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NL-339 (3/77)

A STUDY OF O-METHYLTRANSFERASE SYSTEM IN TOBACCO CELL CULTURE

Yun Fuk Tsang

A Thesis
in
The Department
of
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Montréal, Québec, Canada

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ABSTRACT

A STUDY OF O-METHYLTRANSFERASE SYSTEM IN TOBACCO CELL CULTURE

Yun Fuk Tsang

An O-methyltransferase system, mediating the methylation of a number of phenolic compounds, was isolated and partially purified from tobacco cell culture by ammonium sulphate fractionation and chromatography on DEAE-sepharose, Sephacryl S-200 and hydroxyapatite. Differences in the degree of enzyme purification against various substrates (45-90-fold), variations in specific activity ratios, and results of mixed-substrate experiments, seemed to indicate the presence of two forms of the enzyme. The latter were partially resolved by chromatography on DEAE-cellulose and exhibited distinct meta and para directing activities against caffeic acid and quercetin, respectively, but not to the exclusion of other substrates. The two forms of the enzyme were quite similar in molecular weights (70-74 K) and pI values (6.1-6.3) but differed in pH optima (7.0-7.5 and 8.0-8.5).

The general properties of tobacco culture OMT system have been studied and compared with those from other sources. The role of this enzyme system has been discussed in relation to secondary metabolite biosynthesis.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
CMT	Caffeic:O-methyltransferase
c.p.m.	Counts per minute
2,4-D	2,4-Dichlorophenoxyacetic acid
DEAE-	Diethylaminoethyl-
EDTA	Ethylenediamine-tetraacetate
5HFA	5-Hydroxylferulic acid
IAA	Indoleacetic acid
IUB	International Union of Biochemistry
K_i	Dissociation constant of an enzyme-inhibitor complex
K_m	Michaelis-Menten constant
m-	Meta-
MS medium	Murashige and Skoog medium
M.W.	Molecular weight
o-	Ortho-
OMT	O-methyltransferase
p-	Para-
pCMB	p-Chloromercuribenzoate
pKat	pKatal
PPO	2,5-Diphenyloxazole
QMT	Quercetin:O-methyltransferase
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine

SDS	Sodium dodecylsulphate
TLC	Thin layer chromatography
TLG	Thin layer gel filtration
Tris-	Tris(hydroxymethyl)-aminomethan

INTRODUCTION

O-methylation is an important metabolic reaction in both plant and animal tissues. Extensive studies have been carried out on the enzyme O-methyltransferase (OMT) [E.C.2.1.1.6.] from different tissues of a number of mammalian species. The enzyme is responsible for the methylation of the hydroxyl groups of catechol, and is believed to regulate the levels of both adrenaline and noradrenaline in animal tissues. In plants, however, O-methylation of secondary metabolites has been studied less extensively. It was first reported by Byerrum *et al*. (1954) in connection with lignin formation. Since then, OMT has been detected in a number of species involving the biosynthesis of simple phenolic substances, lignins, alkaloids and flavonoids.

In the presence of a methyl-group donor, such as S-adenosyl-L-methionine, OMT can transfer the methyl-group to o-dihydroxyphenolic substances, such as shown in Figure 1.

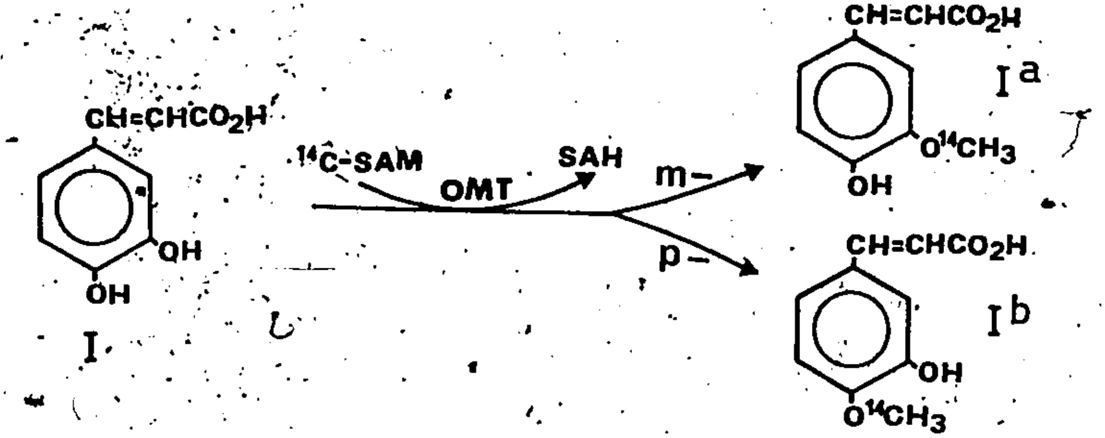
Previous reports showed that some OMT preparations catalyzed the selective methylation of phenolic compounds at either the *para* position (Mann *et al*., 1963; Wat and Towers, 1975; Wengenmayer *et al*., 1974), or the *meta* position (Higuchi *et al*., 1967; Poulton *et al*., 1976a, b). Legrand *et al*. (1976) suggested the presence of three separate OMTs in tobacco leaves, based on their different specificities against various substrates and different meta/para ratios. However, those enzyme fractions have not been further characterized. Recently, two distinct OMTs have been isolated from soybean cell suspension culture (Poulton et al., 1976a, b; 1977): one was specific for substituted cinnamic acids (Poulton et al., 1976a, b) while the other for flavonoids (Poulton et al., 1976a, 1977). Similar results have been reported with tulip anthers (Sütfeld and Wiermann, 1978).

Our preliminary work indicated that both caffeic acid and esculetin were methylated by a crude tobacco OMT preparation to their m- and p-O-methyl derivatives (ferulic and isoferulic; scopoletin and isoscopoletin, respectively). Similar observations have been previously reported with yeast (Müller-Enoch et al., 1976b), plant (Shimada et al., 1972; Legrand et al., 1976; Sütfeld and Wiermann, 1978) and animal tissues. In none of these reports, however, has the problem of distinct enzymes catalyzing the m- and p-methylation been unequivocally resolved.

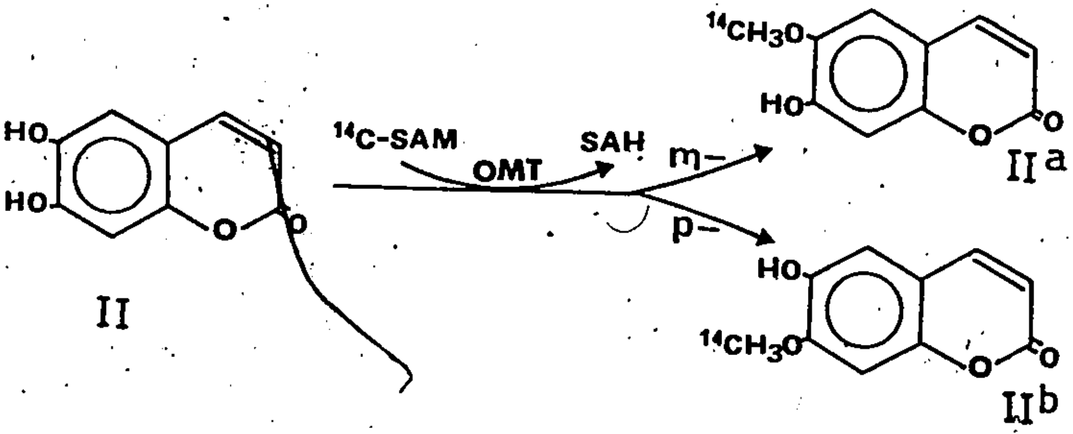
Tobacco cell culture is known to synthesize scopoletin and its glucosides, scopolin as the main phenolic metabolites. OMT activity of the cultured cells wes found to increase concomitantly with the accumulation of phenols during culture growth. It was considered of interest, therefore, to isolate and purify tobacco culture OMT in view of studying its characteristics and its specificity towards m- and p-methylation of phenolic substrates.

Figure 1. *Meta* and *para* *O*-methylation of various substrates by *O*-methyltransferase

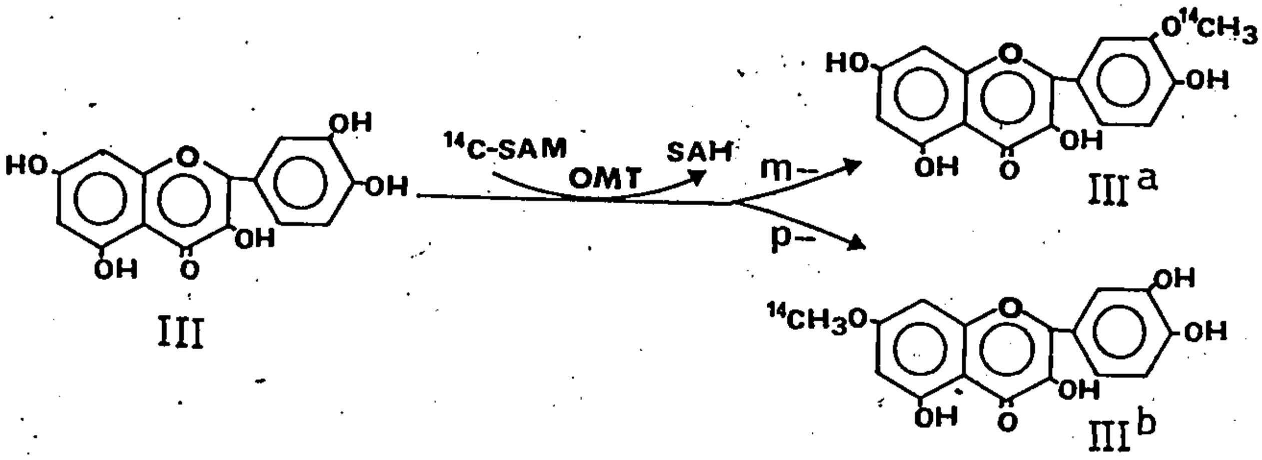
I	caffeic acid
I^{a}	ferulic acid
I^{b}	isoferulic acid
II	esculetin
II^{a}	scopoletin
II^{b}	isoscopoletin
III	quercetin
III^{a}	isorhamnetin
III^{b}	rhamnetin



CH=CHCO2H
OH
OH
I
14C-SAM
OMT
SAH
m-
p-
CH=CHCO2H
O14CH3
OH
I a
CH=CHCO2H
OH
O14CH3
I b



HO
HO
O
O
II
14C-SAM
OMT
SAH
m-
p-
14CH3O
HO
O
O
II a
HO
14CH3O
O
O
II b



HO
O
OH
OH
OH
O
OH
III
14C-SAM
OMT
SAH
m-
p-
HO
O
O14CH3
OH
OH
OH
O
III a
14CH3O
O
OH
OH
OH
OH
O
III b

Section A. LITERATURE REVIEW

A.1. Significance of O-Methyltransferase in Secondary Metabolism

O-methylation is a very common reaction that involves dihydroxy phenolic compounds in both animal and plant tissues. The reaction is catalyzed by the enzyme catechol-O-methyltransferase [E.C.2.1.1.6.] which was first demonstrated in rat liver, kidney and brain tissues (Axelrod, 1957; Axelrod and Tomchick, 1958). In the presence of a methyl group donor the enzyme can O-methylate a number of naturally occurring catechols including adrenaline, noradrenaline, dopa, dopamine and 3,4-dihydroxymandelic acid (Axelrod and Tomchick, 1958). It was suggested, therefore, that this enzyme was important in the metabolism of catecholamines. Noradrenaline and adrenaline present in the blood circulation are primarily inactivated by liver and kidney OMT, thus resulting in the formation of physiologically inactive products - normetaadrenaline and metaadrenaline, respectively (Levin *et al.*, 1970). Flohe *et al.* (1970) claimed that this enzyme might also regulate the levels of these two hormones.

The significance of OMT in plant metabolism has been correlated with the incorporation of the methyl groups of methionine into lignin (Byerrum *et al.*, 1954) which paved the way to numerous studies of the O-methylating enzyme in higher plants. OMT was later reported from the cambial tissues of apple and of pampass grass (Finkle and Nelson, 1963a, b; Finkle and Masri, 1964) as well as several other plant species (See section A.4.2). Higuchi et al. (1967) also found that the increased incorporation of methyl groups was concomitant with increased lignin formation in bamboo shoots and suggested the participation of O-methyltransferase in the biosynthesis of lignin. Furthermore, they pointed out that OMT is a key enzyme which helped to explain the phylogenic difference in the formation of guaiacyl and syringyl units of lignin in angiosperm and gymnosperm species (Higuchi et al., 1967; Shimada et al., 1970, 1972, 1973). Their results indicated that angiosperm OMT catalyzed the formation of both ferulic acid and sinapic acid from caffeic acid and 5HFA, respectively; whereas only caffeic acid was methylated by gymnosperm OMT. It has also been suggested that gymnosperm lignin lacks the syringyl units that are present in angiosperms (Ibrahim et al., 1962).

O-methyltransferase was also found to catalyze the methylation of esculetin to form scopoletin (Hess, 1965a,b; Legrand et al., 1976; Müller-Enoch et al., 1976a). O-methyltransferase is also involved in the biosynthesis of flavonoid compounds. The enzyme has been purified and its properities studied in suspension cultures of parsley (Ebel et al., 1972), Cicer sp. (Wengenmayer et al., 1974), soybean (Poulton et al., 1976a, b, 1977) and Ruta sp. (Thompson et al., 1978):

A.2. The Methyl Group Donor

Borsook and Dubnoff (1945) reported that methionine can serve as a methyl group donor in the enzymic transmethylation. Since then, this amino acid was commonly used as the methyl group donor.

Cantoni and Durell (1957) pointed out that ATP and Mg^{++} were essential to promote the effectiveness of methionine as a methyl group donor, and suggested that methionine is being activated in the presence of ATP and Mg^{++}. It was later shown that the active methionine was actually S-adenosyl-L-methionine (Figure 2).

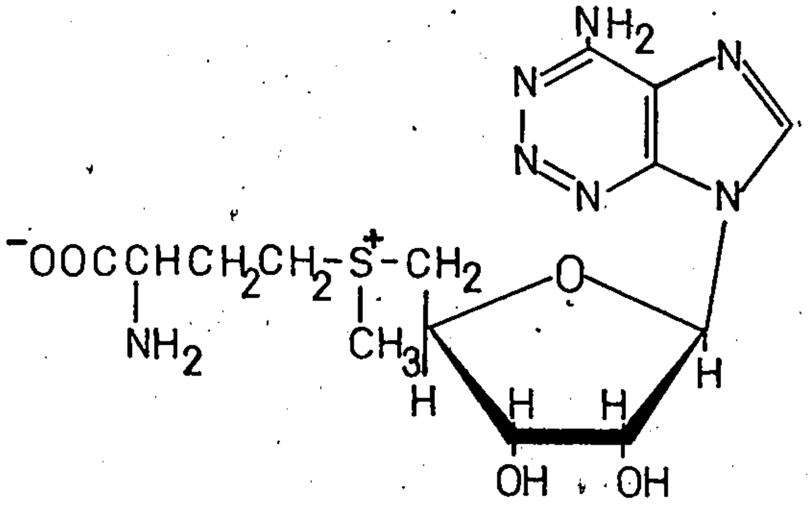


Figure 2. Molecular structure of S-adenosyl-L-methionine.

Futhermore, the enzyme ATP:L-methionine S-adenosyltransferase [E.C.2.5.1.6.] which is responsible for the synthesis of S-adenosyl-methionine was detected in yeast cells (Mudd and Cantoni, 1958) and liver tissues (Mudd and Cantoni, 1962). The mechanism of the enzymic reaction was established as follows:

$$\textit{L}\text{-methionine} + \text{ATP} \xrightarrow{Mg^{++},\ K^{+}} (-)\textit{S}\text{-adenosyl-}\textit{L}\text{-methionine} + \text{PPi} + \text{Pi}$$

Mann *et al.* (1963), in their studies of the steric specificity of *Nerine* OMT for the diastereoisomers of SAM (±), indicated that the (+) SAM is only 10% as effective as the (−) isomer. However, less specificity was shown with regard to the configuration of the α-amino carbon, since (±) − *S*-adenosyl-*D*-methionine was only one-third as effective as the *L*-form.

Haba *et al.* (1959) explained that the ability of SAM to release the methyl group was due to the existence of an energy-rich bond between the sulfonium and the methyl group of the compound.

As the ATP:*L*-methionine-adenosyl-transferase was identified in bamboo shoots (Higuchi *et al.*, 1967) and parsley (Ebel *et al.*, 1972), SAM was recognized as a reasonable methyl group donor of the transfer reaction. Since then, a number of SAM-dependent biological transmethylation reactions have also been deomonstrated in plant tissues (See section A.4.2).

A.3. Isolation of *O*-methyltransferase

A.3.1. Extraction and purification of OMT

O-methyltransferase is generally assumed to be found in the soluble protein fraction of the cell (Pellerin and

D'Iorio, 1958; Molinoff and Axelrod, 1971), therefore, there is no special problem with its extraction or solubility. Grinding with appropriate buffer solution is essential for complete extraction. OMT from rat liver is readily soluble in isotonic KCl (Axelrod and Tomchick, 1958). Tong and D'Iorio (1977) reported that the minor 'particulate' enzyme which was bound to the plasma membrane required acetone treatment for the release of its activity.

Partial purification of OMT from both animal and plant tissues has been achieved by using the classical techniques including differential centrifugation, ammonium sulphate fractionation, ion-exchange and gel filtration chromatography. While these techniques are time consuming, this difficulty is further aggravated by the fact that OMT is a relatively labile enzyme.

The more rapid and efficient purification technique appears to be affinity chromatography. The latter was successfully used in combination with other classical techniques for the purification of rat liver OMT (Borchardt et al., 1975) and resulted in 900-fold purification. Recently, two OMTs were separated by affinity chromatography into o- and m-directing enzymes, though their purification was as low as 50- and 16-fold, respectively (Thompson et al., 1978). Other workers, however, reported no success with the use of affinity chromatography (Darmenton et al., 1976; Brunet and Ibrahim, personal communication).

A.3.2. Electrophoretic behavior and multiplicity of OMT

In order to judge the homogenity of the protein, polyacrylamide gel electrophoresis (Davis, 1964) is generally used. By means of this technique, Shimada et al. (1973) obtained a single-band enzyme from bamboo shoots. The mobility of the pine OMT on polyacrylamide gels was smaller than that of the bamboo OMT. Kuroda et al. (1975) assumed that pine OMT had a lower negative charge as compared with that of bamboo.

Caffeic: OMT (CMT) and quercetin: OMT (QMT) of tulip anthers were reported to locate slighty apart from each other after gel electrophoresis (Sütfeld and Wiermann, 1978), an indication of being two proteins with different molecular weights but similar in electrophoretic mobility

Multiplicity of OMT has been reported with the rat liver enzyme based on electrophoretic evidence (Axelrod and Vesell, 1970). Two separate forms of the enzyme were found to differ in heat stability and kinetic values, though both had the same substrate specificity.

Using isoelectric focussing (Wrigley, 1971) various pI values were reported for different OMTs. It was found that rat liver OMT focussed at pH 5.5 (Darmenton et al., 1976); bamboo OMT at 4.1 (Shimada et al., 1973); CMT and QMT of tulip anthers at 4.8 and 5.0, respectively (Sütfeld and Wiermann, 1978). These data seem to indicate that the enzyme is an acidic protein.

A.4. Distribution of O-Methyltransferase

OMT was detected in many mammalian species (Axelrod and Tomchick, 1958) as well as amphibian tissues (Axelrod et al., 1965). Generally, the highest activity was found in both liver and kidney tissues, where noradrenaline and adrenaline were methylated to their corresponding 3-O-methylamines (Levin et al., 1970). The enzyme activity was also present in the pineal gland (Axelrod and Weissback, 1960) and brain tissue (Inscoe et al., 1965).

Most of OMT activity was reported in the soluble fraction of the cell, though small amounts of activity were associated with the microsomal fraction (Inoscoe et al., 1965) and the plasma membrane (Tong and D'Iorio, 1977). It was also reported that microsomal OMT differed from soluble OMT in its substrate specificity and pH optimum (Inscoe et al., 1965).

O-methyltransferase is widely distributed in almost all plant tissues. The enzyme was partially purified from Nerine bulbs (Mann et al., 1963); bamboo shoot (Higuchi, 1967; Shimada et al., 1972; Kuroda et al., 1975); tulip anthers (Sütfeld and Wiermann, 1978); and a number of cell suspension cultures such as, parsley (Ebel et al., 1972); Cicer sp. (Wengenmayer et al., 1974); soybean (Poulton et al., 1976a,b) and Ruta sp. (Thompson et al., 1978).

In contrast, very few reports have come from work with microorganisms, notably that of Lentinus lepideus (Wat and Towers, 1975) and yeast (Müller-Enoch et al., 1976b).

However, there are no reports of the presence of catechol: OMT in bacteria.

A.5. Properties of O-Methyltransferase

A.5.1. Molecular weight

The molecular weight of OMT has been reported from various plant tissues and was found to range from 48,000 to 110,000 daltons (Table I). Lower value has been reported for the QMT of tulip anthers ca. 35,000 daltons (Sütfeld and Wiermann, 1978). On the other hand, the molecular weights of rat liver and kidney tissue OMTs ranged between 21,500 to 23,000 (Assicot and Bohuon, 1970; Borchardt et al., 1975; Darmenton et al., 1976). The M.W. of the membrane-bound OMT rat liver was similar to that of the soluble enzyme (Tong and D'Iorio, 1977).

A.5.2. Substrate specificity and kinetic constants

In general, both the K_m values and substrate specificities were variable for the enzyme obtained from different sources. The K_m values of the most common substrates are listed in Table II.

A purified OMT from parsley cell culture had a greater affinity for flavonoid than that of phenylpropanoid compounds, even though caffeic acid was a better substrate than luteolin or its 7-O-glucosides (Ebel et al., 1972). Whereas most of the plant OMTs were reported to have no activity with monohydroxy compounds, the enzyme from

Table I. Molecular weights of OMT from different sources

Enzyme Source	M.W.	Substrate Catalysed	Reference
Cicer arietinum	110,000	daidzein	Wengenmayer *et al.* (1974)
Petroselinum hortense (parsley)	48,000	luteolin	Ebel *et al.* (1972)
Pinus thunbergii	67,000	caffeic	Kuroda *et al.* (1975)
Ruta graveoleus	85,000 (*ortho*)	bergaptol	Thompson *et al.* (1978)
	110,000 (*meta*)	xanthotoxol	
Rat liver	24,000	3,4,di-OH-phenylacetate	Assicot and Bohuon (1970)
"	23,000	3,4,di-OH-benzoic	Borchardt *et al.* (1975)
"	21,100	3,4,di-OH-benzoic	Tong and D'Iorio (1977)
Rat kidney	21,500	adrenaline	Darmenton *et al.* (1976)

Lentinus lepideus was found to catalyze the selective O-methylation of p-hydroxycinnate (Wat and Towers, 1975).

A.5.3. pH optimum and its effect

The optimal pH for OMT activity was found to differ in different systems and ranged from pH 6.5 to 9.7 (Table III). Flohe et al. (1972) pointed out that the pH condition could affect the affinity of OMT towards adrenaline. They found that the K_m value for adrenaline decreased significantly with the increase of pH. That for SAM, however, was not affected and remained constant within pH 6.5 to 9.5.

Changes in pH were also reported to affect the enzyme stability as well as the ratio of meta/para methylation. The latter is attributed to changes in the nucleophilicity of hydroxyl groups (see below).

A.5.4. Meta and para methylation

O-methylation is known to occur in vivo at the meta position of o-dihydric acceptors by rat liver OMT (Axelrod and Tomchick, 1958), though the purified enzyme from the same tissue catalyzed the methylation at both the m- and p-positions (Senon et al., 1959). As was mentioned, the pH condition could affect the nucleophilicity of hydroxyl methylation (Senon et al., 1959). These authors found that catechol:OMT normally catalyzed m-O-methylation at neutral pH as a result of the stronger nucleophilic property of the

Table II. K_m values of the most common substrates for OMT

Substrate	K_m (μM)	Source	Reference
Adrenaline	194	rat	Assicot and Bohuon, 1970
Noradrenaline	25	rat	Darmenton *et al.*, 1976
Caffeic acid	16	parsley	Ebel *et al.*, 1972
	133	soybean	Poulton *et al.*, 1976b
Daidzein (7,4'diOH-isoflvone)	80	*Cicer*	Wengenmayer *et al.*, 1974.
Eriodictyol (3',4',5,7-tetraOHflavanone)	12	parsley	Ebel *et al.*, 1972
5-Hydroxyferulic acid	10	bamboo	Shimada *et al.*, 1972
	27	*Pinus*	Kuroda *et al.*, 1975
	55	soybean	Poulton *et al.*, 1976b
Luteolin (3',4',5,7-tetraOHflavone)	46	parsley	Ebel *et al.*, 1972
Luteolin-7-glucoside	31	parsley	Ebel *et al.*, 1972
Norbelladine	100	*Nerine*	Mann *et al.*, 1963
Protocatechuic (3,4,diOHbenzoic)	50	soybean	Poulton *et al.*, 1976b
Quercetin	170	tulip	Sütfeld and Wiermann, 1978
3,4,5,TriOH-cinnamic acid	100	soybean	Poulton *et al.*, 1976b
SAM	10	*Nerine*	Mann *et al.*, 1963
	150	*Cicer*	Wengenmayer *et al.*, 1974.

Table III. Optimal pH of OMTs in different enzyme sources

Enzyme Sources	Optimal pH	Substrates Catalyzed	References
Apple	7.0- 8.0	caffeic acid	Finkle and Nelson, 1963
Beta vulgaris	6.5	caffeic acid	Poulton and Butt, 1975
Cicer arietinum	9.0	daidzein	Wengenmayer *et al.*, 1974
Glycine max (soybean)	6.5- 7.0	caffeic acid	Poulton *et al.*, 1976b
Leutinus lepideus	7.0	methyl-p-coumarate	Wat and Towers, 1975
Nerine bowdenii	8.1	norbelladine	Mann *et al.*,1963
Petroselinum hortense (parsley)	9.6- 9.8	luteolin	Ebel *et al.*,1972
Phyllostachys pubescens (bamboo)	7.5- 8.0	caffeic acid 5HFA	Higuchi *et al.*,1967 Shimada *et al.*,1972
Pinus thunbergii	7.5	caffeic acid	Shimada *et al.*,1972
Ruta graveolens	a) 7.5- 8.0	bergaptol xanthotxol	Thompson *et al.*, 1978
	b) 8.5- 9.7	5-OH-xanthotoxin	" "
Rat liver	7.2	di-OH-phenyl-acetate	Assicot and Bohuon, 1970
Rat liver	7.8- 9.7	adrenaline	Flohe et al.,1970
Rat kidney	7.9- 9.6	adrenaline	Darmenton *et al.*, 1976

m-hydroxyl group. On the other hand, the ratio of m-/p-methylation decreased with increasing pH due to increased nucleophilicity of the p-hydroxyl group.

Most of plant OMTs reported, so far, were found to be meta directing. In contrast, the enzymes from *Nerine* bulbs (Mann *et al.*, 1963), *Lentinus lepideus* (Wat and Towers, 1975) and *Cicer* cell culture (Wengenmayer *et al.*, 1974) were found to catalyze the methylation of para-hydroxyl groups. Recently, an ortho-directing OMT has been reported from *Ruta graveoleus* cell culture tissue (Thompson *et al.*, 1978).

A.5.5. Cofactor requirements

Mg^{++} is generally required for maximum activity of animal catechol:OMT (Molinoff and Axelrod, 1971). Other divalent metal ions, such as Co^{++}, Ca^{++}, Zn^{++} and Ni^{++} have also been reported to substitute for Mg^{++}. A hypothetical OMT-Mg^{++} complex (Fig.3.) has been proposed by Senon *et al.* (1962). They realized that the complex of Mg^{++} and dihydroxyl phenolic compounds and SAM satisfies best the spatial requirement for the enzyme. The primary function of this complex is assumed to bring the substrate and the enzyme together. However, higher concentration of Mg^{++} and Ca^{++} were reported to inhibit OMT activity (Flohe *et al.*, 1972).

In contrast to the animal enzyme, Mg^{++} does not seem to be required for the activity of plant OMTs, though Mn^{++} and

Zn^{++} were reported to inhibit the enzymes of parsley (Ebel et al., 1972) and pine (Kuroda et al., 1975).

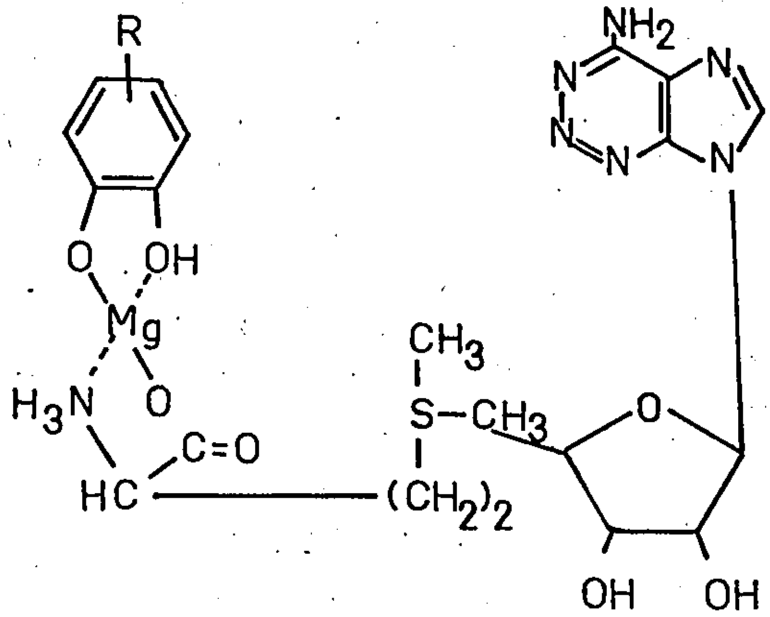


Figure 3 . Hypothetical OMT-Mg^{++}-substrate complex (adapted from Senon et al., 1962)

A.5.6. Inhibitors of O-Methyltransferase

A.5.6.1. Inhibition by S-adenosyl-homocysteine

¶ OMT activity was reported to be strongly inhibited by the other product of the reaction, SAH. Its inhibition was competitive for the methyl group donor (SAM) and its K_i ranged from 0.4 to 30 µM for different OMTs. Generally, the K_i for SAH is smaller than the K_m value for SAM.

Poulton and Butt (1975) claimed that this relationship may function as a regulatory mechanism of the enzyme activity. However, the detection of S-adenosyl-homocysteine hydrolyase [E.C.3.3.1.1.] in plant tissue extracts suggests that SAH may not necessarily inhibit the OMT activity in vivo (Poulton and Butt, 1976).

Borchardt and Wu (1974a, b, 1975) studied the SAH inhibition of OMT using its structural analogues and proposed the binding sites for SAH shown in Figure 4. According to their proposal, the terminal amino group (site b) and the α-amino group of the adenine moiety (site f) were considered particularly important for SAH inhibition.

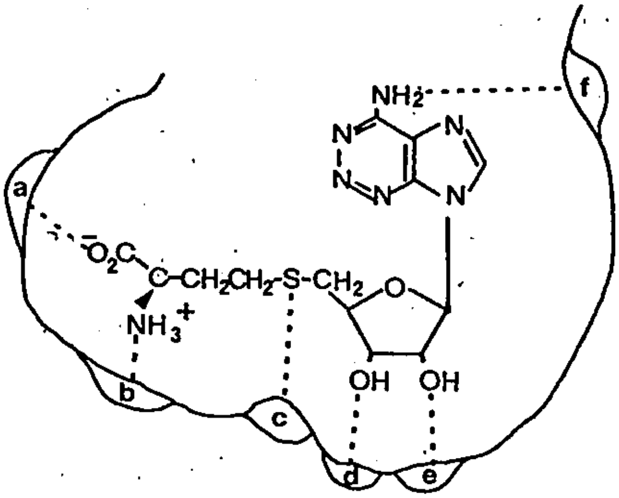


Figure 4. Proposed binding sites for SAH (adopted from Borchardt and Wu, 1975).

A.5.6.2. Other inhibitors

Sulfhydryl group-binding reagents, such as p-chloromercuribenzoate (pCMB) or monoiodoacetate were reported as OMT enzyme inhibitors, which tends to suggest that the enzyme requires -SH groups for activity (Shimada et al., 1972).

The inhibitory effect of flavonoids on rat liver OMT has been reported (Schwabe et al., 1972). Due to structural considerations, kaempferol (a flavanol) which lacks vicinal OH-groups was found to inhibit the enzyme pronouncedly. However, flavones and flavanones which lack the 3-OH group exhibit a less distinct effect (Schwabe et al., 1972).

Tropolones which are isosteric with catechols were reported to act as competitive inhibitors of liver OMT, although they cannot be methylated by the enzyme in presence of SAM (Belleau and Burba, 1961).

A.5.7. Enzyme stability

In most cases, the animal OMTs have been reported to be very unstable (Assicot and Bohuon, 1970). Almost 70% of activity was lost after 24 hours of storage at 4°C. The half-life of the enzyme from kidney tissue was found to be 2 hours at -20°C (Darmenton et al., 1976). The enzyme was extremely heat labile and its activity was lost completely within a 15-minute incubation at 60°C (Assicot and Bohuon, 1970).

SH-group reagents, such as β-mercaptoethanol and dithiothreitol have been reported to serve as OMT stabilizers (Ebel et al., 1972). Assicot and Bohuon (1970) had shown that the loss in enzyme activity could be restored by the addition of dithiothreitol. It was also reported that OMT activity could be maintained over one month in the form of ammonium sulphate precipitate (Tong and D'Iorio, 1977).

Relatively stable enzymes have been reported from *Nerine* bulbs (Mann et al., 1963), parsley (Ebel et al., 1972), soybean (Poulton et al., 1976a, b) and *Ruta* cell cultures (Thompson et al., 1978). It is interesting to note that the caffeic:OMTs isolated from soybean culture (Poulton et al., 1976a) and tulip anthers (Sütfeld and Wiermann, 1978) were more stable than the flavonoid:OMTs from both tissue systems.

A.6. Regulation of O-Methyltransferase Activity

A.6.1. Effect of illumination

Many enzymes involved in the secondary metabolism of plants were found to be stimulated by illumination. Ebel et al. (1972) demonstrated that the flavone:OMT from parsley cell culture was strongly dependent on light treatment. Similarly, a 20-fold increase in OMT activity was reported in potato slices exposed to light for 24 hours (Camm and Towers, 1975). In contrast, illumination appeared to have no significant effect on the isoflavone: OMT from _Cicer_ cell culture (Wengenmayer _et al_., 1974).

A.6.2. Effect of plant hormones

There is very little information on the effect of growth substances on OMT activity. Kinetin was reported to stimulate the tobacco enzyme in relation to lignification and clumping of cells in culture (Yamada and Kuboi, 1976; Kuboi and Yamada, 1976). These results seem to concur with the findings of Bergamann (1964) on the effect of kinetin on the pathways leading to lignin biosynthesis. On the other hand, Thompson _et al_. (1978) reported an inhibitory effect of kinetin on _Ruta_ culture OMT.

Section B. MATERIALS AND METHODS

B.1. Initiation and Maintenance of Tobacco Cell Suspension Culture

B.1.1. Plant material

Nicotiana tabacum L. (CV. Wisconsin #38) was used for the present study. Seeds were obtained by courtesy of Dr. T. A. Thorpe, University of Calgary, and were germinated under greenhouse conditions. The stem pith of two months old plants was used to establish both callus and cell suspension cultures.

B.1.2. Initiation of callus culture

Six-cm-long segments were excised from the middle part of the stem and were surface sterilized by soaking for seven minutes in 1:1 aqueous solution of Javex (10% available Cl_2) then rinsed thoroughly with sterile water. All tissue culture manipulations were carried out aseptically under a laminar-flow transfer hood and using sterile instruments. The stem pith was removed using a 5mm diameter cork borer and then sliced. Pith slices, 5mm thick, were transferred onto the surface of a solidified nutrient-culture medium (Appendix I) in 20 x 5 x 5cm culture bottles. The culture medium (Murashige and Skoog, 1962) was supplemented with 3% surcose, 2 µM indole-3-acetic acid, 1 µM 2,4-dichlorophenoxyacetic acid and 0.1 µM kinetin, and was solidified with 0.7% agar. The pH of the medium was adjusted to 5.7 before being autoclaved at 15 lb p.s.i. for 15 minutes. The cultures were maintained under diffuse light (ca. 100 f.c.) at 24°±1°C.

B.1.3. Initiation of cell suspension culture

A suspension culture which consisted of free cells and small cell aggregates was initiated in the following manner. Several pieces (ca. 10g) of actively growing, friable callus were transferred as aseptically to one-liter nippled flasks, each containing 200 ml of the liquid medium (Appendix I). The flasks were allowed to rotate gently on a rotating wheel at 4-5 r.p.m. which was driven by a motor with changeable gear (Model NSH-12RG, Bodine Electric Co. Chicago, Ill.). The callus masses were transformed into a loose suspension within 2 weeks. Free cells and small cell aggregates were separated from the large clumps by passing the suspension through a sterile nylon wire gauze. The fine cell suspension was used as inoculum for batch cultures which were maintained in 250-ml Erlenmeyer flasks, each containing 50 ml of the liquid medium. The culture flasks were agitated on a gyrotary shaker (Model G-10, New Brunswich Scientific, N.J.) at a rate of 150 r.p.m. under the same light-temperature conditions mentioned above. Growth of the cell suspension was maintained by subculturing at 7-day intervals to the same nutrient medium at a ratio of 1:10. Cell suspension was plated at regular intervals in agar medium and served a stock callus culture. Both cell (Figures 5, 6) and callus cultures were routinely examined under the light microscope for any contamination.

B.[illegible] Determination of Growth Parameters of Tobacco Cell Culture

Duplicate culture samples were taken at daily intervals for the determination of the following growth parameters.

B.2.1. Fresh weight

The contents of 2 or more culture flasks were filtered through a fritted glass funnel (medium porosity) using suction. Air was allowed to pass through the filter for 30 seconds and the cake of cells was weighed. The fresh weight of cells was expressed in terms of gram per culture flask.

B.2.2. Soluble protein

The soluble protein content was determined on aliquots of 0.1 M phosphate buffer (pH 7.5) extracts of fresh cells using the Lowry method (Lowry et al., 1951). Bovine serum albumin, dissolved in the same buffer, was used as standard to establish a calibration curve. Soluble protein content was expressed in mg per gram fresh weight of cells.

B.2.3. Investigation of the phenolic constituents of tobacco cells

A known weight of fresh cells was extracted with 5

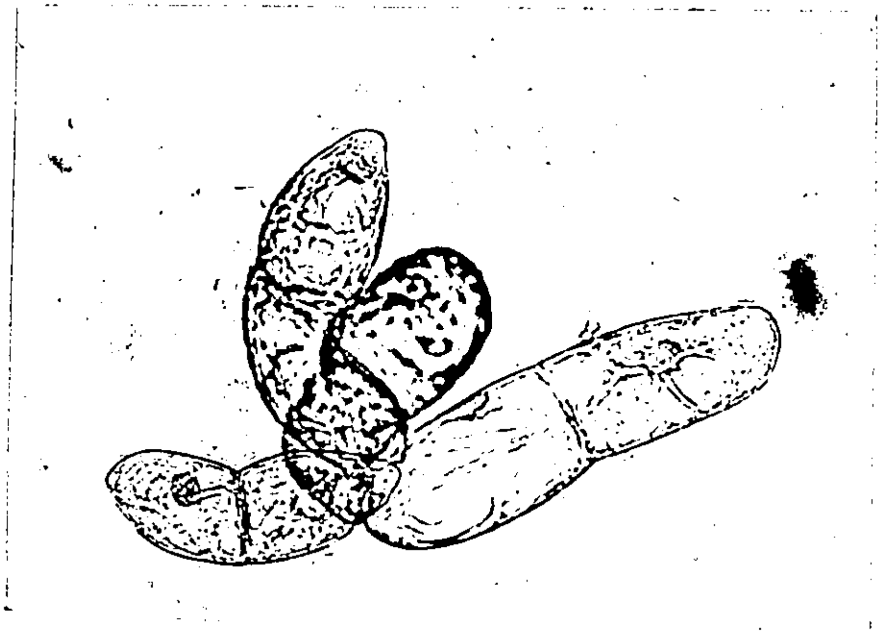


Figure 5 (X100)

Figure 6 (X400)

Figure 5 and 6. Prints of photomicrographs of tobacco cells grown in M.S. medium.

volumes of boiling 85% ethenol twice by refluxing for one hour. The combined alcoholic extracts were filtered and reduced to an aqueous residue by flash evaporation (40°C) under reduced pressure. An aliquot of this extract was chromatographed on cellulose thin-layer plates using n-butanol-acetic acid-water (6:1:2 v/v/v) for the first dimension and 2% HOAc for the second. The dried TLC plates were examined for the presence of phenolic glucosides (see below).

The remaining portion of the aqueous extract was acidified with 2 N HCl and then hydrolyzed for 30 min at 95°C (Ibrahim and Towers, 1960). The acid hydrolyzate was then extracted with diethylether using a continuous liquid-liquid extractor for 6 hours. The ether extract, which contained the free phenols, was evaporated and the residue was taken into a small volume of 85% methanol for chromatography. The latter was carried out on TLC plates coated with cellulose powder or silica gel. The TLC plates were developed with benzene-acetic acid-water (2:2:1, organic layer) for the first dimension and 2% HOAc for the latter. Both free and bound phenolic constituents were visualized in UV-light and their identity was confirmed by co-chromatography with reference compounds and by isolation and determination of their spectral characteristics using the standard methods (Ibrahim, 1961).

One major phenolic compound, scopoletin (6-methoxy-7-

hydroxycoumarin), was found to be the main constituent of the hydrolyzed tobacco cell extracts, and its identity was rigorously confirmed by co-chromatography and UV-spectra.

B.2.4. Quantitative determination of scopoletin

While small amounts of scopoletin were found free in tobacco cells, the bulk of this compound occured bound as its 7-O-glucoside, scopolin. The total scopoletin content was, therefore, determined after acid hydrolysis of tobacco cell extracts. Aliquots of the final ether extract were chromatographed as described in section B.2.3. The blue-violet UV-florescent spot, corresponding to scopoletin, ~~was carefully~~ scraped off the TLC plate, eluted with 85% ethanol and its absorbance was measured at 340 nm in a spectrophotometer (Model Spectronic 700, Bausch and Lomb). The scopoletin content was determined using duplicate tissue. A calibration curve was constructed using standard scopoletin.

B.2.5. O-methyltransferase activity

It was necessary to determine the peak of enzyme activity during growth of tobacco culture. OMT activity was determined using a standard enzyme assay which is described in a following section (B.4.1). Crude enzyme extracts were prepared at daily intervals and were assayed on duplicate tissue samples using caffeic acid and esculetin as substrates. Enzyme units were expressed as pKatals (pmol product formed per second, International Union of Biochemistry, 1973).

B.3. Isolation and Purification of Tobacco Culture O-Methyltransferase

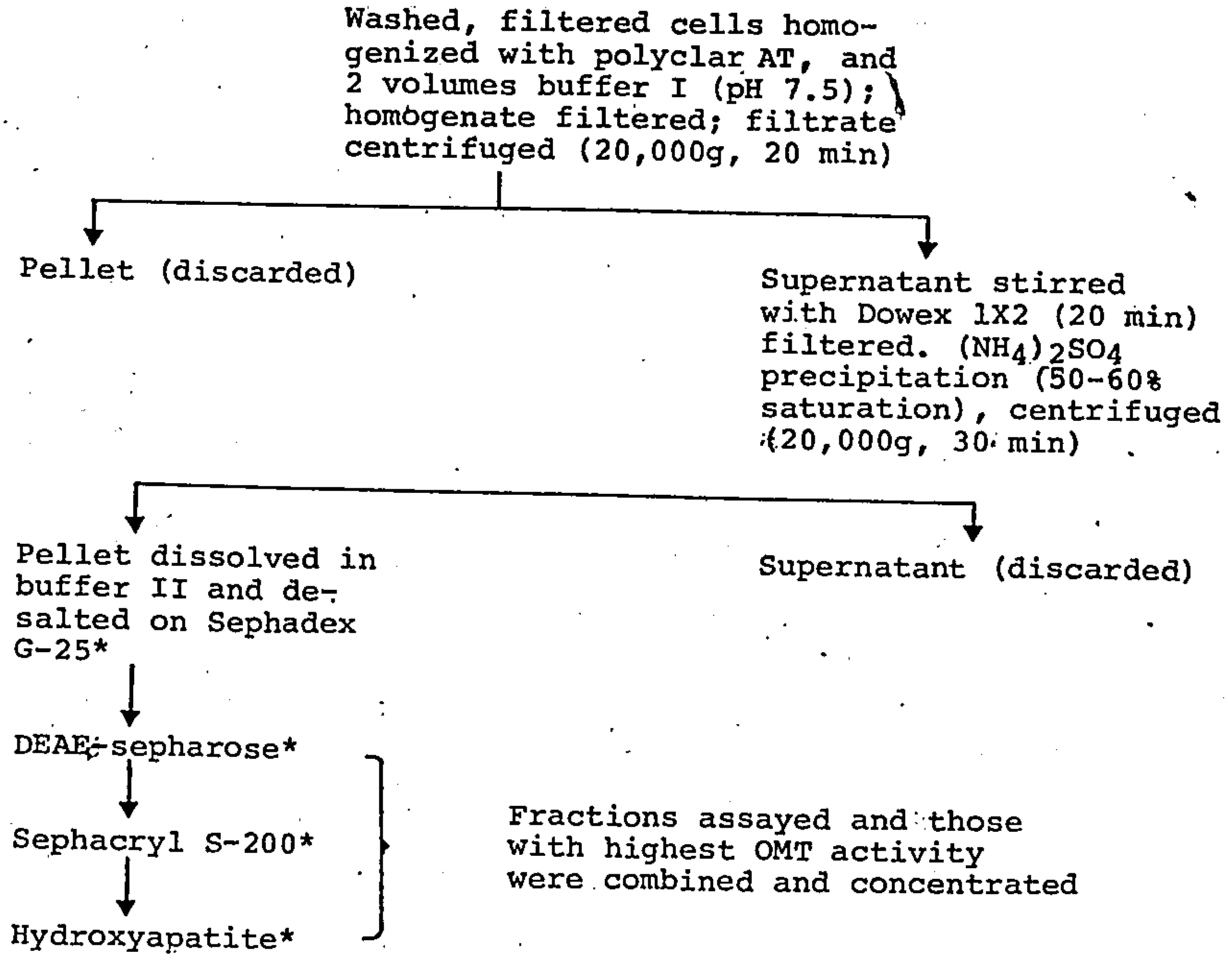
An outline of the procedure of extraction and purification of OMT is illustrated in Figure 7.

B.3.1. Enzyme extraction

The following potassium phosphate buffers, pH 7.5 were used for the extraction and purification of tobacco OMT. Unless otherwise indicated, all buffers contained 5 mM EDTA.

I. 100 mM phosphate buffer
II. 5 mM phosphate buffer
III. Linear gradient phosphate buffer (10-200 mM)

All extraction and purification steps were carried out in a cold laboratory at 2-4°C. The filtered, washed cells were homogenized in a chilled mortar with Polyclar AT (0.1% w/v) (Serva, Germ.) and two volumes of ice-cold buffer I. The homogenate was filtered through a layer of Nitex, pore size 25μ (Tober, Ernst & Traber Inc., Elmsford, New York), and the filtrate was centrifuged at 20,000 g for 15 minutes. The supernatant was stirred for 20 min with Dowex 1 X 2 (Sigma, U.S.A.) which had previously been



* Subjected to acrylamide gel electrophoresis

Figure 7. Summary of enzyme extraction and purification steps

equilibrated with the same buffer and then filtered through glass wool. This filtrate was designated as the 'crude enzyme preparation'.

B.3.2. Ammonium sulphate fractionation

The use of graded concentrations of ammonium sulphate is a common procedure for fractionation of protein mixtures based upon their solubility differences. A preliminary investigation of the distribution of enzyme activity in crude preparation was carried out while increasing the saturation of ammonium sulphate between 0 and 80% by 10-point increments at a time. The protein which precipitated after each treatment was assayed with a number of phenolic substrates. The results indicated that approximately 60% of the enzyme activity was found in the protein fraction which precipitated between 50-60% saturation. Figure 8 shows a typical experiment of ammonium sulphate fractionation. The protein pellets obtained after centrifugation were dissolved in the minimal amount of buffer II and were directly assayed for OMT activity. It can be seen that the highest specific activity of tobacco OMT was attained with the protein which precipitated between 50-60% saturation. Therefore, for enzyme purification the crude extract was brought to 50% saturation by the gradual addition of crystalline ammonium sulphate and continuous stirring while maintaining the pH at 7.5 using dilute KOH. The mixture was allowed to stand for 30 minutes, then centrifuged at

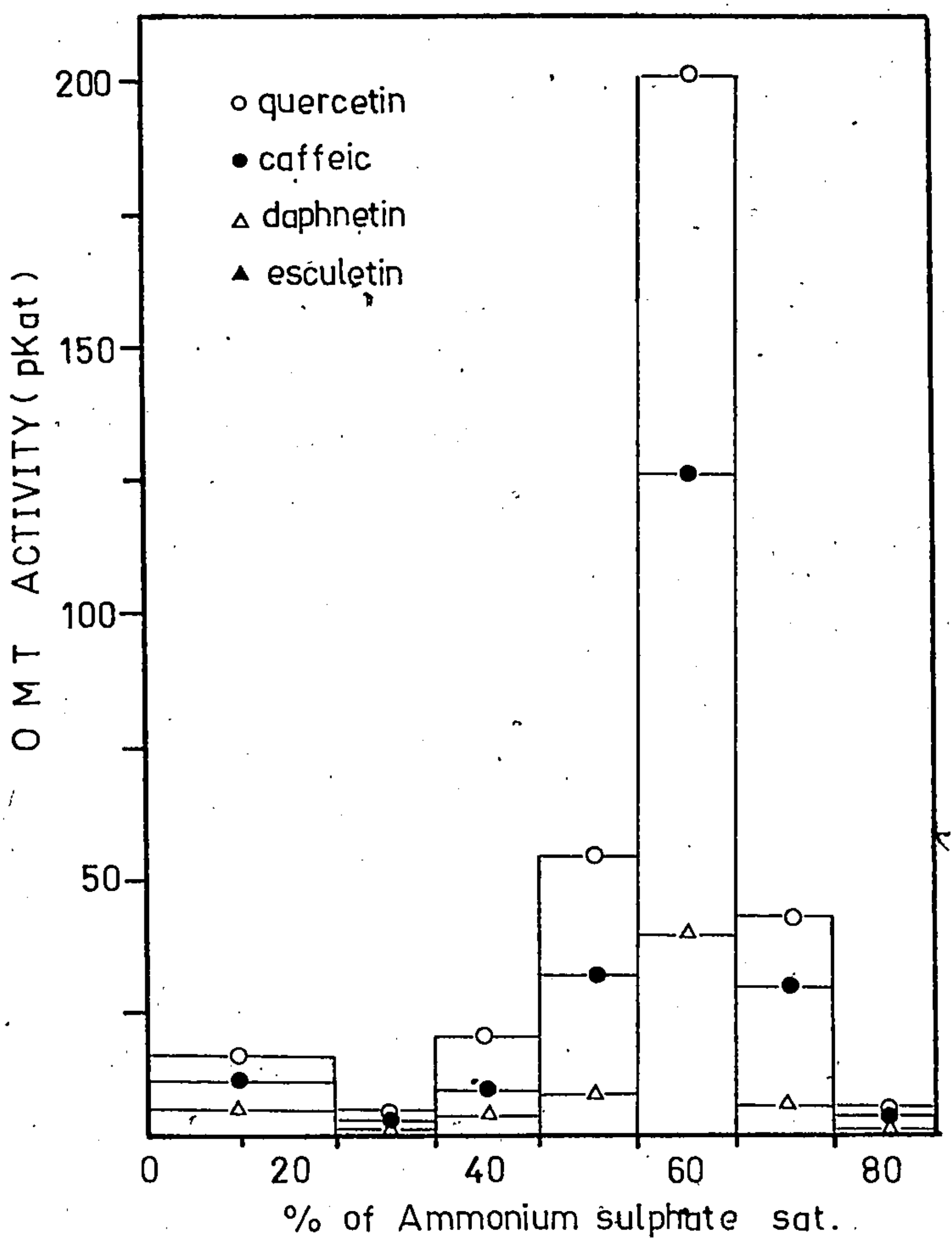


Figure 8. Ammonium sulphate fractionation of tobacco culture O-methyltransferase.

20,000g for 30 minutes and the pellet was discarded. The supernatant was brought to 60% saturation as described before and the protein which precipitated was pelleted by centrifugation and dissolved in the minimal amount of buffer II.

B.3.3. Desalting on Sephadex G-25

A Sephadex G-25 (Pharmacia, Fine Chemicals, Uppsala, Sweden) gel powder was soaked at least three hours in buffer II and the fine particles were eliminated by decantation. The swollen gel was packed into a column (2 x 50 cm) and then washed thoroughly with the same buffer for equilibration. The ammonium sulphate fraction (50-60% saturation), which was solubilized in buffer II, was applied to the column and the protein was eluted with the same buffer solution at a rate of 40 ml/hr. The UV-absorbance of the eluate was monitored at 280 nm using a UV-absorbance monitor (Model 100, Pharmacia, Fine Chemicals, Uppsala, Sweden) and the protein was collected and concentrated by ultrafiltration (Amicon Corp., Mass. U.S.A.) using membrane filter No. XM 50. This fraction was designated as the 'partially-purified' enzyme preparation.

B.3.4. Ion-exchange chromatography

Diethylaminoethyl (DEAE) sepharose CL-6B (Phamacia, Fine Chemicals, Uppsala, Sweden) was prepared according to the manufacturer's instructions. The gel was first diluted with buffer II to a consistency which allowed air bubbles to escape easily. The equilibration and washing process were carried out in the chromatography column (1.5 x 30 cm). The desalted enzyme preparation was applied to the packed column after the latter had been washed with at least three bed-volumes using the same buffer.

The protein was eluted first with 60 ml of buffer II followed by a linear gradient (10-200mM) of NaCl in buffer II, at a flow rate of 15 ml/hr. The eluate was continuously monitored at 280 nm through the UV monitor. The protein profile was recorded on a chart recorder (Servo-Riter II, Texas Inst. Incorp., Texas) at a speed of 1 in/hr. Fractions (4 ml) were collected by means of a fraction collector and were directly assayed against caffeic acid, quercetin, esculetin and daphnetin, as substrates, using the standard enzyme assay as described in section B.4.1. The protein fractions which exhibited the highest enzyme activity were pooled and concentrated by ultrafiltration.

B.3.5. Gel-filtration chromatography

Sephacryl S-200 (superfine, Pharmacia, Fine Chemicals, Uppsala, Sweden) was supplied by the manufacturer in swollen form as a thick suspension. It was first diluted with buffer II and then packed into the chromatographic column (1.5 x 65cm). The column was washed and equilibrated with at least three bed-volumns of the same buffer.

The concentrated DEAE-sepharose eluate (Section B.3.4.) was carefully layered on top of the gel and was eluted with buffer II at a rate of 16 ml/hr. The absorbance of protein was monitored as described before. The fractions collected were assayed against the four phenolic substrates. The most active fractions were combined and reduced in volume by ultrafiltration.

B.3.6. Chromatography on hydroxyapatite

Hydroxyapatite was used as the final step of purification. A suspension of the absorbent was washed three times with buffer II, then poured into a small column (1 x 7cm). After the column had been equilibrated with three bed-volumns of the buffer, the enzyme protein was applied and the latter was eluted first with 10 ml of the same buffer solution, followed by a linear gradient (10-200 mM) of buffer III. Two-ml fractions were collected and assayed for OMT activity with each of the four major substrates.

B.4. Assay of O-Methyltransferase and Identification of the Methylation Products

B.4.1. Enzyme assay

In the present study, OMT activity was determined by a radioactive assay, using ^{14}C-methyl-S-adenosyl-L-methionine (NEN, Boston, Mass.) as the methyl donor. The standard assay mixture consisted of 50 nmoles of the phenolic substrate (dissolved in 10 µl of dimethylsulfoxide), 0.7 nmole of SAM (containing 55,000 c.p.m.), 1.4 µmole β-mercaptoethanol and the enzyme protein. The mixture was added up to a final volume of 250 µl with 0.1M phosphate buffer, pH 7.5. The reaction was carried out in duplicates in 1.5-ml Eppendorff microcentrifuge tubes. The reaction mixture was incubated at 35°C for 30 minutes in a constant temperature water bath with continuous stirring. The reaction was terminated by the addition of 20 µl of 6 N HCl containing 0.25% methylviolet as an organic-aqueous phase indicator.

The methylated products were extracted twice with diethylether by shaking vigorously in an Eppendorff rotary shaker (Model 3200, Brinkmann, N.Y.) for 5 minutes. The organic phase was separated by centrifugation using an Eppendorff microcentrifuge (Model 3300, Brinkmann, N.Y.) at top speed for 2 minutes. The ether layer was transferred into scintillation vials by means of a micro-suction device and then evaporated. Ten ml of the scintillation fluid (5g PPO/l of toluene) was added to each vial and the total radioactivity of the reaction products was determined by liquid scintillation counting using a liquid scintillation spectrometer (Model Unilux II, Nuclear Chicago Inc., Ill.). This represented the total methylating activity of the enzyme. The radioactivity of the reaction products was calculated on the basis that 10^5 c.p.m. was equivalent to 1.27 nmoles of the product formed.

B.4.2. Definition of enzyme units

The enzyme unit used in the present studies was expressed in pKatals as recommended by the International Union of Biochemistry (IUB, 1973). One pKat is defined as the amount of activity which converts one pmol of substrate per second under the assay conditions.

B.4.3. Protein estimation

The protein content of crude preparation and most column eluates was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The protein content of the last column eluate (hydroxyapatite) was determined by the method of Warburg and Christian (1942) and was calculated using the following equation:

$$\text{mg protein/ml} = 1.54\ A_{280} - 0.74\ A_{260}$$

B.4.4. Separation and identification of the reaction products

In order to separate and identify the *m*- and *p*-methylation products, the ether extracts of the enzyme assays were subjected to thin-layer chromatography using a number of supports and solvent systems as shown in Table IV. The developed thin-layer chromatograms were then placed in contact with Kodak No-Screen X-Ray films (8 x 10 in, Eastman Kodak Co., Rochester, New York.) for 3-10 days depending upon the amount of radioactivity in the products, and then developed. The identity of *m*- and

Table IV. R_f values of m- and p-methylation products of some substrates used with the tobacco OMT assay[a)]

Substrate	R_f values of products m-	p-	Solvent system[b)]	Support[c)]
Caffeic acid	Ferulic 0.25	Isoferulic 0.37	A	MNC
Quercetin	Isorhamnetin 0.81	Rhamnetin 0.77	B	EKS
Esculetin	Scopoletin 0.47	Isoscopoletin 0.63	C	MNS
Daphnetin	Hydrangetin			
	0.75	–	D	CA
	0.52	–	C	CA
5-Hydroxyferulic acid	Sinapic 0.27	?[d)] 0.34	A	CA

a) Structures of substrates and products are shown in Figure 15.

b) Solvent systems used: A, n-BuOH-NH_4OH-EtOH-C_6H_6 (5:3:1:1); B, C_6H_6-pyridine-HCOOH (36:18:5); C, n-BuOH-NH_4OH-EtOH-C_6H_6-CCl_4 (5:3:5:4:1); D, C_6H_6-HOAc-H_2O (2:2:1, organic layer).

c) TLC supports used: MNC, MN-cellulose; EKS, Eastman Kodak silica plates; MNS, MN-silica gel; CA, cellulose-Avicel (FMC Corp., Pa.).

d) Unidentified product.

p-methylation products was confirmed by co-chromatography with reference compounds and visualization in UV-light (366 nm) (Model C5, Ultra-Violet Product Inc., Calif.).

The labelled products were carefully scrapped off the TLC plates and transferred to the scintillation vials. 20 mg Cab-O-Sil powder (Cabot Corp., Boston, Mass.) and 10 ml scintillation fluid were added before being counted for radioactivity.

B.5. Acrylamide Gel Electrophoresis

B.5.1. Analytical disc gel electrophoresis

During the various steps of enzyme purification (Sections B.3.3.-3.6.) the protein profiles were monitored by the use of acrylamide gel electrophoresis. Standard, 7.5% polyacrylamide gels (pH 8.9) were prepared according to the method of Davis (1964), but without sample gel. Except for ammonium persulphate, all essential stock solutions were stored in brown glass bottles at 2-4°C and solutions were freshly prepared when required for use.

The glass columns were 7 cm long with an inner diameter of 6 mm. Prior to use they were soaked in cleaning solution, then rinsed first in distilled water and then in 0.5% solution of Kodak Photo-Flo solution. For a run of 12 gels, 18 ml of the separating gel solution was used. Each tube was filled with 1.5 ml of the solution. Onto the top of the gel solution, a layer of distilled water was placed carefully by means of a micropipet. The gels were allowed to polymerize under a day-light fluorescent tube for about 15-20 minutes. An interface could be seen indicating that the gel had solidified. 200 µl of the stacking gel (3.1%) was added and polymerized on the top of the separating gel. The gel columns were placed in the electrophoretic chamber and electrode buffer reservoirs were filled with Tris-glycine buffer, pH 8.3 and the trapped air bubbles were removed.

The enzyme preparation containing a known quantity of protein (100-200 µg) was desalted and freeze dried. It was redissolved in 100 µl of the sample buffer (10% sucrose in buffer II) and loaded underneath the buffer onto the stacking gel. One ml of 0.001% bromophenol blue was stirred into the upper buffer and served as tracking dye.

An electric current from a power supply (Buchler Instruments Division, N.J.) was connected, the cathode (-) to the upper reservoir and the anode (+) to the lower one. Electrophoresis was carried out at 5 mA/gel and was completed when the dye migrated to a distance of approximately 5 mm from the bottom of gels.

Immediately after stopping electrophoresis, the gel columns were removed from the glass tubes and fix-stained with 1% Amido-black (in 7% aqueous acetic acid) for a minimum of one hour. The gels were then destained electrophoretically using 7% aqueous acetic acid for 2-4 hours. They were then removed from the columns and stored in 7% acetic acid.

B.5.2. Gel isoelectric focussing

The method for isoelectric focussing employed here was similar to that described by Vindogradov *et al.* (1973). Carrier ampholyte solution, pH 3-10 (40% w/w) was obtained from LKB-produkter AB, Sweden. Gels containing 7.5% w/w acrylamide in 0.4 x 10 cm columns were prepared by mixing the reagents in the following proportions:

a. 8 ml distilled water

b. 0.3 ml carrier ampholyte solution

c. 3 ml acrylamide solution (30g acrylamide and 1g N,N'-methylene bisacrylamide in 100 ml water)

d. 0.7 ml ammonium persulphate (1%)

All gels were preelectrophoresed for 2 hours prior to application of the samples to remove excess persulphate. Monoethanolamide (4% v/v) was used as the cathode electrolyte (top) while the anode electrolyte (bottom) was 2% (v/v) phosphoric acid. Protein samples, containing 1% ampholyte in 20% surcose solution were applied on top of the gels and were covered with 100 µl of 10% surcose solution containing 1% ampholyte. Electrofocussing was carried out at 4°C at a constant voltage of 150V for about 16 hr. At the end of the run, focussed gels were fix-stained overnight in a solution containing 0.5% $CuSO_4$, 0.05% Coomassie blue, 10% of acetic acid and 27% ethanol (Otavsky and Drysdale, 1975). They were then soaked in a solution of 0.01% Coomassie blue in 10% acetic acid and 25% ethanol for 6 hr. The gels were destained in a solution of 10% acetic acid and 10% ethanol and stored in 10% acetic acid.

For pH gradient determination, 0.5 cm segments of the gel were cut, extracted with one ml of double distilled water and the pH was measured.

B.5.3. Sodium dodecylsulphate gel electrophoresis

7.5% SDS-polyacrylamide gels (0.4 x 6cm) were prepared as described by Weber and Osborn (1969). The gels were polymerized in 0.1 M phosphate buffer pH 7.5, containing 0.1% SDS. To prepare the sample, each 50 µl of protein was mixed with 3 µl of 0.05% bromphenol, 1 drop of glycerol, 5 µl of β-mercaptoethanol and 50 µl of SDS-phosphate buffer. After mixing for 10 min, the samples were applied onto the gels. Electrophoresis was performed at a constant current of 4 mA per gel for 4-5 hr. The gels were stained for 6 hr with 0.25% Coomassie blue in 50% methanol, 10% acetic acid, and were destained with 5% methanol in 7.5% acetic acid. The gels were then stored in 7.5% acetic acid solution.

B.6. Molecular Weight Determination

The molecular weight of the purified enzyme was determined by gel filtration on a calibrated column of Sephacryl S-200 (Andrews, 1965) and by thin-layer gel filtration using Sephadex G-150 (Radola, 1968).

B.6.1. Gel-filtration chromatography

For gel filtration, a Sephacryl S-200 (superfine) column (1.5 x 65cm) was used that has been calibrated with a number of standard proteins (Table V). The void volume of the column was determined by elution of a sample of blue dextran (Andrews, 1965). The K_{av} values for the proteins used were plotted against their molecular weights on a logarithmic scale. The molecular weight of the enzyme protein was then calculated using the equation

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where, Vt = total volume
Vo = void volume
Ve = elution volume

B.6.2. Thin-layer gel filtration

Sephadex G-150 (superfine) gel suspension was prepared according to the manufacturer's instructions. The gel (4.8 grams) was soaked in 100 ml 0.1 M NaCl in 0.05 M phosphate buffer, pH 7.5. The slurry was degased briefly before being used to coat the plates. The glass plates (20 x 40cm) were coated with 0.8 mm thick-layer by means of a thin-layer spreader. The coated plates were transferred immediately to the moist chamber of TLG apparatus. Two 20 x 6cm strips of Whatman No. 3 filter paper were used to establish contact between the buffer solution and the gel layer at both ends. The gel was allowed to equilibrate within the chamber overnight.

For application of the samples, a starting mark-line was drawn on the back of the glass plate at a distance of 3 cm from the upper end. The enzyme and reference proteins (1-2%) including cytochrome c, which served as marker protein, were applied as 5 µl spots directly along the marker line on the plate. The angle of the apparatus was adjusted at 10° and the run was completed within a period of 5-6 hours.

At the end of the run, the plate was removed from the chamber immediately and a piece (20 x 37cm) of filter paper (Whatman No.3) was rolled onto the gel layer for 30-60 second in order to absorb the liquid phase containing the proteins. This paper chromatogram was fix-stained with 0.25% Coomassie blue (in 9:1 v/v methanol-glacial acetic acid) for 10 minutes, rinsed with tap water until no dye could be washed off, then destained with a mixture of methanol-acetic acid-water (5:1:5 v/v/v).

For the calculation of molecular weight, the distance travelled from the starting line to the middle of each spot was accurately measured. The results were expressed in terms of the R_c value which is defined as the ratio of the migration distance of cytochrome c (dc) to that of the test protein (dp). Therefore,

$R_c = dc / dp$

In order to construct a calibration curve, the R_c values of the reference proteins were plotted against their molecular weights on a logarithmic scale.

Table V. Standard proteins used in the calibration of gel filtration column and TLG for M.W. determination.

Proteins	M.W. (dalton)	Source
1 Cytochrome c	11,700	Sigma
2 Ribonuclease	13,700	Pharmacia
3 Chymotrysinogen A	25,700	"
4 Ovalbumin	45,000	"
5 BSA (monomer)	68,000	Calbiochem
6 BSA (dimer)	136,000	"
7 Aldolase	158,000	Pharmacia

B.6.3. Separation of subcellular fractions

Subcellular fractionation of tobacco cell homogenates was carried out using the method of Russel et al. (1971), after some modification as illustrated in Figure 9. Filtered cells were ground in a chilled mortar with 2 volumes of extraction buffer I. The homogenate was filtered through a layer of Nitex and the residue was washed twice with 2 volumes of the same buffer. The combined filtrates were centrifuged at 500g for 10 minutes and the residue was combined with the original cell debris. Both were designated as the 'cell wall fraction'. The supernatant was further centrifuged at 12,000g in an ultracentrifuge for 10 minutes to obtain a particulate fraction which was designated as 'the 12,000g residue' and was mainly made up of mitochondria. The 12,000g supernatant was again centrifuged at 105,000g for one hour whereby the 'soluble fraction' and a high-speed 'particulate fraction' were obtained. The particulate fractions were washed twice with buffer I and then resuspensed in measured aliquots of the same buffer for enzyme assay using the standard method described before (Section B.4.1).

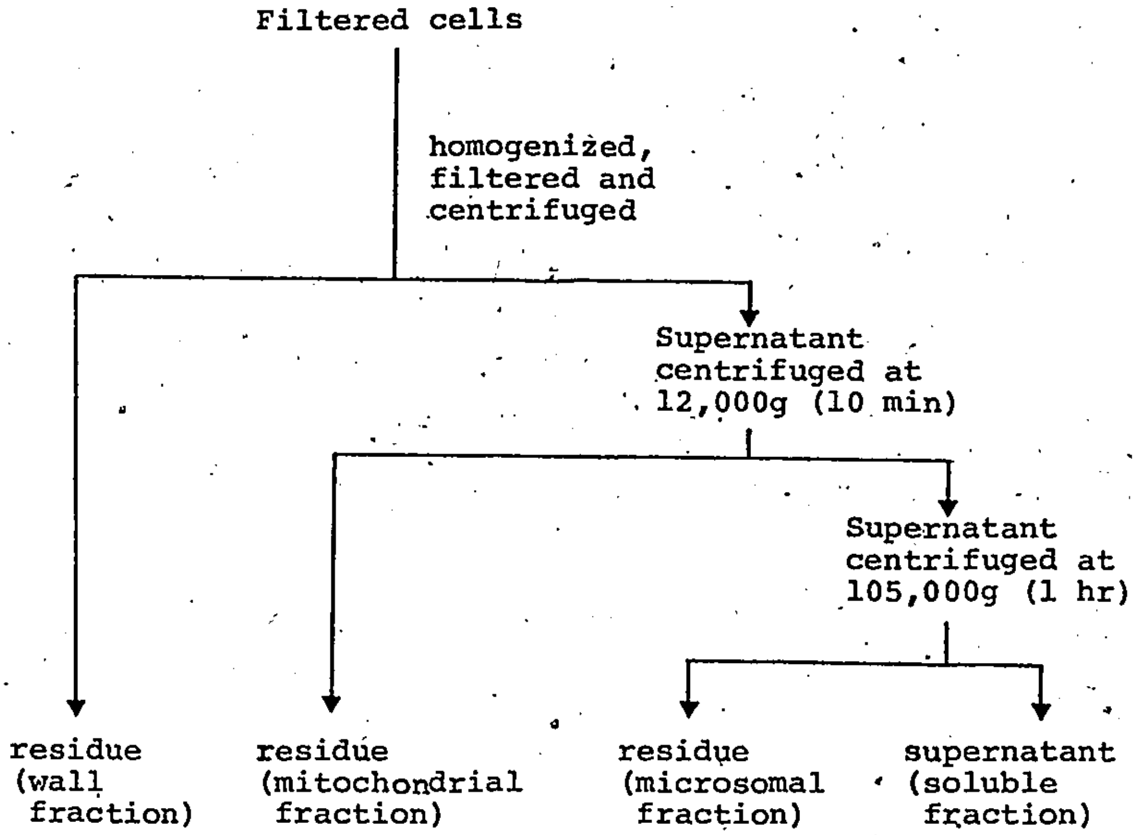


Figure 9. Outline of the procedure for subcellular fractionation of cell homogenate*

* Adapted from Russel *et al.*,1971

Section C. RESULTS.

C.1. Growth of Tobacco Suspension Culture

The growth of tobacco culture, which was maintained on a defined nutrient-medium, appeared as a fine suspension consisting of a mixture of free cells and small cell clumps as shown in Figure 5 and 6, the fresh weight growth curve (Figure 10) exhibited an initial lag phase which lasted for 2-3 days. The lag phase was characterized by active protein synthesis and low OMT activity against both caffeic acid and esculetin as substrates. This was followed by a period of rapid growth that was associated with increasing OMT activity. The latter reached a maximum after 7 days culture growth. It is interesting to note that the activity of OMT was concomitant with the accumulation of scopoletin and its glucoside, scopolin (shown combined in Figure 10).

C.2. Optimization of O-Methyltransferase Assay

Preliminary experiments were carried out to optimize the conditions of the enzyme assay using crude and partially purified (Sephadex G-25 fraction) enzyme preparations which were prepared as previously described in Section B.3.1.

C.2.1. Choice of the form of L-methionine as a methyl group donor

The results given in Table VI show that despite the presence of ATP, methionine was a poor donor of methyl

Figure 10. Growth parameters of tobacco cell suspension culture. Time course of the change in O-methyltransferase activity with caffeic acid (▲) and esculetin (△) as substrates, and the accumulation of scopoletin (□) during growth of tobacco cell culture as measured by fresh weight (●) and soluble protein content (○) of the cells. Free and glucoside-bound scopoletin were determined after acid hydrolysis of cell extracts, partition in diethyl ether and chromatography. The amount of total scopoletin was determined colorimetrically using the Folin reagent and a calibration curve.

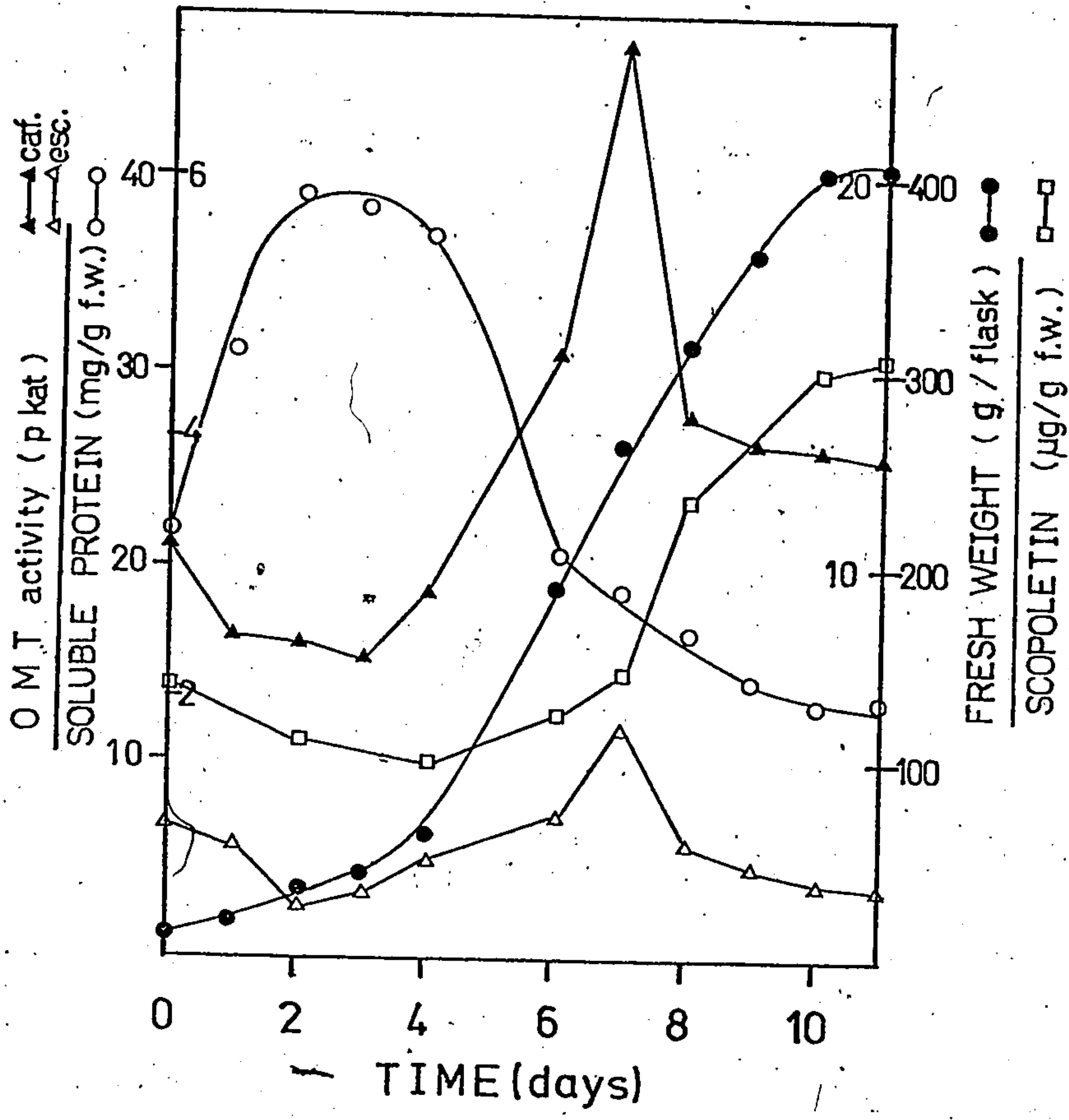

O M.T activity (p kat)
SOLUBLE PROTEIN (mg/g f.w.)
▲—▲ caf.
△—△ esc.
○—○
40
30
20
10
6
4
2
FRESH WEIGHT (g / flask) ●—●
SCOPOLETIN (µg/g f.w.) □—□
20
10
400
300
200
100
0
2
4
6
8
10
TIME(days)

Table VI. Effects of different methyl group donors on tobacco OMT

Assay conditions[a]	OMT activity[d] (c.p.m./assay)	
	Crude enzyme[b]	Partially purified enzyme[c]
$^{14}CH_3$-methionine (0.7 nmol)	2142	4003
$^{14}CH_3$-methionine + ATP (0.7 nmol)	4748	5367
$^{14}CH_3$-SAM (0.7 nmol)	17629	20284

a) The enzyme assay described in section B.4.1. was used.

b) Prepared as described in section B.3.1.

c) Sephadex G-25 eluate of the ammonium sulphate (50-60% saturation) protein fraction.

d) Assayed against caffeic acid as substrate, c.p.m. represents total methylation as means of duplicate determinations.

groups as compared with S-adenosyl-L-methionine which resulted in 4-fold increase of activity.

C.2.2. Effect of β-mercaptoethanol

The effect of β-mercaptoethanol on OMT activity is shown in Table VII. When 1.4 µmole (5.6 mM) of freshly prepared β-mercaptoethanol was added to the reaction mixture, a considerable increase in enzyme activity was detected with both the crude and partially purified enzyme preparations. The reaction rate was found to increase by approximately 75 to 90% above that of the control.

C.2.3. Effect of substrate concentration

A freshly prepared enzyme preparation (in phosphate buffer, pH 7.5) containing approximately 100µg protein per assay was used for the study of the effect of substrate concentration on OMT activity. The standard assay described in Section B.4.1. was used except that varying amounts of caffeic or esculetin were added, while maintaining the same concentration of $^{14}CH_3$-SAM. The results (Figure 11) show a linear relationship between the concentration of either substrate and the rate of reaction up to 0.1 mM. While the reaction was saturated at 0.2 mM esculetin, however, the saturation point was not reached at that concentration with caffeic acid.

C.2.4. Effect of temperature

The temperature tested on enzyme activity ranged from

Table VII. Effect of β-mercaptoethanol on tobacco OMT activity[a)]

Enzyme preparation	Substrates	Enzyme activity (c.p.m.)[b)]		% increase above control
		Control	Plus 1.4 µmole β-mercapto-ethanol	
Crude	esculetin	2127	3807	79
	caffeic acid	5320	10267	93
Sephadex-G-25 fraction	esculetin	2352	4116	75
	caffeic acid	8012	14292	78

a) Using the standard assay described in section B.4.1.

b) Counts are the net differences between assays with and without added substrates, in order to eliminate the possibility of methylation of β-mercaptoethanol.

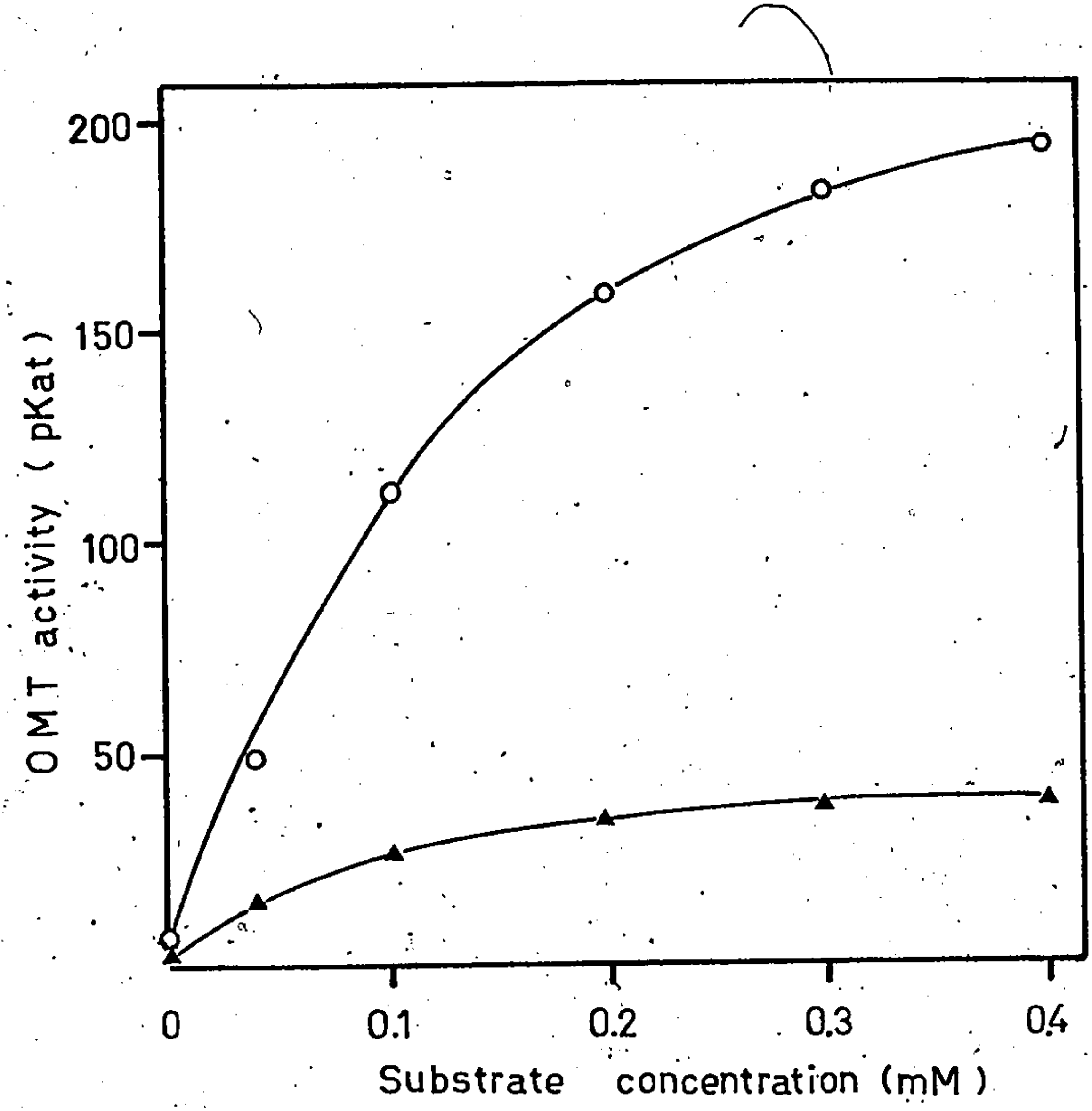


Figure 11. Effect of substrate concentration on O.M.T. activity against caffeic acid (o-o) and esculetin (▲-▲). The reaction mixture contained approximately 100 µg protein in 200 µl phosphate buffer, pH 7.5, $^{14}CH_3$-SAM and 1.4 µmole β-mercaptoethanol. The enzyme activity was measured as described in the Methods section.

25-50°C, with 5 increments. The results, presented in Figure 12. show that the rate of reaction increased with increasing temperatures between 25-35°C, after which the activity declined rapidly. Therefore, the optimal temperature for the assay of tobacco OMT was considered to be 35°C, and was routinely used in further experiments.

C.2.5. Time course of the reaction catalyzed by <u>O</u>-methyltransferase

In this experiment, the concentration of substrates (caffeic acid and esculetin) was fixed at 0.2 mM per assay and the reaction mixtures were incubated at 35°C for different periods of time. The results, presented in Figure 13, show a linear relationship between the reaction rate and time of incubation up to 45 minutes. For further assays, 30-min incubation time was used.

C.2.6. pH optimum

Using the standard assay with caffeic acid and esculetin as substrates the results, shown in Figure 14a, b, respectively, indicated that the pH optimum for the methylation of both substrates was 7-7.5 when assayed in 0.1 M phosphate buffer.

C.3. Purification of Tobacco Culture <u>O</u>-Methyltransferase

Tobacco culture OMT was isolated from 7-day-old cells. The procedure outlined in Figure 7 for the purification of the enzyme consisted mainly of :

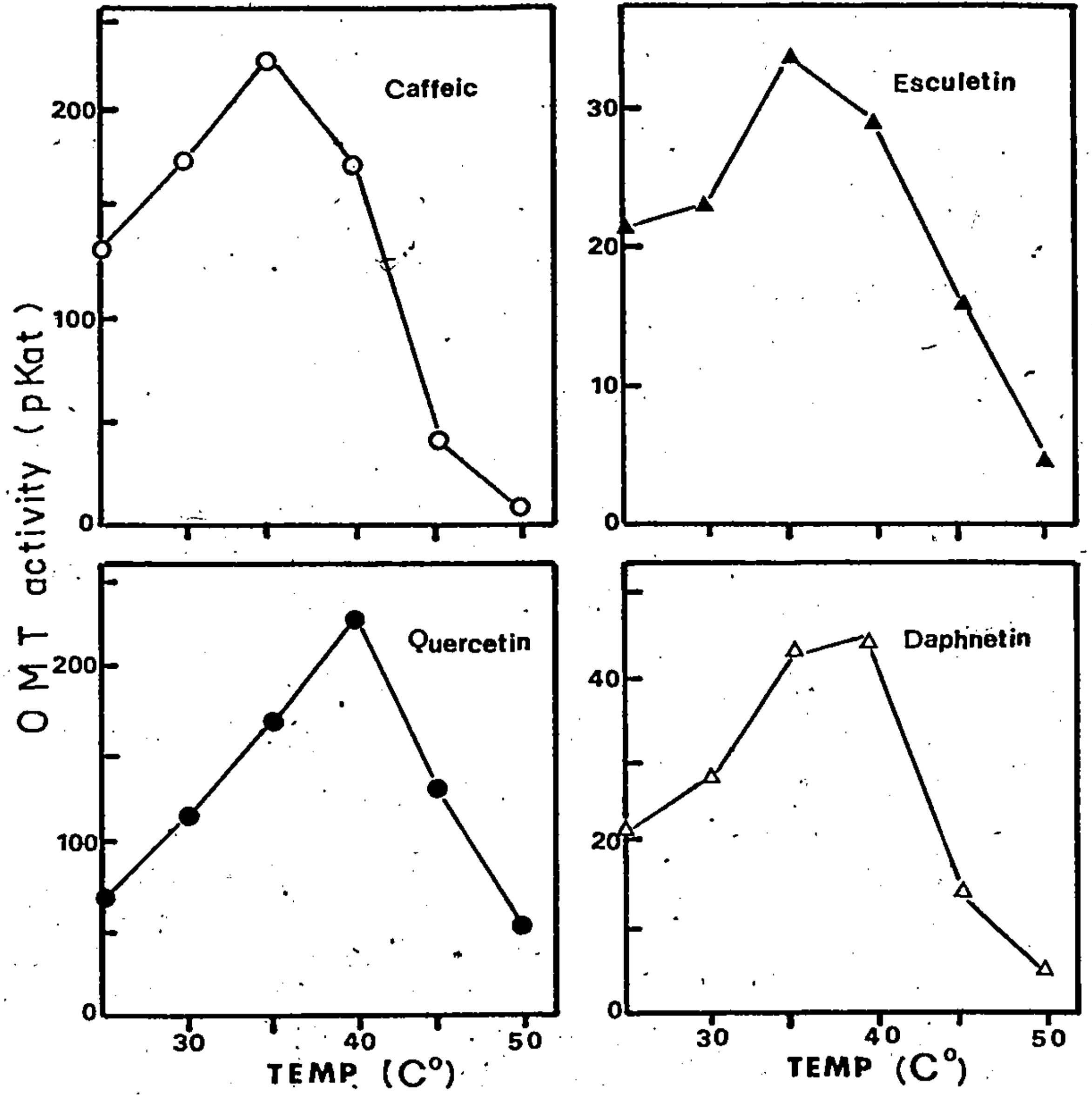


Figure 12. Effect of temperature on O.M.T. activity against four substrates: caffeic acid (○), esculetin (▲), quercetin (●) and daphnetin (△). The reaction mixtures were incubated at different temperatures for 30 minutes. The enzyme activities were measured as described in the Methods section.

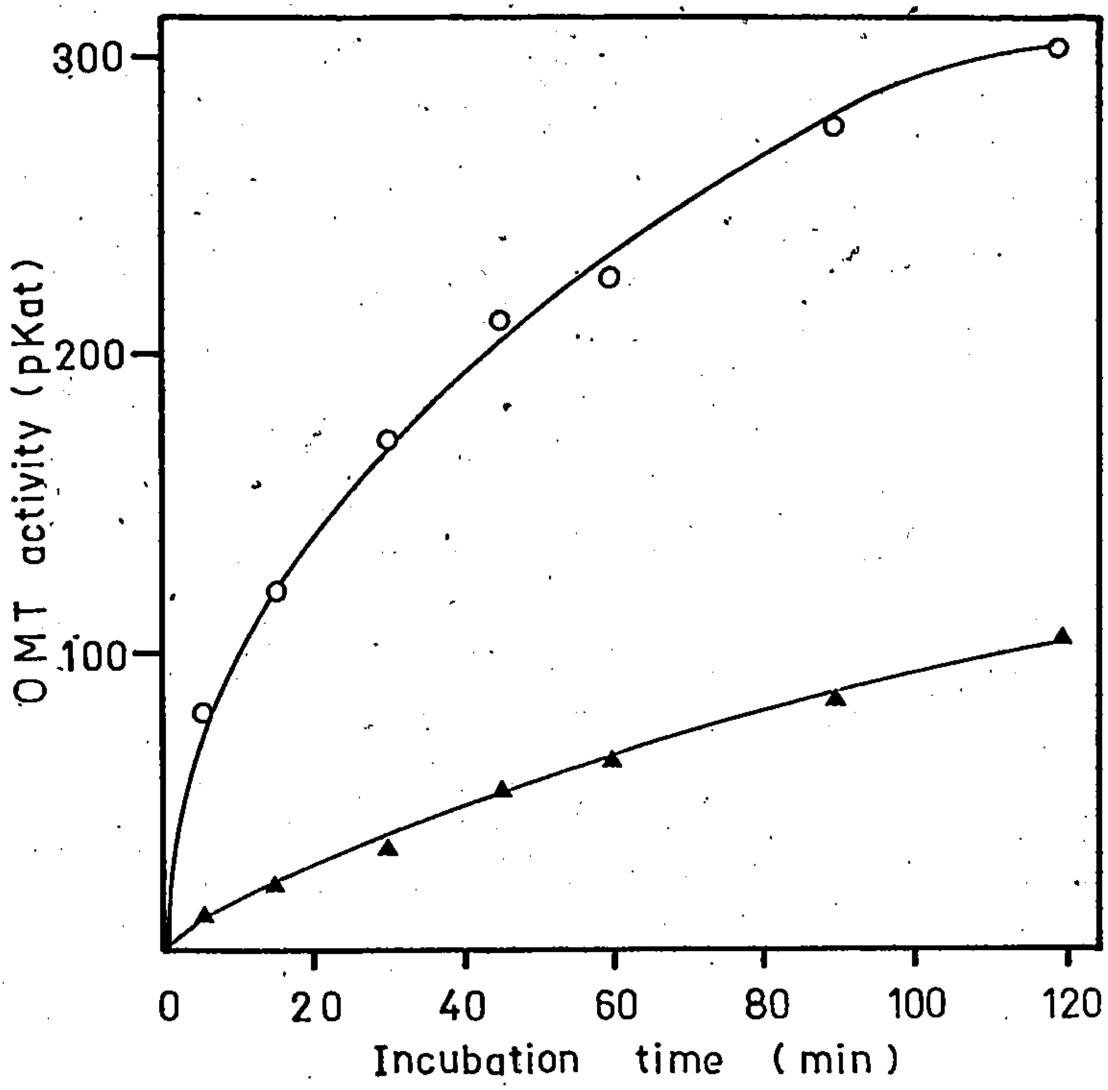


Figure 13. Time course of the reaction catalyzed by tobacco O-methyltransferase, incubation at 35°C, ○-○ caffeic acid, ▲-▲ esculetin.

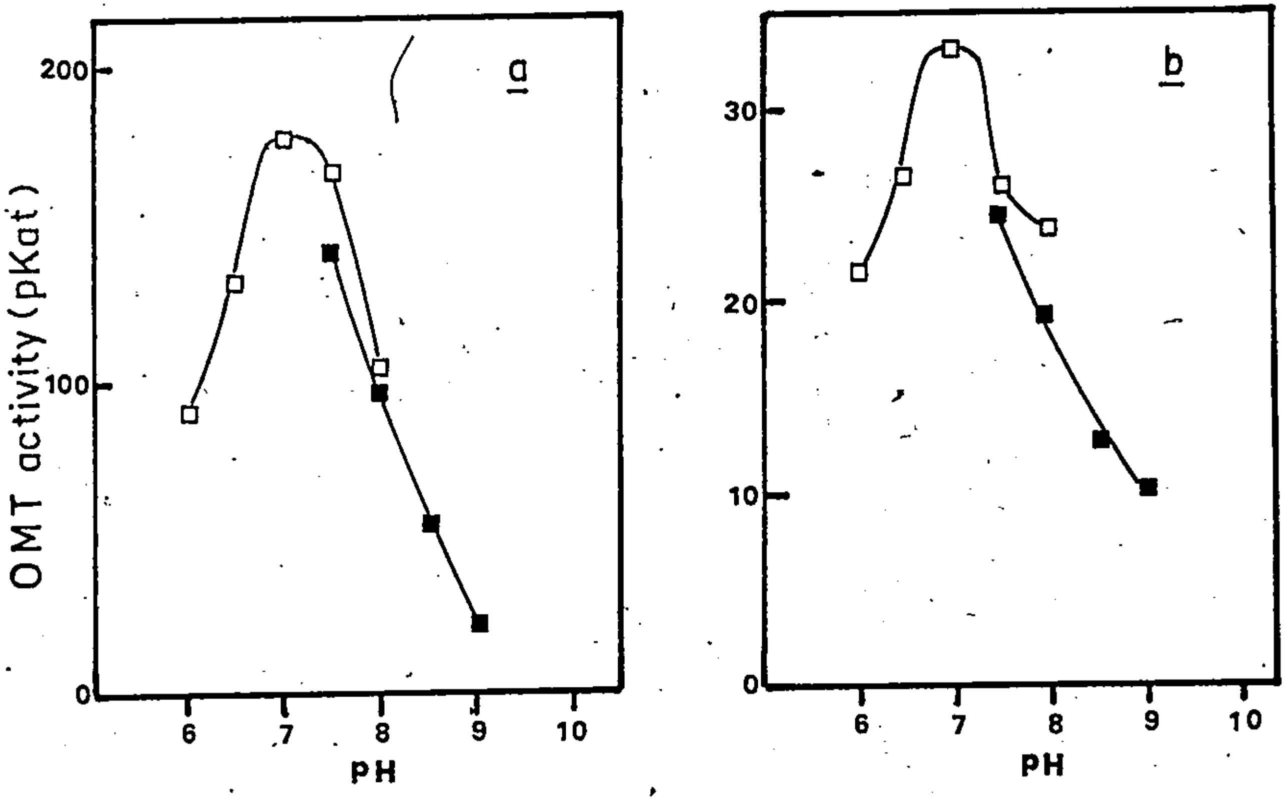


Figure 14a and b. The pH optimum of the partially purified O-methyltransferase assayed against caffeic acid (a) and esculetin (b). Reaction mixtures were adjusted to different pH values using 100mM KH_2PO_4-NaOH(□) or Tris-HCl buffer(■)

a) Fractional precipitation of the protein using solid ammonium sulphate (50-60% saturation) (See Figure 8).

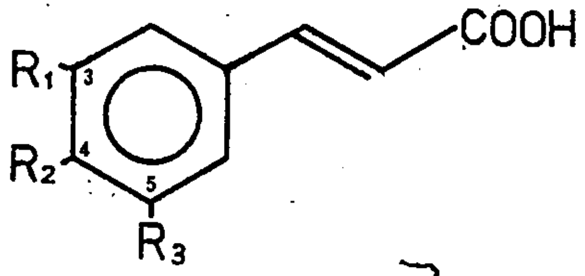
b) Desalting the protein fraction by chromatography on a Sephadex G-25 column.

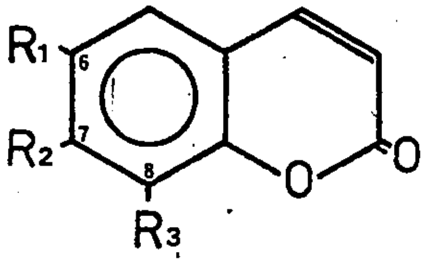
c) Further purification of the desalted protein by successive chromatography on DEAE-sepharose, Sephacryl S-200 and hydroxyapatite columns.

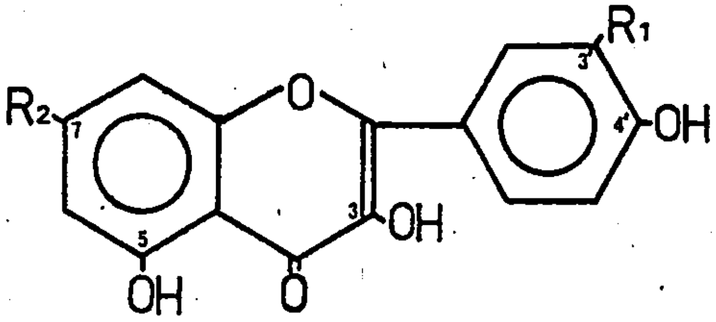
Preliminary work indicated that the additon of 5 mM EDTA to the buffers used in enzyme extraction, equilibration of chromatographic columns and elution of proteins helped to stabilize the enzyme and improved its activity during the assays.

Throughout the different steps of purification, fractions from column eluates were routinely assayed against four major substrates - caffeic acid, esculetin, daphnetin and quercetin; using the standard assay previously described in Section B.4.1. The choice of substrates was meant to represent a cinnamic acid (caffeic), two coumarins (esculetin and daphnetin) and a flavonol (quercetin), all of which share the common property of having o-dihydroxy groupings (Figure 15).

Furthermore, the protein profiles of the active fractions, obtained from column eluates, were monitored for purity by acylamide gel electrophoresis.







	R₁	R₂	R₃
CAFFEIC ACID	OH	OH	H
FERULIC ACID	OMe	OH	H
ISOFERULIC ACID	OH	OMe	H
5-HYDROXYFERULIC ACID	OMe	OH	OH
SINAPIC ACID	OMe	OH	OMe
ESCULETIN	OH	OH	H
SCOPOLETIN	OMe	OH	H
ISOSCOPOLETIN	OH	OMe	H
DAPHNETIN	H	OH	OH
HYDRANGETIN	H	OH	OMe
QUERCETIN	OH	OH	
ISORHAMNETIN	OMe	OH	
RHAMNETIN	OH	OMe	

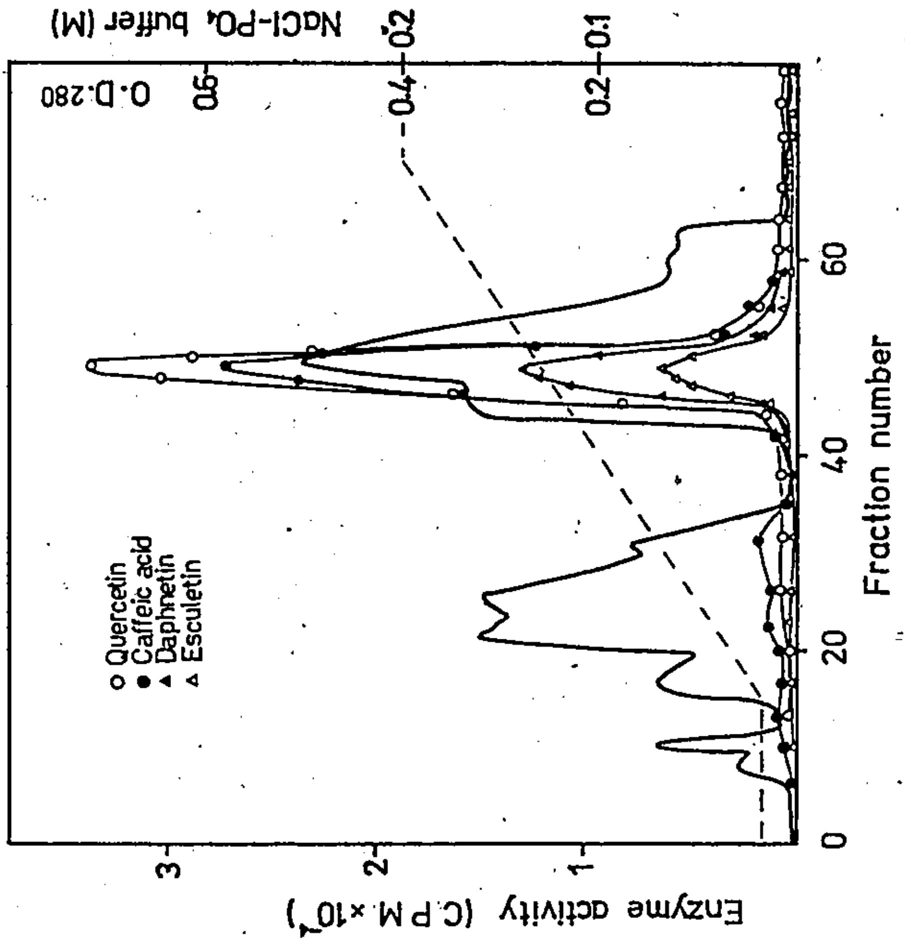
Figure 15. Structures of the major substrates and their methylated products.

C.3.1. Elution profiles and purification data

The elution profiles of the enzyme protein from DEAE-sepharose, Sephacryl-S200 and hydroxyapatite columns are shown in Figures 16, 17 and 18. Most of the enzyme activity was eluted from the DEAE-sepharose with 0.1-0.14 M NaCl in buffer II (Figure 16). It is interesting to note that the enzyme activities against the four substrates fell within one pronounced peak during the purification process though another minor peak of activity was observed after elution from hydroxyapatite. Furthermore, there was no significant change in the relative activity of the enzyme towards these substrates.

The data presented in Table VIII show that the procedure used for enzyme purification resulted in 80-, 90- and 45-fold increase in enzyme activity against caffeic acid, esculetin and quercetin, respectively. This was made possible by using a narrow range (50-60% saturation) of ammonium sulphate precipitation (Figure 8) which resulted in the elimination of approximately 95% of the other protein contaminations and a 6-12 fold increase in the specific activity as compared with that of crude extract. The highly purified enzyme protein had a specific acitivty of 4.07, 1.5 and 4.4 nKat/mg for caffeic acid, esculetin and quercetin, respectively (Table VIII). Furthermore, the specific activity ratios of esculetin to caffeic acid did not change appreciably during the different steps of enzyme purification.

Figure 16. Chromatography of tobacco culture O-methyltransferase on a DEAE-sepharose column (30 x 1.5 cm). Protein (———) fractions, 4 ml each, were eluted at 15 ml/hr with a linear gradient (10-200 mM) of NaCl in 0.05M phosphate buffer, pH 7.5 (..........) and assayed with four substrates as described in the Methods section.



Enzyme activity (C.P.M.×10⁴)
3
2
1
0
20
40
60
Fraction number
O.D.280
0.6
0.4
0.2
NaCl-PO₄ buffer (M)
0.2
0.1
○ Quercetin
● Caffeic acid
▲ Daphnetin
△ Esculetin

Figure 17. Chromatography of tobacco culture O-methyltransferase on Sephacryl S-200 column (1.5 x 65 cm). Protein (——) fractions, 4 ml each, were eluted at 15 ml/hr with 0.05M phosphate buffer, pH 7.5 and assayed with four substrates as described in the Methods section.

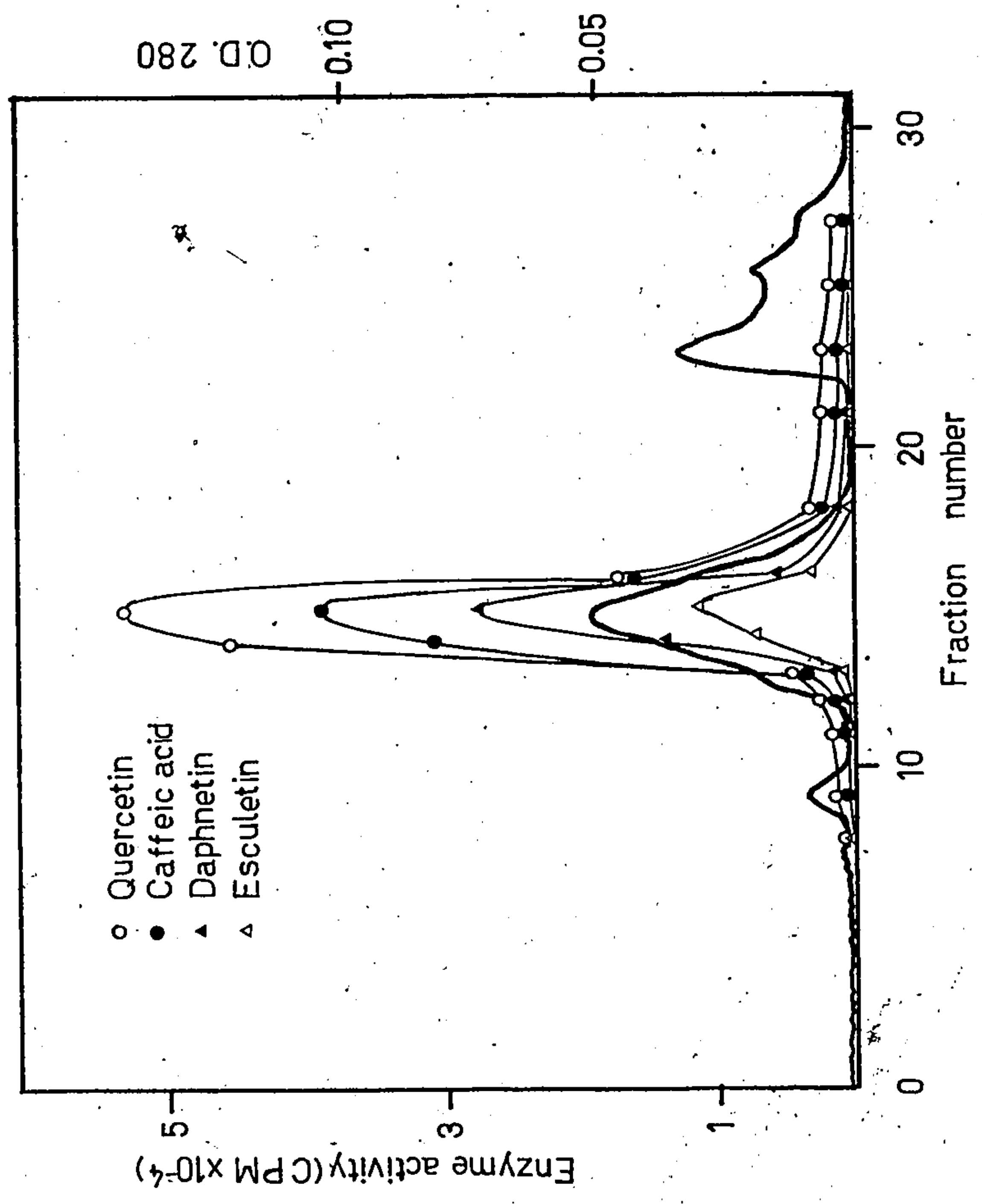

○ Quercetin
● Caffeic acid
▲ Daphnetin
△ Esculetin
Enzyme activity (C PM x10-4)
5
3
1
O.D. 280
0.10
0.05
0
10
20
30
Fraction number

Figure 18. Chromatography of tobacco culture O-methyltransferase on a hydroxyapatite column (7 x 1 cm). The enzyme protein (———) was eluted in 1.5 ml fractions at 10 ml/hr using a linear gradient (10-200 mM) phosphate buffer, pH 7.5 (.........). Fractions were assayed with two substrates as described in the Methods section.

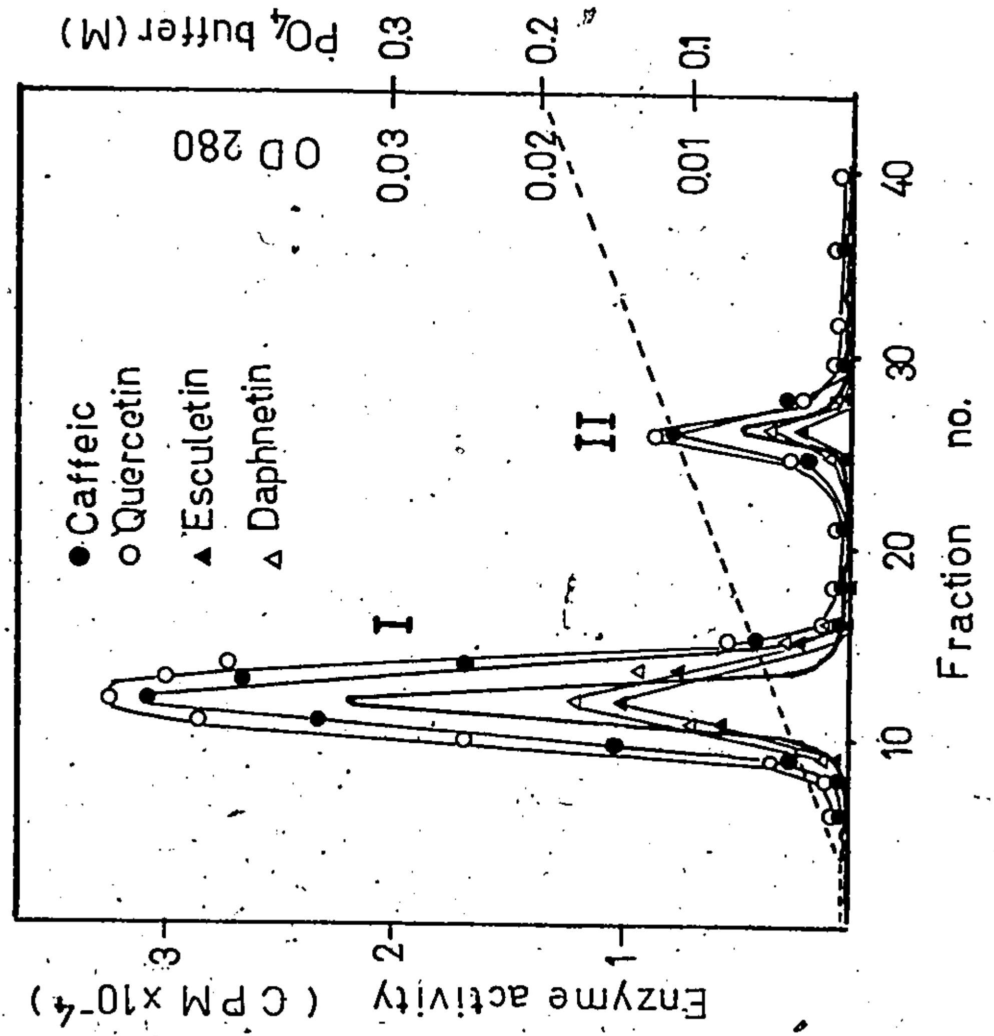

● Caffeic
○ Quercetin
▲ Esculetin
△ Daphnetin
I
II
Fraction no.
10
20
30
40
Enzyme activity (C P M x10⁻⁴)
1
2
3
O D 280
0.01
0.02
0.03
PO₄ buffer (M)
0.1
0.2
0.3

Table VIII. Purification data of tobacco culture OMT against caffeic acid, esculetin and quercetin*

Fraction	Total protein (mg)	Total activity (nkat)			Specific activity (nkat/mg)			Purification (fold)			Ratio of specific activity	
		Caf.	Esc.	Que.	Caf.	Esc.	Que.	Caf.	Esc.	Que.	Esc./Caf.	Que./Caf.
Crude extract	503.8	29.9	8.2	44.3	0.05	0.016	0.09	-	-	-	0.27	1.5
Amonium sulphate (50-60% sat.)	26.3	14.6	3.3	14.2	0.56	0.13	0.55	9.3	8.1	6.1	0.23	0.92
Sephadex G-25	22.8	13.2	3.6	13.2	0.58	0.16	0.57	9.7	10.0	6.3	0.27	0.95
DEAE-sepharose	10.9	11.3	3.1	12.4	1.04	0.28	1.14	17.3	17.5	12.7	0.28	1.10
Sephacryl S-200	2.3	3.8	1.1	5.2	1.65	0.48	2.26	27.5	30.0	26.1	0.29	1.40
Hydroxyapatite	0.27	1.1	0.4	1.2	4.07	1.48	4.44	81.4	92.5	45.8	0.36	1.09

* Details of the purification steps and assay conditions are described in the Method section.

However, that of quercetin/caffeic acid fluctuated significantly during the purification process. The purification data for the enzyme against daphnetin (not shown) was quite similar to that of caffeic acid and attained approximately 90-fold purification.

C.3.2. Acrylamide gel profiles

Figure 19 shows the electrophoretic protein patterns of the enzyme preparation during the different steps of purification. Examination of the gel profiles shows a major protein band (A_1) constituting about 25% of the partially purified preparation. During the purification process, there was parallel disappearance of this major protein and appearance of 2 other protein bands (2 and 3, gels B-D). When tested for OMT activity, the three protein bands were active against caffeic acid and quercetin, as substrates. It was considered, therefore, that the major protein (peak I) eluted from hydroxyapatite column (Figure 18), represents the dissociated form of the enzyme, whereas peak II and band E4 (Figure 19) represent the remaining undissociated form of the enzyme. Up to this stage of investigation, the significance of both protein bands could not be determined. Therefore, the pooled fractions of peak I were used for the study of enzyme characteristics.

C.4. Subcellular Distribution of O-Methyltransferase Activity

Figure 20 shows the subcellular distribution of OMT

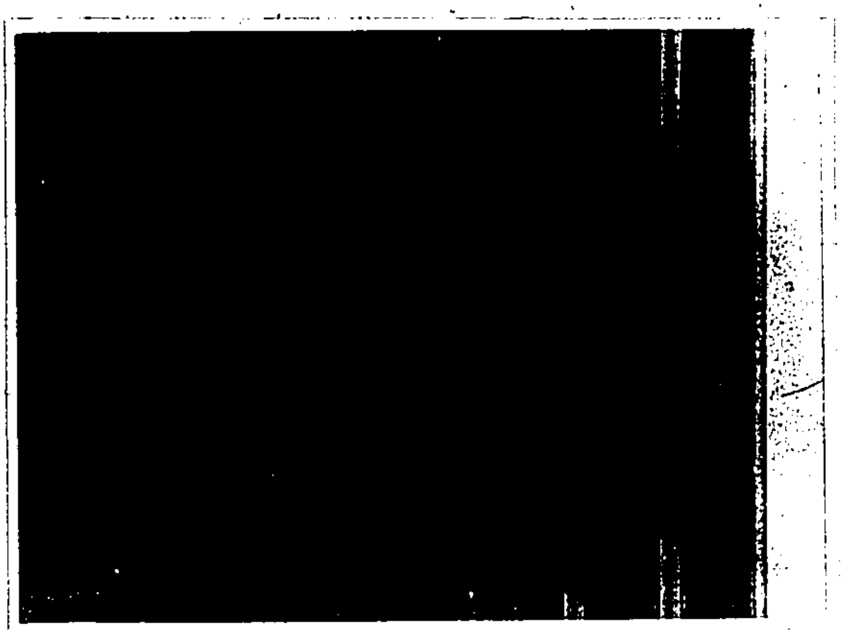


Figure 19. Acrylamide gel electrophoresis of tobacco culture O-methyltransferase during purification steps. Protein samples (50-100 µg in 30 µl of 10% surcose) were applied directly to standard 7.5% acrylamide gels prepared according to the method of Davis (1964), but without sample gel. Electrophoresis was conducted in Tris-glycine buffer, pH 8.3 using 5 mA/tube for 45-60 min and bromophenol blue as marker. The gels were stained with 1% Amido Schwartz in aq. 7% acetic acid and destained electrophoretically. A. $(NH_4)_2SO_4$ (50-60% sat.) fraction after being desalted on Sephadex G-25; B. DEAE-sepharose eluate; C. Sephacryl S-200 eluate; D. Hydroxyapatite eluate (peak I); E. Hydroxyapatite eluate (peak II); F. Hydroxyapatite eluate (peak I) on SDS gel electrophoresis.

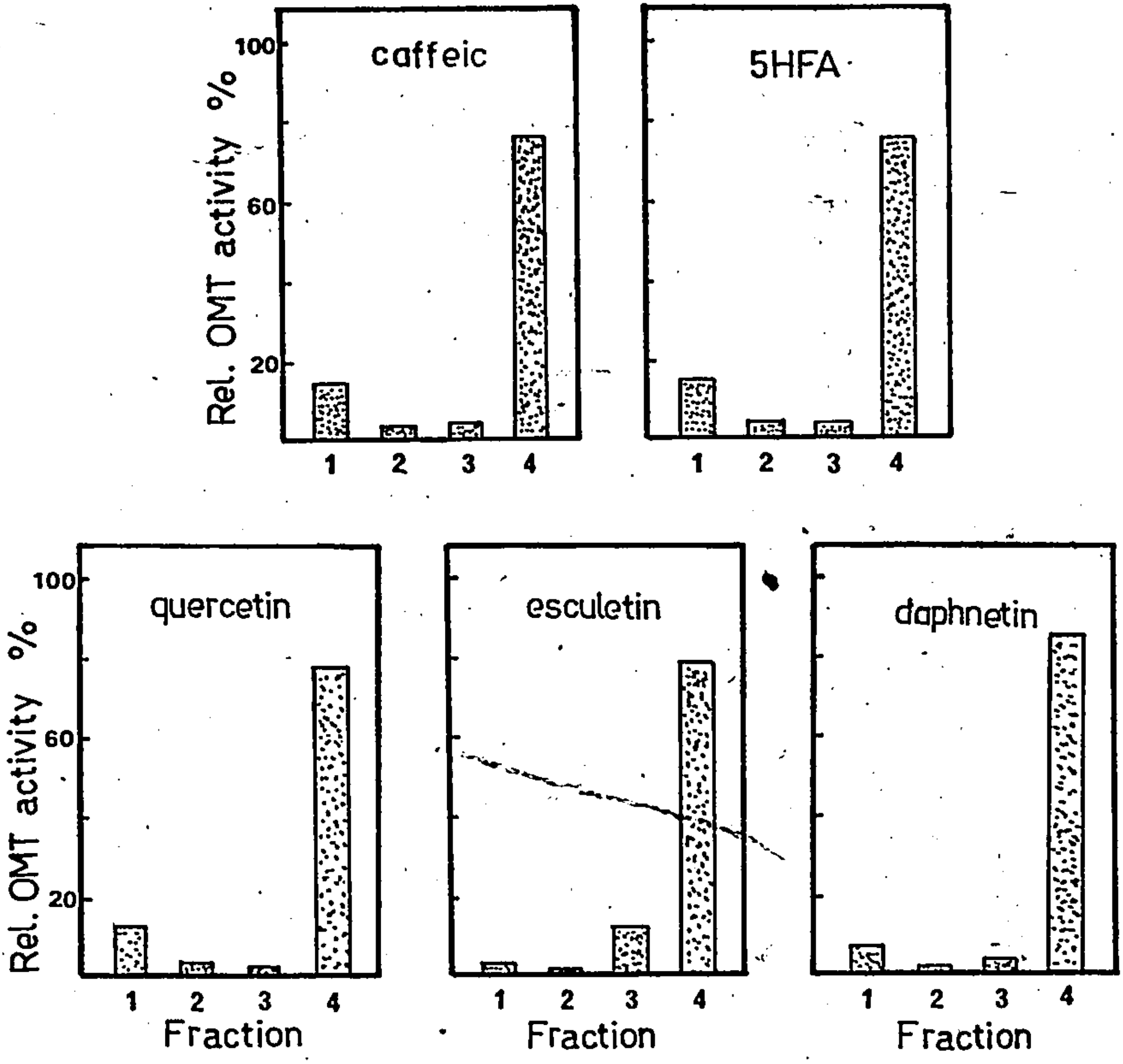


Figure 20. Subcellular distribution of tobacco culture OMT activity against five substrates. The method was described in Section B.6.3. Fractions 1, cell wall; 2, mitochondrial; 3, microsomal; 4, soluble protein.

activity in cultured tobacco cells. The buffer extract of the cell wall fraction was found to contain approximately 3.5 to 16.7% of total enzyme activity when various substrates were used. The supernatant, which represents the soluble fraction, contained most of the activity (75-85%). Approximately 3 to 5% of the total activity was associated with the mitochondrial and microsomal fractions. The results obtained conform well with the observations of Tong and D'Iorio (1977) on the distribution of rat liver enzyme.

C.5. Properties of the Purified Enzyme

C.5.1. Stability

Stability of the partially purified preparation was studied by mixing equal volumes of the enzyme preparation with solutions of test stabilizers dissolved in buffer II. The effect of a number of stabilizing agents on the stability of the enzyme was studied. The mixtures were stored at 2-4°C and the enzyme activity was assayed immediately after mixing, after 48 hours and after 120 hours of storage.

As shown in Table IX, storage of the enzyme preparation in phosphate buffer, pH 7.5 at 2-4°C resulted in a 50% loss of activity within 48 hours. The stability of the enzyme improved slightly in the presence of 5% ethylene glycol, but not with 5% glycerol. Most of these substances have been reported to stabilize many enzymes (Storme, 1967; Byrne, 1974). In sharp contrast with other OMTs (Kuroda, 1975; Poulton and Butt, 1975) the addition of 5mM EDTA to tobacco culture enzyme was found to stabilize and improve its activity. β-mercaptoethanol (5 mM), dithiothreitol (5 mM) and bovine serum albumin (0.5%) were found to have some protective effect on enzyme activity (Table IX). However, since both SH-group reagents and albumin markedly affect protein determination by the Lowry method, their use was discarded and was replaced by 5 mM EDTA.

The thermostability of the purified enzyme was studied at 50°C for different periods of time. After rapid cooling, the enzyme activity was assayed against the four major substrates. The results (Figure 21) show that about 90% of OMT activity was lost in one minute and the enzyme was completely inactivated after 20-25min incubation at 50°C.

C.5.2. Substrate concentration and kinetic constants

In order to evaluate the relative affinities of OMT activity towards the major substrates, the methylation rates were determined using a constant amount of the purified enzyme preparation and increasing substrate concentrations (40-800 μM). The maximum velocities obtained with the substrates used are shown in Table X. The apparent K_m values were plotted according to the method of Lineweaver-Burk as shown in Figure 22. Vmax values and the corresponding K_m values are given in Table X. Based on the V/K_m ratio, the efficiency of m-methylation appears to follow

Table IX. Stability of the partially purified OMT from tobacco cells[a)]

Addition	Relative activity (%)[b)]	
	After 48 hr	After 120 hr
None	52	20
5 mM β-mercaptoethanol	104	76
5 mM dithiothreitol	82	78
5 mM EDTA	98	79
0.5% bovine serum ablumin	90	85
5% ethylene glycol	67	55
5% glycerol	52	23

a) Sephadex G-25 eluate was prepared as previously described in the Methods section.

b) Compared with control assays using caffeic acid as substrate at zero time = 100%, after 48 and 120 hr storage at 2-4°C.

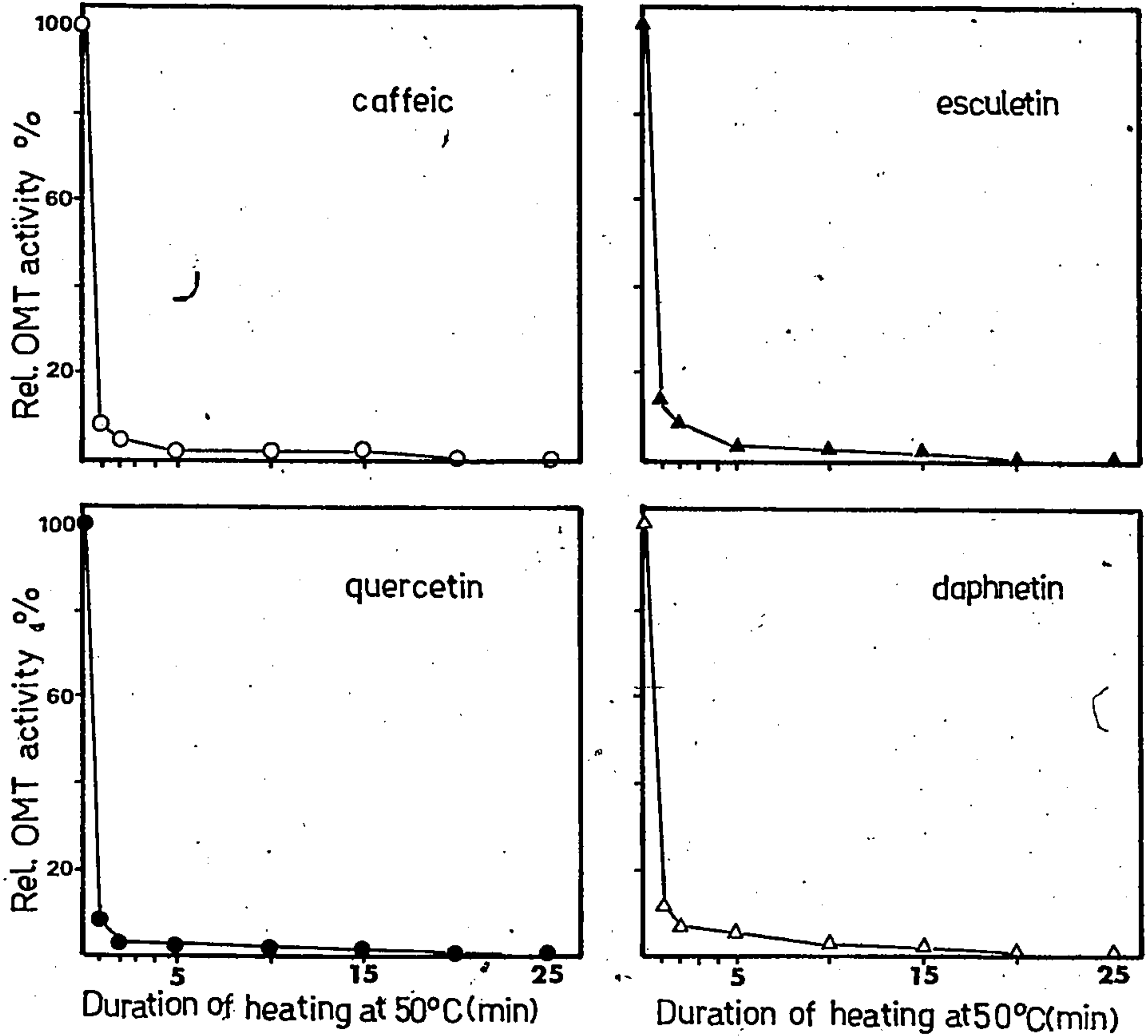


Figure 21. Thermal stability of tobacco culture O-methyltransferase. Enzyme activities were measured after heating at 50°C for different periods in the absence of substrates.

Table X. Some characteristics of tobacco culture O-methyltransferase against five different substrates[a].

Characteristic	Caffeic acid	5HFA	Esculetin	Daphnetin	Quercetin
$V_{total\ methylation}$ (pkat)	169.9	230.6	33.1	60.0	190.5
$V_{m\text{-}methylation}$ (pkat)	153.3	153.3	18.7	60.0	37.7
m/p-methylation[a]	9.4	1.9	1.3	-	0.33
K_m substrate (μM)[b]	100.0	100.0	45.0	45.0	45.0
10^{-6} x V/K_m (pkat M^{-1})	1.53	1.53	0.42	1.33	0.84
K_m SAM (μM)[c]	4.0	n.d.	4.0	4.0	4.4
K_i SAH (μM)[d]	2.5	n.d.	2.5	2.5	2.8

a) The reaction products were chromatographed in different solvent systems as described in Table IV and the autoradioactivity in m- and p-methylation products was determined as described in the Methods section. Daphnetin was methylated in the m-position only. The methylation of quercetin resulted in isorhamnetin, rhamnetin (Figure 25) and five other minor products in 1:3:1:1 ratio, respectively.

b) Values calculated from Lineweaver-Burk plots (Figure 22).

c),d) Values calculated from Lineweaver-Burk plots in absence of SAH and in presence of 10 μM SAH, respectively.

n.d. Not determined.

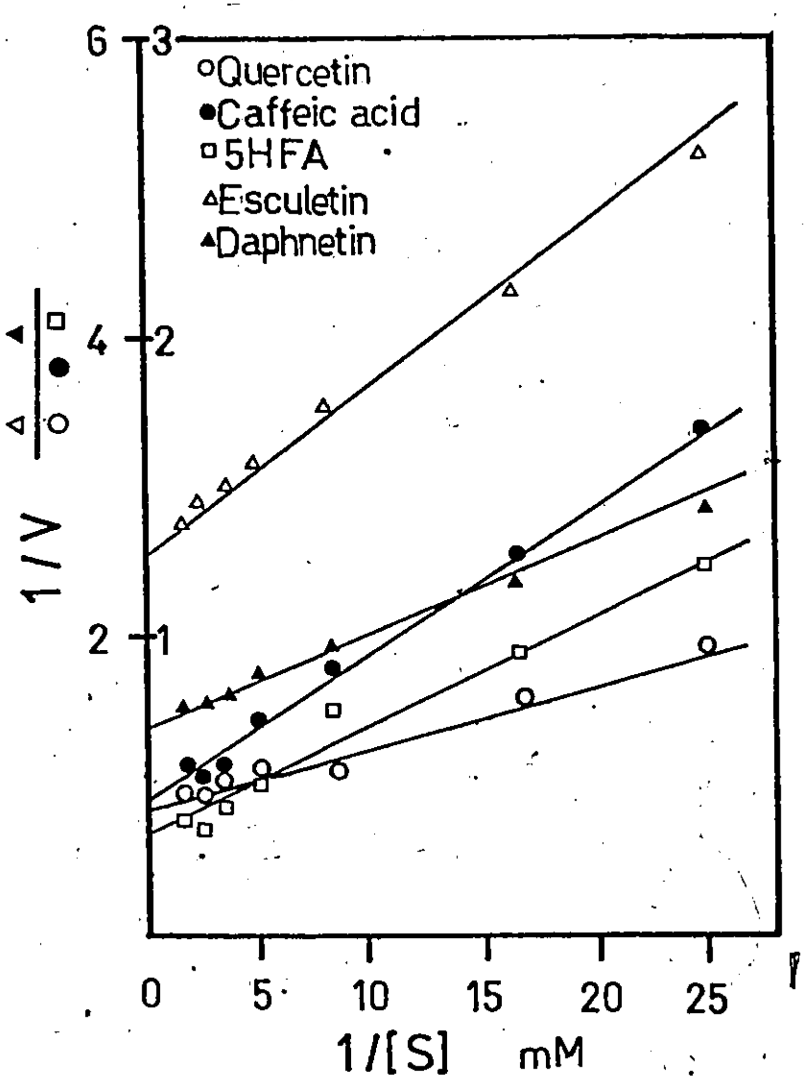


Figure 22. Lineweaver-Burk plots of enzyme activity (V) and substrate concentration (S). The standard assay, described in the Methods section was used except the substrate concentration was varied as indicated.

the order:-

caffeic acid > 5HFA > daphnetin > quercetin > esculetin.

The apparent K_m for SAM was determined in the presence of saturating concentration of the major substrates. The Lineweaver-Burk plots of the data obtained show K_m values which varied between 4 and 4.4 µM.

C.5.3. Effect of inhibitors

C.5.3.1. Inhibition by products of phenolic substrates

Ferulic acid, scopoletin, sinapic acid and rhamnetin - the methylated products of caffeic acid, esculetin, 5HFA and quercetin, respectively, were chosed to investigate the possible inhibition of the enzyme reaction by its products. The results (Table XI) show that the rate of methylation of all substates used was inhibited by 23-55% in the presence of the methylated compounds tested.

C.5.3.2. Inhibition by S-adenosyl-L-homocysteine

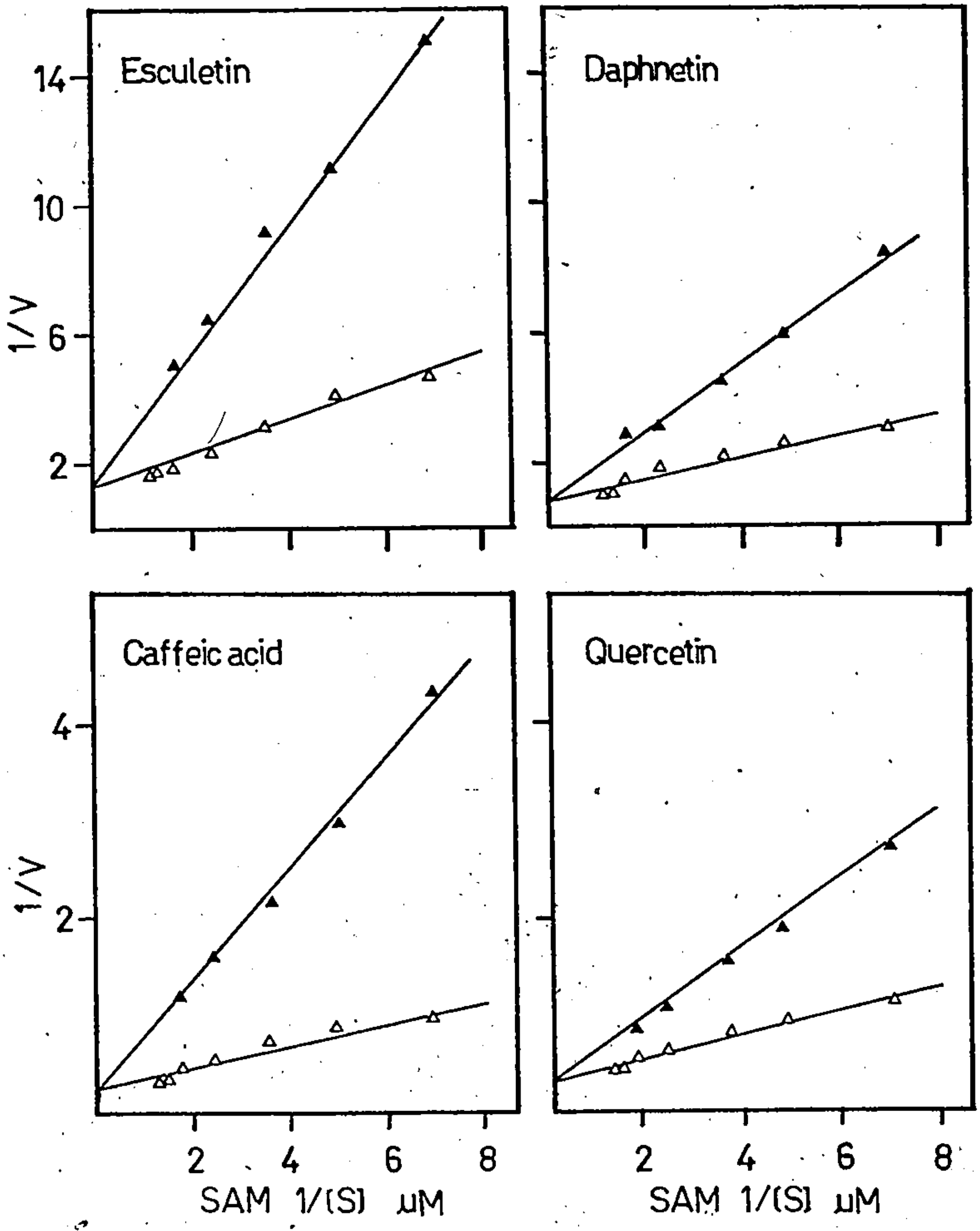
The second product of the reaction SAH (S-adenosyl-L-homocysteine) was found to be a potent competitive inhibitor of the enzyme reaction, as has been shown with other OMTs (Flohe *et al.*, 1972; Poulton *et al.*, 1976b). The Lineweaver-Burk plots of the data obtained with the major substrates gave apparent K_i values of 2.5-2.8 µM (Figure 23). These values compare well with those obtained for spinach OMT (Poulton and Butt, 1975).

Table XI. Inhibitory effects of products of phenolic substrates*

Inhibitors	Percentage inhibition				
	Caffeic acid	5HFA	Esculetin	Daphnetin	Quercetin
Ferulic acid	30.8	27.3	23.6	26.3	25.1
Scopoletin	30.2	33.1	34.3	26.3	21.6
Sinapic acid	35.0	43.0	47.7	55.7	40.7
Rhamnetin	37.8	26.0	42.0	43.0	25.5

* The rate of reaction was measured with addition of 50 nmole inhibitors, using the standard assay described in section B.4.1.

Figure 23. SAH as a competitive inhibitor of tobacco culture OMT. Lineweaver-Burk plots of the enzyme activity (V) and SAM concentration (S) with four substrates. The rate of the reaction was measured without added SAH (△) and in the presence of 10 µM SAH (▲).


Esculetin
Daphnetin
Caffeic acid
Quercetin
1/V
14
10
6
2
1/V
4
2
2
4
6
8
SAM 1/(S) µM
2
4
6
8
SAM 1/(S) µM

C.5.3.3. Effect of divalent-metal ions

Mg^{++}, Mn^{++} and Zn^{++} were tested for their effect on the rate of methylation. The results shown in Table XII indicate that both the Mn^{++} and Zn^{++} inhibited the rate of methylation of the 3 substrates tested but to different extents. In the absence of EDTA, $MnCl_2$ (1 mM) inhibited the rate of methylation of the above substrates by 87-96% (Table XII). However, this inhibitory effect was not significant with Mg^{++}, except at a high concentration (10 mM). This result is in contrast with those reported for other OMTs which were slightly stimulated (Shimada et al., 1970; 1972; Ebel et al., 1972; Thompson et al., 1978) or required Mg^{++} (Molinoff and Axelrod, 1971; Poulton et al., 1977) for maximal activity. Mn^{++} and Zn^{++}, however were reported to inhibit the parsley (Ebel et al., 1972) and pine (Shimada et al., 1972; Kuroda et al., 1975) enzymes.

C.5.3.4. Inhibitory effect of sulfhydryl-binding reagents

In view of the effect of β-mercaptoethanol on enzyme stability and reactivity (see Sections C.2.2. and 2.3.), it was considered important to investigate the effect of sulfhydryl-binding reagents on tobacco culture OMT. The data given in Table XII show that p-chloromercuribenzoate (1 mM) markedly inhibited the rate of methylation of caffeic acid, esculetin and quercetin. However, almost 50% of this inhibition was mitigiated by the addition of 5 mM β-mercaptoethanol. These results strongly suggest that an

Table XII. Effect of divalent ions and other reagents on tobacco culture O-methyltransferase

Reagent and concentration	Relative OMT activity (% of control)*		
	Caffeic acid	Esculetin	Quercetin
Divalent ions:			
$MgCl_2$ (1.0 mM)	94	98	90
(10.0 mM)	82	86	76
$MnCl_2$ (1.0 mM)	13	8	4
$ZnCl_2$ (0.5 mM)	67	60	50
EDTA (10 mM)	127	121	117
p-Chloromercuribenzoate			
(1 mM) **	22	27	68
(1 mM + 5mM β-mercaptoethanol	67	62	85
Iodoacetate (20 mM) **	73	59	20
Tropolone (1 mM)	98	77	97
(10 mM)	30	28	56

* The standard enzyme assay was used as described in the Methods section. EDTA was not included when metal ion effect was studied. Control activities (100%) were 15,500 c.p.m. with caffeic acid, 3,940 c.p.m. with esculetin and 18,700 c.p.m. with quercetin, as substrates.

** β-mercaptoethanol was not added.

SH-group was essential for the enzyme activity. Iodoacetate (20 mM), on the other hand, inhibited the methylation of quercetin more effectively (by 80%) than that of caffeic acid or esculetin which were inhibited by 27% and 41%, respectively (Table XII).

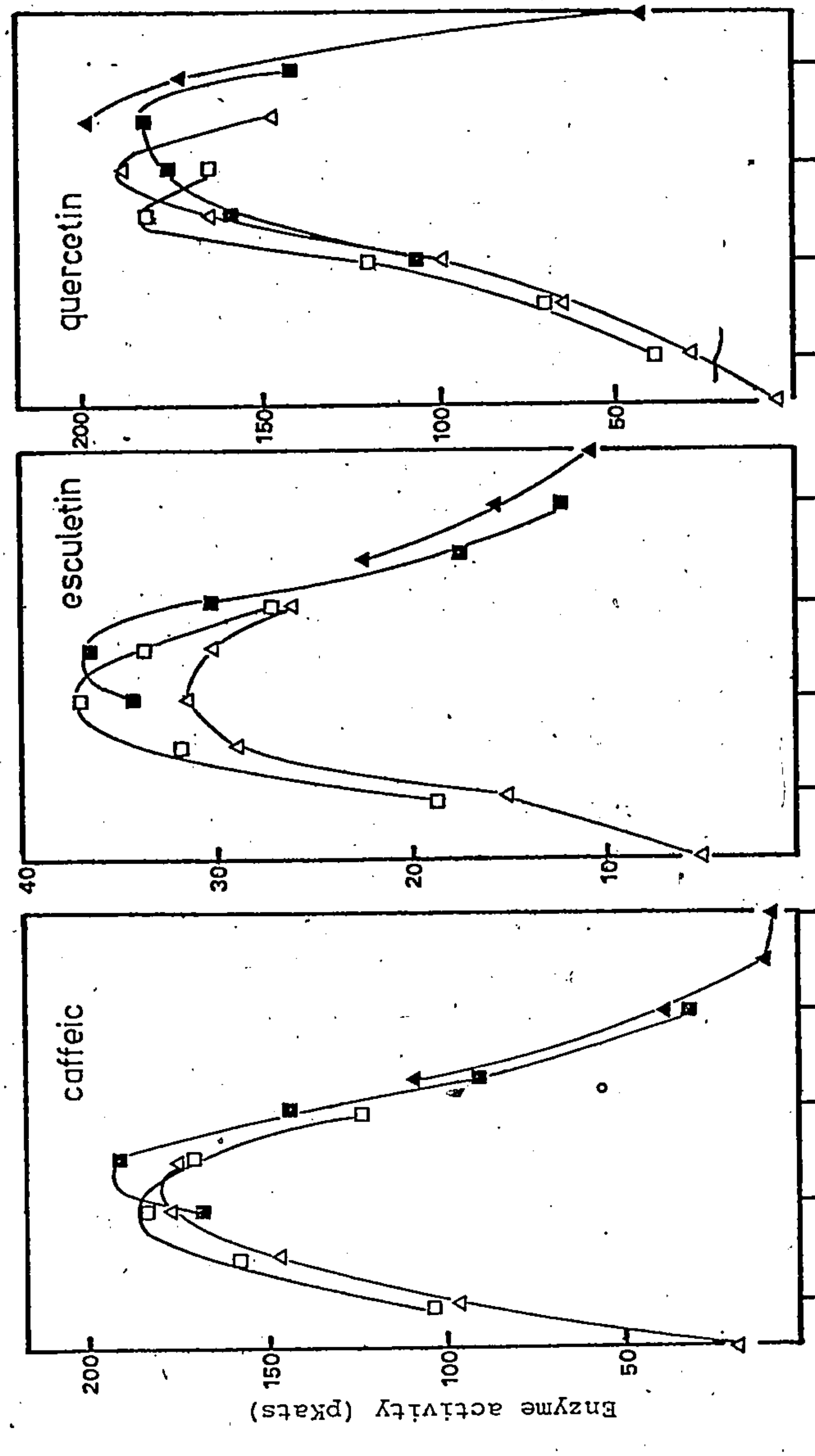
C.5.3.5. Inhibitory effect of tropolone

Tropolone is a 7-carbon ring structure with two o-dihydroxyl groups and has been considered as isosteric inhibitor of rat liver OMT (Belleau and Burba, 1961). The effect of tropolone (1 mM and 10 mM) on OMT, shown in Table XII, indicates that our enzyme is less susceptible to tropolone inhibition as compared with that of the rat liver enzyme.

C.5.4. pH optimum

The effect of pH on the rate of methylation of caffeic acid, esculetin and quercetin was studied using four different buffer systems: tris-maleate, phosphate, tris-HCl and glycine-NaOH buffers, and caffeic acid or esculetin as substrates. The pH optimum of purified OMT was found to be 7-7.5 in tris-HCl and phosphate buffers, respectively (Figures 24a, b). However, the pH optimum for quercetin was 8.0 in Tris-HCl buffer (Figure 24c). Almost 80% of the maximum activity was realized in the ranges between pH 6-8.5 and 7-9 for both types of substrates, respectively.

Figure 24a, b, c. The pH optimum of the most purified tobacco OMT (peak I of O-H-apat.) assayed against the three substrates. Reaction mixtures were adjusted to different pH using 100mM KH_2PO_4-NaOH (□-□), tris-HCl (■—■), tris-maleate (△—△) and glycin-NaOH (▲—▲).


caffeic
esculetin
quercetin
Enzyme activity (pKats)
pH
pH
pH
200
150
100
50
40
30
20
10
200
150
100
50
6
7
8
9
10
6
7
8
9
6
7
8
9

C.5.5. Substrate specificity

Table XIII lists the various phenolic substrates that were used for their methyl acceptor ability by tobacco culture OMT. The relative activities shown here represent the total (*m*- and *p*-) methylation of these compounds. The results show that 5HFA, caffeic and quercetin have the highest rate of methylation. 3,4,5-Trihydroxycinnamic acid, daphnetin, esculetin and luteolin were also methylated but to a lesser exte[illegible] The monohydroxyphenylpropanoids (*p*- and *m*-coumaric acids and umbelliferone) were poor methyl acceptors. Furthermore, there was no significant methylation of ferulic acid, isoferulic acid, scopoletin, or isoscopoletin, indicating that an *o*-dihydroxy substitution is required for the enzyme activity. However, unlike the pine OMT (Kuroda *et al.*, 1975), it is surprizing to note that none of the dihydroxybenzoic acids or 3,4-dihydroxyphenylacetic acid were methylated to any significant extent (Table XII). Other flavanoid compounds, such as cyanidin, genistein, kaempferol, apigenin, naringenin, were also found to be poor methyl acceptors.

It is interesting to note that the caffeoyl ester-chlorogenic acid - and the two glucosides, caffeic-4-glucoside and esculin, were very poor substrates as compared with their parent phenols. Similar results have been reported for the poor methylation of caffeoyl CoA ester (Poulton *et al.*, 1977) and chlorogenic acid (Legrand *et al.*, 1976), whereas, the methylation of the latter

Table XIII. Substrate specificity of tobacco culture O-methyltransferase

Substrate	Relative activity a) (%)
Caffeic (3,4-diOH-cinnamic) acid	100.0
5-Hydroxyferulic (3-MeO-4,5-diOH-cinnamic) acid	122.0
3,4,5-TriOH-cinnamic acid	44.0
Caffeic-4-O-glucoside	9.2
Chlorogenic (3-O-caffeoylquinic) acid	8.0
Ferulic (3-MeO-4-OH-cinnamic) acid	7.5
Isoferulic (3-OH-4MeO-cinnamic) acid	5.8
p-Coumaric acid	6.9
m-Coumaric acid	5.1
Daphnetin (7,8-diOH-coumarin)	38.8
Esculetin (6,7-diOH-coumarin)	25.4
Scopoletin (6-MeO-7-OH-coumarin)	6.7
Isoscopoletin (6-OH-7-MeO-coumarin)	3.5
Esculin (esculetin-7-O-glucoside)	6.4
3,4-Dihydroxyphenylacetic acid	8.7
Protocatechuic (3,4-diOH-benzoic) acid	10.3
o-Pyrocatechuic (2,3-diOH-benzoic) acid	8.2
Gallic (3,4,5-triOH-benzoic) acid	8.9
Quercetin (3,3',4',5,7-pentaOH-flavone)	121.0
Luteolin (3',4',5,7-tetraOH-flavone)	23.6
Cyanidin (3,3',4',5,7-pentaOH-flavylium)	11.5
Genistein (4',5,7-triOH-isoflavone)	8.6
Kaempferol (3,4',5,7-tetraOH-flavone)	7.0
Apigenin (4',5,7-triOH-flavone)	4.3
Naringenin (4',5,7-triOH-flavanone)	3.5

a) The potential substrates were supplied at 0.2 mM final concentration (dissolved in 10 µl of dimethyl sulphoxide) with 0.1 ml enzyme in phosphate buffer, pH 7.5 and the reaction was terminated after 30 min at 35°C. Radioactivity of the products was determined as described in the Methods section, except for glucosides, which was determined after acid hydrolysis.

substrate has been demonstrated with the bamboo enzyme (Shimada *et al.*, 1970).

C.5.6. *m*- and *p*-Methylating activity

The partially purified tobacco OMT was found to catalyze both *m*- and *p*-methylation of caffeic acid, 5HFA, esculetin and qurecetin with *m*/*p* ratios of 9.4, 1.9, 1.3 and 0.33, respectively (Table X). However, daphnetin (7,8-dihydroxycoumarin) was methylated exclusively at the *m*-position. The isomeric products of the major substrates used were sucessfully separated by various TLC systems used (Table IV) and were identified by co-chromatography with reference compounds as previously discussed in section B.4.4. The autoradiograms of the reaction products are shown in Fig.25. Caffeic acid gave ferulic and isoferulic acids; esculetin gave scopoletin and isoscopoletin; 5HFA gave sinapic acid and another unidentified product; quercetin gave mainly rhamnetin and isorhamnetin.

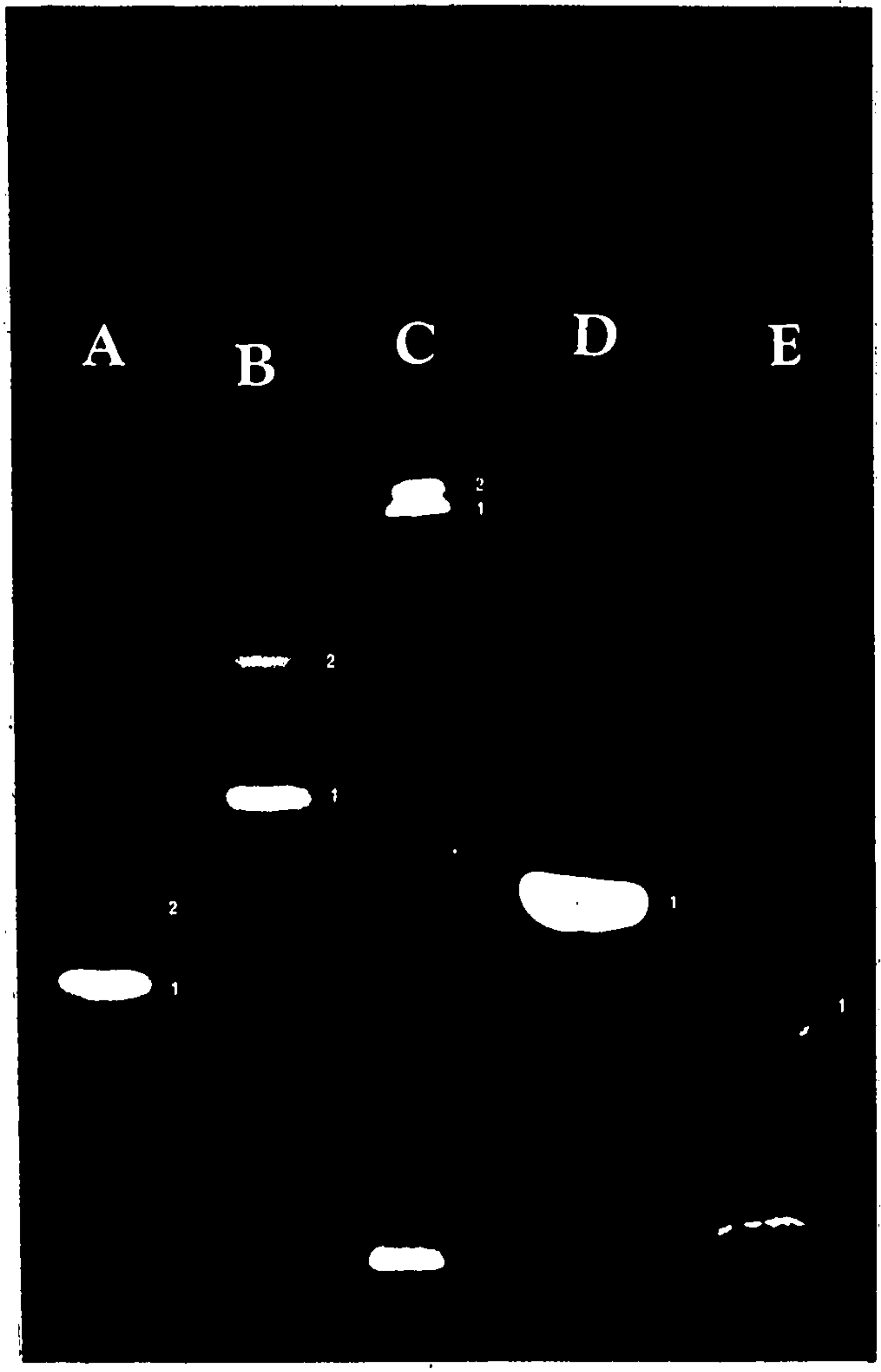
Since no *p*-*O*-methyl derivatives (isoferulic acid, isoscopoletin) are known to occur in cultured tobacco cells, it appears, therefore, that *p*-methylation occurs exclusively *in vivo*.

C.5.7. Further purification of *O*-methyltransferase

At this stage of investigation, however, it became evident that tobacco culture OMT system apparently consisted of two 'forms' of the enzyme: one catalyzed the *O*-methylation of caffeic acid and daphnetin almost predominantly

Figure 25. The combined autoradiogram of methylation products of different substrates: A. caffeic acid; B. esculetin; C. quercetin; D. daphnetin; E. 5HFA. The solvent systems and TLC supports, and R_f values were described in Table IV.

A1: ferulic acid A2: isoferulic acid
B1: scopoletin B2: isoscopoletin
C1: rhamnetin C2: isorhamnetin
D1: hydrangetin
E1: sinapic acid


A
B
C
D
E
2
1
2
1
2
1
1
1

at the meta-position, and the other methylated quercetin to a large extent at the para-position (Table X). A partial, but fair separation of the two forms of the enzyme was further achieved by chromatography of the Sephadex G-25 fraction on a DEAE-cellulose column. The latter, when used with a shallow gradient of the eluent buffer, had double the ion-exchange capacity as that of DEAE-sepharose (Pharmacia Co., personal communication). The elution profile of the OMT system and its activity against caffeic acid and quercetin are shown in Figure 26.

The purest fractions of both forms of the enzyme were analyzed for m- and p- activity against caffeic acid, esculetin and quercetin. The results given in Table XIV clearly show the m- and p-directing abilities of the two forms of the enzyme; though the latter appeared to be less specific than the former form.

C.5.8. Molecular weight

The molecular weight of the partially purified OMT was determined by gel filtration on a calibrated Sephacryl S-200 (superfine) column (not shown) and by thin-layer gel filtration on Sephadex G-150 (superfine) (Figure 27) using protein standards. The results indicate that the enzyme had a K_{av} and R_c values corresponding to a molecular weight of 75,000 dalton. This molecular size appears to be intermediate between those reported for OMTs of *Cicer* (Wengenmayer *et al.*, 1974) and parsley (Ebel *et al.*, 1972)

Figure 26. Chromatography of tobacco culture OMT on a DEAE-cellulose column (30 x 2 cm). DEAE-cellulose was pretreated with 1 M NaOH and 0.1M HCl. It was then suspended in extraction buffer and equilibrated for 24 hr prior to packing into the column. Protein (———) fractions, 5 ml each, were eluted at 15 ml/hr with a linear gradient (10-150 mM) of NaCl in 0.05 M phosphate buffer, pH 7.5 (----) and assayed with 2 substrates as described in the Methods section.

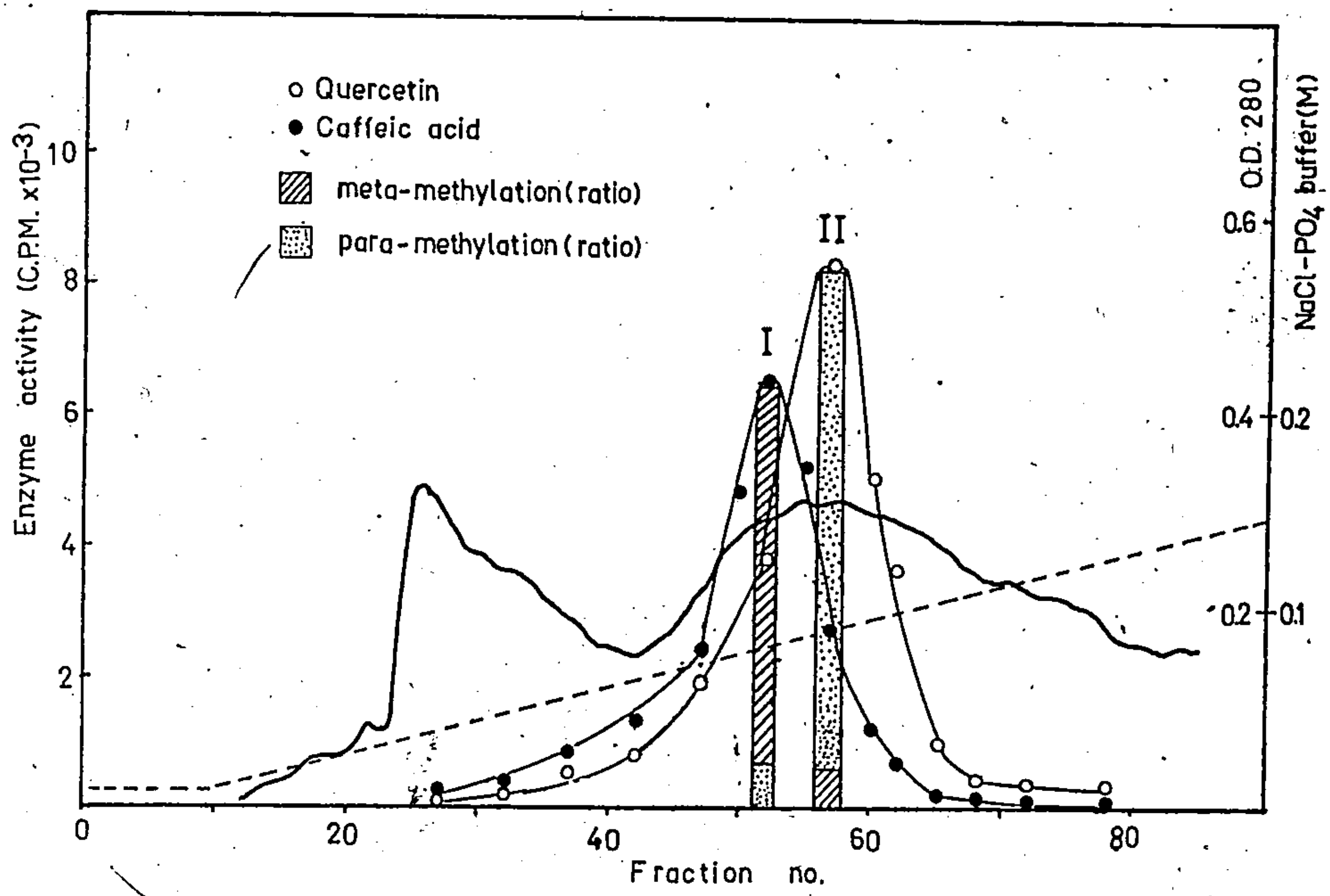
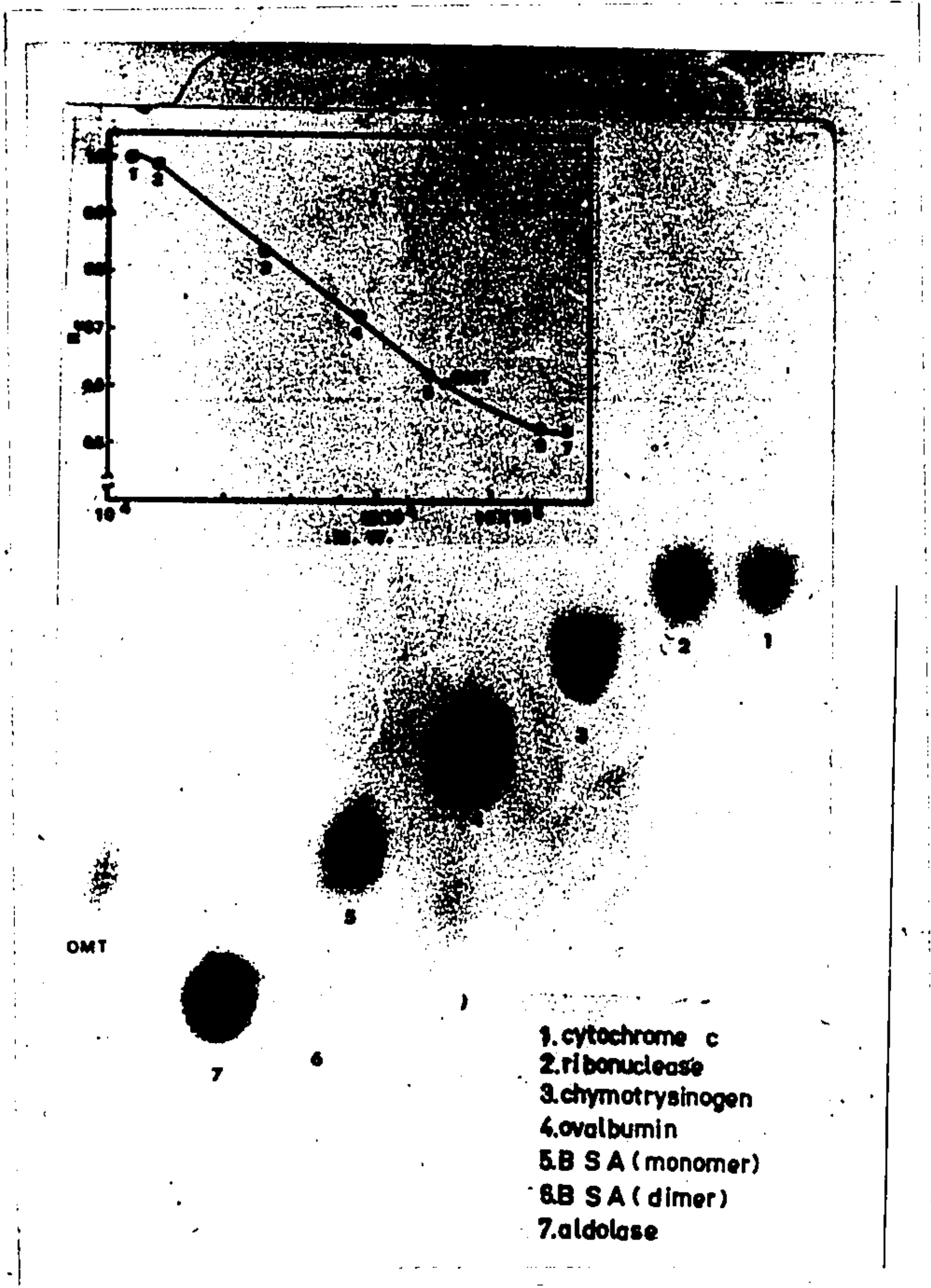

○ Quercetin
● Caffeic acid
▨ meta-methylation (ratio)
░ para-methylation (ratio)
I
II
Enzyme activity (C.P.M. x10-3)
10
8
6
4
2
0
20
40
60
80
Fraction no.
O.D. 280
0.6
0.4
0.2
NaCl-PO4 buffer(M)
0.2
0.1

Table XIV. *Meta* and *para* activity ratios of OMT fractions eluted from DEAE-cellulose* and some of their characteristics

Characteristics	Fraction I			Fraction II		
M.W.	74,000			70,000		
pI	6.1			6.3		
pH optimum	7.25			8.25		
	Caffeic acid	Esculetin	Quercetin	Caffeic acid	Esculetin	Quercetin
m-/*p*- methylation ratio	11.13	1.59	0.15	3.32	0.68	0.07
p-/*m*- methylation ratio	0.09	0.63	6.78	0.30	1.46	14.80

* The standard assay was used with saturating substrate concentrations. Products were separated by TLC and *m*- and *p*- isomers were counted for radioactivity.

Figure 27. Molecular weight of purified OMT (OH-apatite eluate) as determined by TLG on Sephadex G-150 (superfine). The method was described in Section B.6.2.


1
2
3
4
5
6
7
10^4
OMT
1
2
3
5
6
7
1. cytochrome c
2. ribonuclease
3. chymotrysinogen
4. ovalbumin
5. B S A (monomer)
6. B S A (dimer)
7. aldolase

cell cultures and that of pine seedlings (Kuroda *et al.*, 1975).

The molecular weight of the two forms of the enzyme (Figure 25 and Section C.5.7) was determined by TLG using standard proteins and found to be 74,000 and 70,000 (±7%) for the *m*- and *p*-directing forms, respectively.

Isoelectric focussing of the two separated fractions of OMT was carried out as described in Section B.5.2. The pI values for the *m*- and *p*- forms were 6.1 and 6.3, respectively (Table XV).

DISCUSSION

The experiments described here have demonstrated the presence of an enzyme system in tobacco cell suspension culture that mediated the transfer of the methyl groups of S-adenosyl-L-methionine to the meta and para hydroxyls of several dihydroxy phenolic compounds. Tobacco culture OMT system was purified by ammonium sulphate precipitation and chromatography on DEAE-sepharose 6B, sephacryl S-200 and hydroxyapatite columns. The enzyme activity was increased by 80-fold against caffeic acid, 90-fold against esculetin and 45-fold against quercetin as substrates. Furthermore, acrylamide gel electrophoresis of the different fractions indicated that the native enzyme (Band 1, Figure 19A), which exhibited OMT activity (Figure 28), has dissociated into two protein bands (Bands 2 and 3, Figure 19 B-D) during the purification process; both of which were active against caffeic acid and quercetin (Figure 29). Tobacco OMT was partially separated, by chromatography on DEAE-cellulose, into two fractions which exhibited distinct meta and para O-methylation against caffeic acid and quercetin, respectively; but not to the exclusion of other substrates. For unknown reasons, the DEAE-cellulose eluates were unstable and did not lend themselves to further purification. That was the reason why DEAE-sepharose was chosen, despite its poor ion-exchange capacity.

Despite the small differences observed in molecular weights and pI values of the two forms of tobacco OMT, there

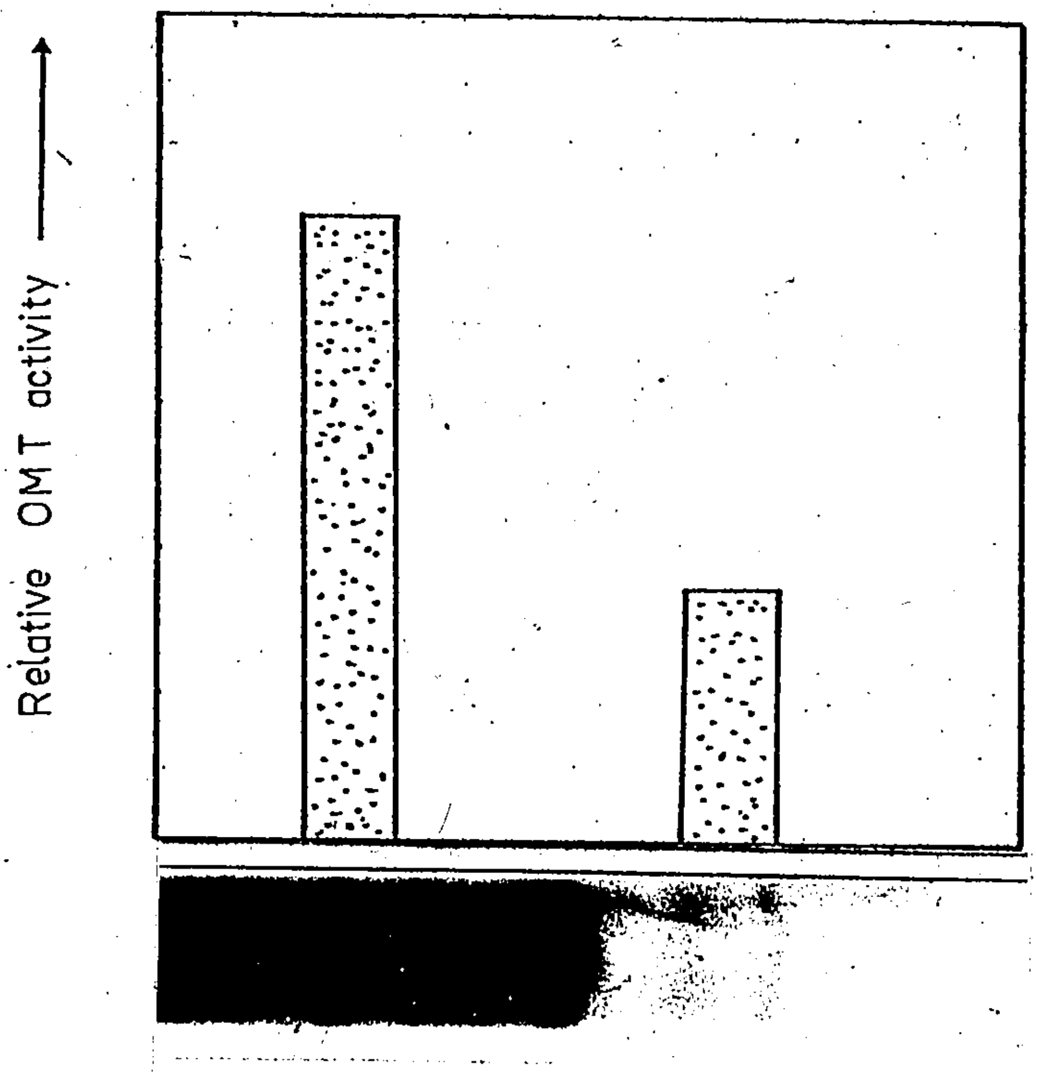


Figure 28. Polyacrylamide gel electrophoresis of OMT activity (G-25 eluate). Following electrophoresis, the regions of the gel corresponding to Band 1 and 2+3 were sliced and OMT was eluted by incubating the sections for 1 hr in 200 µl 0.1 M phosphate buffer, pH 7.5. The eluate was used directly for enzyme assay against caffeic acid.

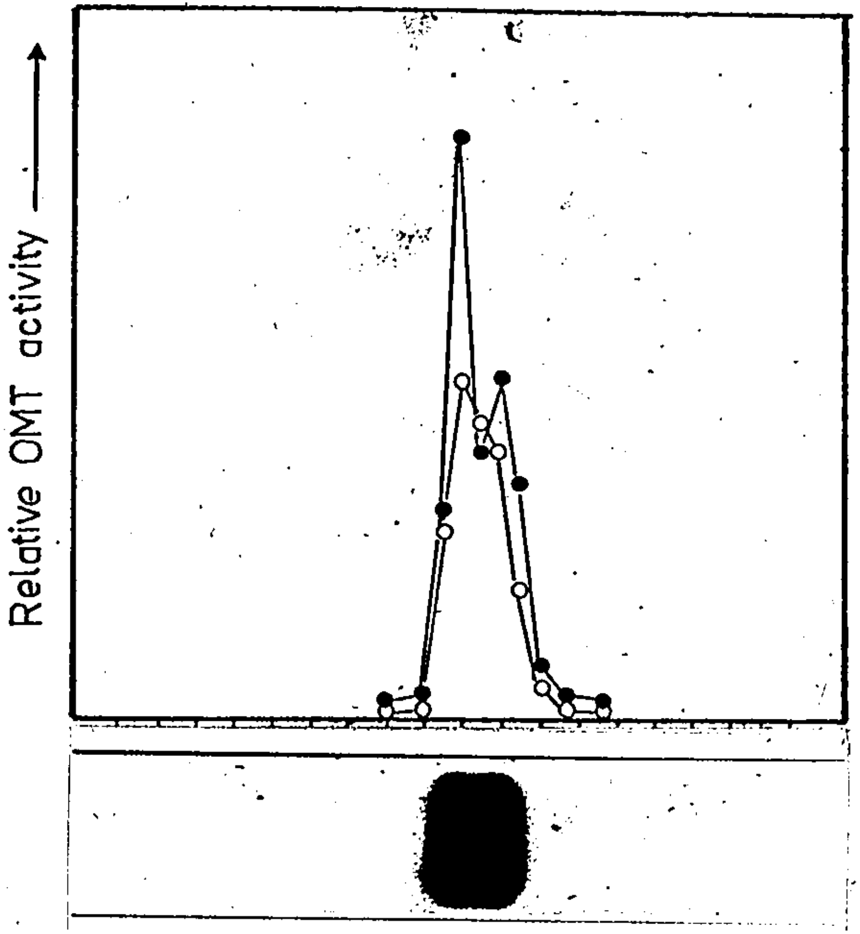


Figure 29. Polyacrylamide gel electrophoresis of OMT activity (OH-apatite eluate, peak I). After electrophoresis, gels were sliced into 1.0mm thick sections. The enzyme protein was eluted and the relative activities against caffeic acid (●) and quercetin (○) were measured using standard assay method described in Section B.4.1.

are several properties indicative of the existence of two distinct enzymes acting at the meta and para positions:

a) The elution profile of OMT system from DEAE-cellulose was resolved into two activity peaks: One of which catalyzed, almost exclusively, the O-methylation of caffeic acid at the meta position, and the other, quercetin at the para position; though the latter was less substrate-specific than its meta counterpart, to the extent of being able to catalyze significant methylation of both esculetin and caffeic acid (Table XIV).

b) The differences in the degree of enzyme purification against caffeic acid, daphnetin and esculetin (80-90-fold) on the one hand, and that of quercetin (45-fold) on the other, tends to suggest that the para directing enzyme is less stable than its meta counterpart; though both forms were heat labile. This result concurs with the relative activities of both protein bands after acrylamide gel electrophoresis against caffeic acid and quercetin (Figure 27).

c) The failure of the specific activity ratios of quercetin/caffeic to remain constant during purification, as compared with that of esculetin/caffeic (Table VIII), may also be taken to indicate the existence of two distinct forms of the enzyme.

d) SDS-acrylamide gel electrophoresis of protein bands 2 and 3 (Figure 19F) seems to indicate that the latter are not charge-isomers or isoenzymes, but two distinct proteins with similar molecular weights, each of which appears as a single unit, with an electrophoretic mobility corresponding to the 70-75 K range. The faint bands with higher mobility seem to be polypeptide degradation products that may have resulted from SDS treatment.

e) The differences observed in the effects of divalent cations and SH-group inhibitors on enzyme activity, as well as the differences in pH optima against caffeic acid and quercetin (Table XIV) also support this view.

f) More conclusive evidence for the existence of two discrete enzymes acting at the meta and para positions was obtained from mixed-substrate experiments using a purified, yet unresolved OMT system (peak I, Figure 18). The results are shown in Table XV. The additive effect observed when quercetin was added to caffeic acid is in sharp contrast with the decreased activity obtained after the addition of either esculetin or daphnetin. This result is in agreement with the kinetic theory (Dixon and Webb, 1964) which demonstrates that, at near saturating substrate concentrations, a higher activity is observable in the presence of two substrates than with either one alone, if the system contains separate enzymes mediating the reactions of both substrates.

To our knowledge, this is the first reported instance where meta and para directing OMTs have been isolated and characterized. Several workers have observed OMT activity at both meta and para hydroxyls of dihydroxy phenolic compounds in tobacco leaves (Legrand et al., 1976), in rat

Table XV. Activity of purified OMT (OH-apatite, peak I) against mixed substrates*

Substrate / Concentration	Relative activity (c.p.m./assay)		
	0.0 mM	0.08 mM	0.2 mM
Quercetin	16,484 (100%)	23,540 (143%)	34,150 (207%)
Esculetin	16,660 (100%)	11,640 (70%)	9,630 (58%)
Daphnetin	16,850 (100%)	13,650 (83%)	10,750 (64%)

* Standard assay contained 0.2 mM caffeic acid (control = 100%) to which the indicated substrate concentrations were added.

liver tissue (Müller-Enoch et al., 1976a), in yeast (Müller-Enoch et al., 1976b) and in pampas grass (Finkle and Kelly, 1974). In none of these cases, however, does the question of whether discrete enzymes mediate the reaction at the meta and para hydroxyls to have been unequivocally resolved. Very recently, Thompson et al. (1978) reported on the occurance, in Ruta graveolens cell culture, of two distinct enzymes which mediated the O-methylation of linear furanocoumarins at the meta and ortho hydroxyls. The latter report, together with our finding add to our knowledge of O-methylation of different hydroxyls of phenolic compounds.

As to the role of tobacco culture OMT, the efficiency with which it catalyzed the O-methylation of caffeic acid and 5HFA, especially the former substrate by a highly specific meta directing enzyme, suggests that this enzyme is involved in the sequence of reactions leading to lignin formation, as has been shown with other OMTs (Shimada et al., 1973; Poulton et al., 1976b). The methylated products of the above substrates, ferulic and sinapic acids, respectively, have been shown to be efficient substrates for the other enzymes involved in lignin biosynthesis, namely p-coumarate: CoA ligase (Knobloch and Hahlbrock, 1975), cinnamyl CoA reductase (Wengenmayer et al., 1976), cinnamyl alcohol dehydrogenase (Wyrambik and Grisebach, 1975) and coniferyl alcohol glucosyltransferase (Ibrahim and Grisebach, 1976). The activity of the latter enzyme has been demonstrated in tobacco callus and shoots (Ibrahim, 1977). An increase in

OMT activity, that was concomitant with lignification, has also been reported in both tobacco callus and cell cultures (Kuboi and Yamada, 1976).

Tobacco culture OMT also catalyzed the O-methylation of two dihydroxycoumarins, namely esculetin and daphnetin. Despite its low K_m value, the rate of methylation of esculetin was lower than that of caffeic acid or daphnetin, which may be related to the differences in nucleophilicity of the hydroxyl groups on the phenolic ring system (Senon et al., 1959). However, the in vitro O-methylation of esculetin suggests that this enzyme is involved in coumarin biosynthesis. Substituted coumarins were thought to be formed by lactonization of their corresponding cinnamic acids (Towers, 1964; Brown, 1966). Reznik and Urban (1957) showed that scopoletin may be formed from ferulic acid, and Steck (1967a, b) using tobacco leaves and a number of labelled precursors, gave isotopical evidence to suggest that scopoletin was formed as scopoletin glucoside (scopolin) through the methylation of caffeic acid to glucosidoferulic acid as follows: caffeic ⟶ ferulic ⟶ 4-OH-glucosidoferulic ⟶ scopolin ⟶ scopoletin. A similar pathway has been demonstrated by Fritig et al. (1970), except that scopoletin was formed directly from free ferulic acid and not from its glucosidic form. In both proposed pathways, the O-methylation step was considered to precede lactone ring formation. Both the in vitro methylation of esculetin and the accumulation of its methylated products, scopoletin and scopolin, tend to suggest an alternative pathway for scopoletin biosynthesis where: caffeic → esculetin → scopoletin → scopolin. The poor methylation of caffeoyl esters and glucosides by tobacco culture OMT (Table XIII) strongly supports this view. Esculetin has been reported to occur in the tobacco plant (Dieterman *et al.*, 1959). Although it could not be detected in tobacco cell culture, however, its presence in catalytic amounts cannot be excluded. The recent discovery, in tobacco cell culture, of a glucosyltransferase which mediates the transfer of glucose from UDP-glucose to scopoletin with the formation of scopolin (Tsang and Ibrahim, unpublished results), supports the proposed pathway for scopoletin formation and is in agreement with the hypothesis that glucosylation is a final step in phenolic biosynthesis (Hahlbrock and Grisebach, 1975).

Although tobacco culture OMT catalyzed the efficient methylation of quercetin by a specific *para*-directing enzyme, the *in vivo* physiological role of this reaction is yet unclear, since neither the substrate or product(s) is a metabolite of tobacco cell culture. The quercetin glycoside, rutin, is a natural constituent of the tobacco plant (Couch, 1944; Griffith, 1955) and it is conceivable that tobacco cells may preserve the ability to methylate quercetin in *in vitro* culture. However the extent to which tobacco OMT catalyzed the *para* methylation of ring-A of quercetin is remarkable, especially in view of the absence of vicinal hydroxyls on the phenolic ring. Recently, Poulton *et al.*

(1977) reported ring-A methylation of texasin (6,7-dihydroxy-4'-methoxyisoflavone) by a purified soybean OMT, though the exact position of methylation still remains to be determined.

Whereas tobacco culture O-methyltransferase appears similar to other plant OMTs in its general properties, it differs, however, in many respects. Tobacco OMT system exhibited a fairly wide range of substrate specificity. It catalyzed the efficient O-methylation of caffeic acid, 5HFA, and quercetin, but not to the exclusion of the coumarins, daphnetin and esculetin. The two forms of the enzyme, however, exhibited distinct meta and para-directing activities towards caffeic acid and quercetin, respectively. The results obtained with the studies of substrate specificity indicate that the substrates must satisfy certain structural requirements for maximal activity of the tobacco enzyme, among which are:

a) vicinal dihydroxy-substituted phenolic compounds; though the para-directing enzyme could methylate efficiently the 7-OH group of ring-A of quercetin, but not monohydroxy cinnamic acids or coumarins.

b) a 3-carbon side chain attached to the phenolic ring and containing a double bond, since neither dihydroxyphenylacetic or benzoic acids nor dihydrocaffeic acid or dihydroquercetin were methylated to any significant extent.

c) a free carboxyl group for phenylpropanoid compounds; esterification of that group (as in chlorogenic acid) or lactonization (as in coumarins) significantly reduced the enzyme activity.

d) a gama pyrone ring with flavonoid substrates having a free 3-OH group, since an anthocyanin and a flavone were poor methyl acceptors.

e) finally, it was observed that phenolic gluco-/glycosides were very poor methyl acceptors as compared with their aglycones; this has been shown with esculin, caffeic-4-glucoside and rutin.

A number of O-methyltransferases, on the other hand, have been reported to exhibit high specificity towards certain substrates. The pine seedling enzyme had highest affinity for caffeic acid (Kuroda et al., 1975), while that of bamboo shoots catalyzed the methylation of both caffeic acid and 5HFA (Shimada et al., 1973); thus establishing the ability of the latter tissue to synthesize both guaiacyl and syringyl residues of lignin. Among other OMTs reported from cell suspension cultures, the parsley enzyme was specific to flavones (Ebel et al., 1972); Cicer to isoflavones (Wengenmayer et al., 1974); two distinct enzymes from soybean were specific to phenylpropanoids (caffeic:OMT) and flavonoids (quercetin:OMT) (Poulton et al., 1976a, b), as were those of tulip anthers (Sütfeld and Wiermann, 1978). Very recently, a novel substrate specificity towards ortho and meta hydroxyls of linear, dihydroxyfuranocoumarins was reported for two distinct OMTs in Ruta graveolens cell suspension culture (Thompson et al., 1978). The discovery of an ortho directing OMT, in the latter culture, and a para directing enzyme in tobacco cells, completes the knowledge of O-methylation at the different positions of the phenolic ring structure. The only fungal enzyme purified from Lentinus lepidus (Wat and Towers, 1975), which was found to be specific for p-coumarate, that is, methylation para to the side chain, has no counterpart in higher plants.

As to the molecular weight of tobacco culture OMT, the two forms of the enzyme had very similar values, 70-74 K daltons for the meta and para directing forms, respectively. This molecular size is similar to that reported for the pine enzyme (67 K) and is intermediate between those of parsley (48 K) and Cicer (110 K) enzymes. Apparently, there exists a large variation in the molecular weights of OMTs isolated from different sources. Whereas, the M.Ws. of caffeic:OMT and quercetin:OMT of tulip anthers were reported to be 35 K and 50 K, respectively (Sütfeld and Wiermann, 1978), those of Ruta OMT system were 85-110 K (Thompson et al., 1978).

Except for the parsley enzyme (Ebel et al., 1972), there is no sufficient information, in the literature, on the extent of OMT inhibition by its methylated products. The activity of tobacco culture OMT, however, was inhibited by 22-50% in the presence of ferulic acid, sinapic acid, scopoletin and rhamnetin (0.2 mM final concentration) the methylation products of caffeic acid, 5HFA, esculetin and quercetin, respectively. This end-product inhibition may act as a mechanism for the regulation of enzyme activity in vivo and hence the biosynthesis of lignin precursors and other phenolic metabolites. The potent competitive inhibition of tobacco culture OMT by its other reaction product, SAH, appears similar to those reported for the sugar beet (Poulton and Butt, 1975) and soybean culture (Poulton et al., 1976a, b, 1977) enzymes.

Mg^{++} was not necessarily required for the activity of tobacco culture OMT. Furthermore, it had an inhibitory effect on enzyme activity at a higher concentration (up to 10 mM). This result is in sharp contrast with those reported for other OMTs, which were stimulated (Shimada et al., 1972; Ebel et al., 1972; Poulton et al., 1977; Thompson et al., 1978) by Mg^{++}. The inhibition of OMT activity by other divalent cations, Mn^{++} and Zn^{++} was similar to that observed with the parsley (Ebel et al., 1972) and pine (Shimada et al., 1972; Kuroda et al., 1975) enzymes.

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Appendix I. Components of culture medium (Murashige and Skoog, 1962).

Macro-elements	mg/l
$CaCl_2.2H_2O$	440.0
KH_2PO_4	170.0
KNO_3	1900.0
$MgSO_4.7H_2O$	370.0
NH_4NO_3	1650.0
Micro-elements	**mg/l**
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	27.800
H_3BO_3	6.200
KI	0.830
$MnSO_4.4H_2O$	22.300
Na-EDTA	37.300
$Na_2MoO_4.2H_2O$	0.250
$ZnSO_4.7H_2O$	8.600
Vitamins	**mg/l**
glycin	2.0
nicotinic acid	0.5
pyridoxine-HCl	0.5
thiamin-HCl	0.1
Growth hormones	**µM**
2,4-D	1.0
IAA	2.0
Kinetin	0.1
Others	**g/l**
myo-inositol	0.1
casein-hydrolysate	1.0
surcose	30.0
agar-agar*	7.0
pH	5.8±0.1

* only for solid culture medium