Appendix I.

Figure 5.   Linearity with time for the 3-, 6- and 4'-OMT

assays.

In A, C and E the concentration of SAM was constant
at 8.3 µM (0.05 µCi); whereas for A the concentration
of quercetin was 0.5, 1, 3, and 10 µM; for C the
concentration of 3,7-dimethylquercetin was 0.6 and
6 µM; for E the concentration of 3,7,3'-trimethyl-
quercetagetin was 0.6 and 6 µM.

In B, D and F the concentration of SAM was constant
at 116.6 µM (0.1 µCi), whereas the concentrations
of flavonol substrates were identical to A, C and
E, respectively.

G.2. Appendix II: Statistical Tables.

Table I. Substrate interaction kinetics; estimation of kinetic parameters

for the 3-, 6-, 8- and 4'-OMTs.

Varied Substrates			Parameter		
			Value (standard deviation) (µM)		
A	B	Term	Complete Model (Equation I: intersecting)		Reduced Model (Equation II: parallel)
SAM	Q[a]	K_a	114	(33)	195 (52)
		K_b	12	(3.3)	19 (5.3)
		$K_{ia}K_b$	85	(34)	-- --
		V	24	(4.6)	34 (8)
SAM	3,7,3'Qg	K_a	51	(27)	411 (478)
		K_b	18	(6)	87 (103)
		$K_{ia}K_b$	214	(55)	-- --
		V	20	(5.5)	82 (93)
SAM	3,7Q'	K_a	130	(25)	176 (29)
		K_b	15	(2.6)	19 (3.2)
		$K_{ia}K_b$	73	(33)	-- --
		V	29	(3.9)	36 (5)
SAM	8-OHK	K_a	53	(10)	142 (41)
		K_b	1.33	(0.27)	3.68 (1.1)
		$K_{ia}K_b$	45	(6.6)	-- --
		V	3.6	(0.4)	6.3 (1.5)

[a] Q, 3,7,3'Qg, 3,7Q and 8-OHK represent quercetin, 3,7,3'-trimethyl-

tagetin, 3,7-dimethylquercetin and 8-hydroxykaempferol, respectively,

and were used for the 3-, 6-, 4'- and 8-OMTs as the respective flav-

onoid substrate.

Table II. Product inhibition kinetics; estimation of kinetic parameters
for the 3-OMT.

Substrate	Inhibitor		Parameter			
			Value (standard deviation) (μM)			
A	I	Term	Complete Model (Equation III:non-competitive)		Reduced Model (Eq. IV or V Competitive(C) or uncompetitive(U))	
Q^a	SAH	K_b	$--^b$		(U) 1.25	(0.12)
		K_{is}	--		--	--
		K_{ii}	--		8.4	(0.6)
		V	--		1.8	(0.08)
Q	3Q	K_b	4.5	(0.31)	(C) 4.1	(0.36)
		K_{is}	27	(2.8)	20	(1.5)
		K_{ii}	128	(36)	--	--
		V	2.5	(0.09)	6	(0.4)
SAM	3Q	K_a	44	(4.5)	(U) 60	(10)
		K_{is}	29	(5.3	--	--
		K_{ii}	65	(9)	32	(3.6)
		V	5.5	(0.18)	6	(0.38)
SAM	SAH	K_a	$--^b$		25	(5.7)
		K_{is}	--		4.5	(0.9)
		K_{ii}	--		--	--
		V	--		4.7	(0.23)

[a] Q and 3Q represent quercetin and 3-methylquercetin .

[b] The data could not be fitted to this equation.

Table III. Product inhibition kinetics; estimation of kinetic parameters for the 6-OMT.

| Substrate | Inhibitor | | Parameter | | | | |
| | | | Value (standard deviation) (µM) | | | | |
A	I	Term	Complete Model (Equation III:non-competitive)		Reduced Model (Eq. IV or V Competitive(C) or uncompetitive(U))		
SAM	3,6,7,3'Qg[a]	K_a	36	(2.2)	(U)	44	(3.7)
		K_{is}	74	(13.5)		--	--
		K_{ii}	81	(6)		56	(3.5)
		V	5	(0.09)		5.2	(0.15)
SAM	SAH	K_a	--[b]	--	(C)	26	(2.3)
		K_{is}	--	--		16	(1.3)
		K_{ii}	--	--		--	--
		V	--	--		2.4	(0.08)
3,7,3'Qg	3,6,7,3'Qg	K_b	9.4	(0.7)	(U)	10.3	(0.64)
		K_{is}	1413	(820)		--	--
		K_{ii}	204	(31)		167	(16)
		V	3.1	(0.14)		3.2	(0.13)
3,3,3'Qg	SAH	--[c]	--	--		--	--

[a] 3,6,7,3'Qg and 3,7,3'Qg represent 3,6,7,3'-tetramethylated and 3,7,3'-trimethylated quercetagetin derivatives.

[b] The data could not be fitted to this equation.

[c] The data did not give linear double reciprocal plots and could not be fitted to any of the equations.

Table IV. Product inhibition kinetics; estimationof kinetic parameters
for the 4'-OMT.

Substrate	Inhibitor		Parameter				
			Value (standard deviation) (µM)				
A	I	Term	Complete Model (Equation III:non-competitive)		Reduced Model (Eq. IV or V Competitive(C) or uncompetitive(U))		
SAM	3,7,4'Q	K_a	27	(1.2)	(C)	25	(2.2)
		K_{is}	23	(0.8)		16	(1.2)
		K_{ii}	140	(32)		--	--
		V	5.5	(0.14)		5.3	(0.15)
SAM	SAH	K_a	--[b]	--	(C)	21	(2.8)
		K_{is}	--	--		4.4	(0.44)
		K_{ii}	--	--		--	--
		V	--	--		5.3	(0.21)
3,7Q	3,7,4'Q	K_a	1.4	(0.08)	(C)	1.3	(0.14)
		K_{is}	3	(0.33)		1.5	(0.15)
		K_{ii}	10	(1.4)		--	--
		V	4	(0.09)		4	(0.16)
3,7Q	SAH	K_a	--[b]	--	(U)	1.1	(0.24)
		K_{is}	--	--		--	--
		K_{ii}	--	--		10	(1.9)
		V	--	--		1.4	(0.13)

[a] 3,7,4'Q and 3,7Q represent 3,7,4'-trimethyl and 3,7-dimethylated quercetin.

[b] The data could not be fitted to this equation.

Table V. Product inhibition kinetics; estimation of kinetic parameters

for the 8-OMT.

Substrate	Inhibitor		Parameter				
			Value (standard deviation) (µM)				
A	I	Term	Complete Model (Equation III:non-competitive)			Reduced Model (Eq. IV or V Competitive(C) or uncompetitive(U))	
SAM	8MeK[a]	K_a	14.	(3)	(C)	7.5	(2.7)
		K_{is}	14	(6)		3.9	(1.4)
		K_{ii}	28	(7.5)		--	--
		V	0.47	(0.03)		0.4	(0.03)
SAM	SAH	K_a	20	(2.2)	(C)	16	(2.7)
		K_{is}	4	(1.1)		2.5	(0.8)
		K_{ii}	15	(6.2)		--	--
		V	0.72	(0.03)		0.07	(0.02)
8OHK	8MeK	K_a	1.34	(0.15)	(C)	1.31	(0.13)
		K_{is}	6.2	(0.96)		5.9	(0.53)
		K_{ii}	215	(518)		--	--
		V	1	(0.05)		1	(0.05)
8OHK	SAH	K_a	1.7	(0.14)	(C)	1.2	(0.16)
		K_{is}	3.8	(0.82)		1.3	(0.21)
		K_{ii}	2.7	(0.42)		--	--
		V	0.84	(0.04)		0.71	(0.04)

[a] 8MeK and 8OHK represent 8-methoxykaempferol and 8-hydroxykaempferol.

Table VI. Variance ratio test. Evaluation of the $K_{ia}K_b$ and K_{ii} terms in the complete model for the 3-, 6-, 8- and 4'-OMTs.

Enzyme	Experiment	Residual Sum of Squares		Calculated	(Degrees of freedom	Tables ($\alpha = 99$)
		Complete Model	Reduced Model			
3-OMT	SAM/Q[a]	0.4207	0.5237	4.76	(1;20)	8.1
	SAM/SAH	--	0.8007	--	(1,16)	--
	SAM/3Q	0.2334	1.0053	53	(1,16)	8.53
	Q/SAH	--	0.0362	--	(1,16)	--
	Q/3Q	0.0144	0.2765	14.8	(1,16)	8.53
6-OMT	SAM/3,7,3'Qg	0.2432	0.4005	13.3	(1,20)	8.1
	SAM/SAH	--	0.0505	--	(1,16)	--
	SAM/3,6,7,3'Qg	0.0623	0.2262	58	(1,16)	8.53
	3,7,3'Qg/SAH	--	0.0505	--	(1,16)	--
	3,7,3,Qg/3,6,7,3'Qg	0.0182	0.0213	3.4	(1,20)	8.1
4'-OMT	SAM/37Q	0.4471	0.5392	4	(1,20)	8.1
	SAM/SAH	--	0.4943	--	(1,26)	--
	SAM/3,7,4'Q	0.0421	0.2352	92	(1,20)	8.1
	3,7Q/SAH	--	0.2013	--	(1,20)	--
	3,7Q/3,7,4'Q	0.0574	0.2110	53	(1,20)	8.1
8-OMT	SAM/8OHK	0.0574	0.1706	39.3	(1,21)	8.01
	SAM/SAH	0.0045	0.0091	17	(1,16)	8.53
	SAM/8MeK	0.0041	0.0111	17	(1,12)	9.93
	8OHK/SAH	0.0034	0.0139	64	(1,21)	8.01
	8OHK/8MeK	0.0069	0.0069	.0.16	(1,16)	8.53

[a] Tables I to V have the abbreviations for the substrates and products.

G.3. Appendix III: Perspectives for future work.

III. Perspective for future work.

The OMT system of C. americanum has proven to be ideal in the study of closely related enzymes involved in the sequential O-methylation of flavonoids. Therefore, this tissue unlike that of spinach (72) should prove to be ideal for studies on the in situ localisation of these enzymes. Apart from the localisation studies described earlier in this thesis, a more direct approach would involve the localisation of OMT antigens at the electron microscope level using specific OMT antibodies. The methodology for the latter technique is well established in plant systems (134), given that the antibody used is prepared from pure antigen.

The procedures reported in this thesis involved the partial purification and isolation of separate OMTs from Chrysosplenium. Affinity chromatography with SAH-Agarose could be useful in further purifying these enzymes. Preliminary work by this author demonstrated that the 3-, 6-, 7- and 4'-OMTs of Chrysosplenium did bind to SAH-Agarose and were not eluted with SAM (1 mM) Appendix, Fig. 6). However it was found that the 3-, 6-, 7- and 4'-OMTs could be partially separated from each other by a 0-100 mM NaCl gradient. This demonstrates the potential use of affinity chromatography on SAH-Agarose in the purification of individual OMTs. Hopefully, the refinement of this technique may allow the preparation of pure OMTs for antibody production.

Another potential use of Chrysosplenium could involve studies on the in vivo regulation of O-methylation in this tissue. It has been shown that the levels of SAM can be modified in plant tissues by
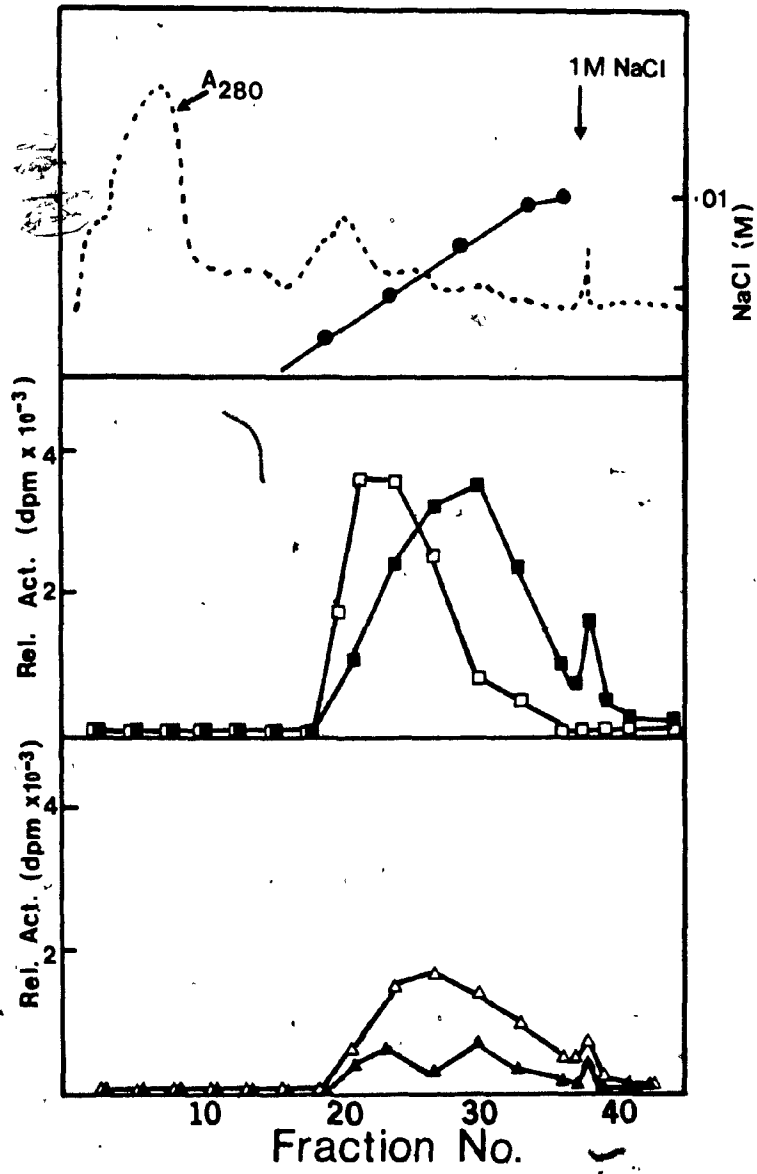
Appendix III.

Figure 6.   Chromatography on SAH Agarose (1 x 4 cm) of
             active fractions from Sephadex G-100 (as
             described in figure 9).

             OMT activity was assayed using quercetin (-□-),
             3-methylquercetin (-▲-), 3,7-dimethylquercetin
             (-■-) and 3,7,3'-trimethylquercetagetin (-△-)
             as substrates for the 3-, 7-, 4'- and 6-OMTs,
             respectively.

simply placing the latter in a solution containing methionine. It was
shown that addition of methionine to the growth medium of Lemna
paucicostata (56) increased the steady state concentration of SAM
between 10- to 20-fold. Similarly, turnip discs incubated with
methionine accumulated SAM to approximately 200 μM (135). It would be
interesting, therefore, to raise the levels of SAM in Chrysosplenium
before the administration of $[2-^{14}C]$-cinnamic acid and determine if
the rate of partially methylated flavonoid synthesis could be enhanced
(Appendix, Fig. 1). This may provide indirect evidence for the effect
of SAM/SAH ratio on the biosynthesis of such compounds.

Photoaffinity labelling can be useful in the further
characterization of Chrysosplenium OMTs as demonstrated with the
catechol OMT of porcine liver (136). It was shown that in the absence
of UV light, the 8-Azido analog of S-adenosyl-L-$[^{35}S]$-methionine
could serve as a methyl donor for porcine catechol-OMT, whereas
photolysis of this analog in the presence of the enzyme resulted in
covalent binding. The specificity of the incorporation indicated that
it occurred at the SAM binding site on the catechol-OMT.

Preliminary work by this author demonstrated that in the absence
of light 8-Azido-S-adenosyl-L-$[C^3H_3]$-methionine (80 Ci/mmol, New
England Nuclear) also served as a methyl donor with the 3-, 6- and
4'-OMTs of Chrysosplenium. The rate of methylation when 8-Azido-SAM
was the methyl donor was similar for the 3- and 6-OMTs whereas the
rate of incorporation with the 4' OMT was very low. However, the rate
of incorporation from $[^{14}CH_3]$-SAM was highest with the 4'-OMT followed
by the 3-OMT and the 6-OMT. This demonstrates different efficiency

of incorporation of this analog for each of the <u>Chrysosplenium</u> OMTs as well as a possible tool for further characterization of these enzymes. The fact that this analog acted as a methyl donor with the three OMTs, indicates that these enzymes could also be subjected to photoaffinity labelling for covalent binding to their respective active sites. Radioactively labelled enzymes could be subjected to SDS-PAGE followed by fluorography and identification of the 3-, 6- or 4'-OMTs. The latter could be submitted to proteolytic digestion in order to isolate the polypeptide fragment containing the active site and subsequent amino acid analysis. The 'finger prints' of each fragment could be compared and perhaps determine which differences may confer specificity to these enzymes.

These are only a few of the potential studies which could be carried out with the OMT system of <u>Chrysosplenium</u>. However, these examples serve to illustrate the fundamentally interesting biochemistry which can be carried out with the advent of technological innovations and the knowledge that plants like <u>Chrysosplenium</u> do exist.