NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, tests publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

Canada

Enzymatic Aspects of O-Glucosylation of Anthraquinones and O-Methylation of Flavonoids in Plant Tissues

Henry E. Khouri

A Thesis

in

The Department

of

Chemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy at
Concordia University
Montréal, Québec, Canada

January 1988

ISBN 0-315-41662-9

ABSTRACT

Enzymatic Aspects of O-Glucosylation of Anthraquinones and O-Methylation of Flavonoids in Plant Tissues

Henry E. Khouri, Ph.D.
Concordia University, 1988

Cell suspension cultures of Cinchona succirubra produce a number of anthraquinone glucosides. The effect of nutritional and hormonal factors on growth and anthraquinone production were investigated in order to study the enzyme-catalyzed glucosylation of these metabolites. Five anthraquinone-specific glucosyltransferases were resolved, by chromatofocusing, with apparent pI values of 5.3, 4.8, 4.5, 4.3 and 4.1. They accepted emodin, anthrapurpurin, quinizarin, 2,6-dihydroxyanthraquinone and 1,8-dihydroxyanthraquinone as the best substrates, respectively. These enzymes exhibited similar characteristics as to pH optimum (pH 7) in histidine/HCl buffer, M_r 50,000, had no cation requirement and were inhibited by various SH-group reagents. The K_m values of the respective anthraquinones for either of the five enzymes was 10 μM.

An isoflavone 5-O-methyltransferase from Lupinus luteus and two, position-specific flavonol O-methyltransferases (2′-/5′- and 7-) from Chysosplenium americanum were partially purified and characterized. These enzymes had pH optima between 7 and 8, M_r 55,000, no cation requirement and were inhibited by various SH-group reagents. The steady state kinetic behavior of the 5- and 7-O-methyltransferases suggested that these enzymes proceeded by an ordered bi bi mechanism with S-

adenosyl-L-methionine and S-adenosyl-L-homocysteine as leading reaction partners.

The effect of various parameters on the recovery and enzyme activity of five, position-specific O-methyltransferases of C. americanum, by chromatofocusing, was investigated using fast protein liquid chromatography. These parameters included the nature and pH of the starting buffer, the dilution and flow-rate of the Polybuffer and the presence of zwitterions.

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

To my mother for being

my inspiration

To Johann Sebastian Bach, Giacomo Puccini and Jean Sebelius

whose music kept me from losing my sanity

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. R. K. Ibrahim, without whom this thesis would simply not be, for his friendship, guidance and the keen interest he has shown throughout this work. I will forever be indebted to him for providing me with the moral and academic support which have been my major motivation thoughout this thesis. His encouragements, critisisims, and financial support are deeply appreciated. He is a true scientist and a gentleman.

Special thanks should be extended to Dr. S. Tahara, Hokkaido University for his generous gift of the isoflavone compounds used in this study; and to Dr. M. Rideau, University of Tours, for supplying the callus cultures of C. succirubra used in this work.

Appreciation is expressed to Dr. L. Colebrook, Dr. B. Hill and Dr. R. Townshend for their helpful advice and assistance as supervisory committee members.

I must also thank Mr. Chris Boer, for his technical assistance; and my collegues, Denis Barron, Louise Brisson, Emidio De Carolis, Lilian Latchinian, Jacynthe Seguin and Luc Varin, for their moral support and encouragement.

Finally, I would like to thank the Chemistry Department for a teaching assistantship during the initial part of this work; and the Department of Higher Education, Government of Quebec (F.C.A.R.) for a postgraduate scholarship as well as the Natural Sciences and Engineering Research Council of Canada for their financial support of this project.

TABLE OF CONTENTS

	PAGE
ABSTRACT	iii
ACKNOWLEDGMENTS	vi
TABLE OF CONTENTS	1
LIST OF TABLES	4
LIST OF FIGURES	6
LIST OF APPENDICES	9
ABBREVIATIONS	10
A. INTRODUCTION	11
B. REVIEW OF LITERATURE	16
1. Anthraquinones	16
1.1. Occurrence and distribution	16
1.2. Productivity in tissue culture	16
1.3. Biosynthesis	22
1.3.1. The acetate-polymalonate pathway	22
1.3.2. The shikimic acid o-succinylbenzoic acid pathway	22
1.4. Enzymatic synthesis	22
1.5. Significance	27
2. Flavonoids	28
2.1. Occurrence and distribution	28
2.2. Biosynthesis	28
2.3. Enzymatic synthesis	33
2.3.1. Enzymes of general phenylpropanoid metabolism	33
2.3.2. Chalcone synthase	33
2.3.3. Chacone isomerase	34
2.3.4. Hydroxylases	34
2.3.5. O-Methyltransferases	35
2.3.6. O-Glucosyltransferases	36
2.4. Significance	36

		PAGE
C.	MATERIALS AND METHODS	37
1.	Plant material	37
1.1.	Cinchona succirubra	37
1.2.	Chrysosplenium americanum	42
1.3.	Lupinus luteus	42
2.	Chemicals	45
3.	Extraction of anthraquinones	45
4.	Estimation of anthraquinone content	46
5.	Characterization of anthraquinones	46
6.	Protein extraction	47
7.	Enzyme purification	47
7.1.	Buffers	47
7.2.	Ion exchange chromatography	48
7.3.	Chromatofocusing	48
8.	Fast protein liquid chromatography system	48
9.	O-Glucosyltransferase assay	49
10.	O-Methyltransferase assay	49
11.	Identification of reaction products	50
12.	Molecular weight determination	50
13.	Determination of pI values	50
14.	Analysis of kinetic data	51
15.	Protein estimation	52
16.	Definition of enzyme units	52
D.	RESULTS	53
1.	Anthraquinones	53
1.1.	Growth of Cinchona succirubra cell culture	53
1.2.	Characterization of anthraquinone glucosides	53
1.3.	Separation of anthraquinones by HPLC	58
1.4.	Anthraquinone profile of callus and cell suspension cultures	58
1.5.	Anthraquinone profile as a function of growth in culture	58
1.6.	Effect of nutritional factors	66
1.6.1.	Effect of sucrose	66
1.6.2.	Effect of medium composition	66
1.7.	Effect of growth regulators	69
1.8.	Changes in glucosyltransferase activity during culture growth	73
1.9.	Purification of GT activity	73
1.10.	Characterization of GT activity	73
1.10.1.	Substrate specificity	73
1.10.2.	Effect of pH	79
1.10.3.	Linearity of the glucosylation reaction	79
1.10.4.	Enzyme stability	85
1.10.5.	Effect of divalent cations and sulfhydryl group reagents	85
1.10.6.	Other properties	85

	PAGE
2. Flavonoid O-methyltransferases (OMT)	87
2.1. Purification of the 7-OMT	90
2.2. Properties of the purified 7-OMT	90
2.2.1. Substrate specificity	90
2.2.2. Effect of divalent cations and sulfhydryl group reagents	98
2.3. Kinetic analysis of the 7-OMT	98
2.3.1. Substrate interaction kinetics	98
2.3.2. Product inhibition studies	103
2.4. Purification of the 2´-/5´-OMT	109
2.5. Characterization of the 2´-/5´-OMT	109
2.5.1. Substrate specificity	109
2.5.2. Linearity of the methylation reaction	113
2.5.3. pH optimum	113
2.5.4. Effect of divalent cations and sulfhydryl group reagents	113
2.5.5. Other properties	117
2.6. Resolution of the Chrysosplenium OMT system	117
2.6.1. Nature and pH of starting buffer	118
2.6.2. Dilution and flow-rate of Polybuffer	118
2.6.3. Presence of zwitterions	120
2.7. The Lupinus luteus system	124
2.8. Tissue localization of the 5-OMT	124
2.9. Purification of the 5-OMT	124
2.10. Enzyme properties	130
2.10.1. Substrate specificity	130
2.10.2. Linearity of the methylation reaction	130
2.10.3. pH optimum	130
2.10.4. Stability of the enzyme	137
2.10.5. Effect of divalent cations and sulfhydryl group reagents	137
2.10.6. Other properties	137
2.11. Kinetic analysis	137
2.11.1. Substrate interaction kinetics	141
2.11.2. Product inhibition studies	141
E. DISCUSSION	149
1. Anthraquinones	149
2. Flavonoid O-methyltransferases	152
F. CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE	166
G. PERSPECTIVE FOR FUTURE WORK	167
H. REFERENCES	169
I. APPENDICES	182

LIST OF TABLES

TABLE		PAGE
1	Some common naturally occurring anthraquinones	19
2.	Type and source of anthraquinone-producing tissue cultures	21
3	Anthraquinone content of Cinchona succirubra cell suspension as a function of day in culture	65
4	Effect of sucrose concentration on growth and anthraquinone production	67
5	Effect of different media on growth and anthraquinone production	68
6	Effect of growth regulators on cell growth and anthraquinone production of C. succirubra cultures	70
7	Effect of increasing concentration of cytokinins on growth and anthraquinone production	71
8	Effect of increasing concentration of auxins on cell growth and anthraquinone production of C. succirubra cultures	72
9	Purification of C. succirubra glucosyltransferase activity	78
10	Substrate specificity of C. succirubra glucosyltransferases	80
11	Effect of divalent cations and sulfhydryl group reagents on C. succirubra glucosyltransferase activity	86
12	Purification data of the 7-OMT from Chrysosplenium americanum	97
13	Substrate specificty of the 7-OMT from C. americanum	99
14	Effect of divalent cations and SH-group reagents on the 7-OMT activity	100
15	Kinetic constants of the 7-OMT from C. americanum	108
16	Purification of Chrysosplenium 2′-/5′-OMT	112
17	Effect of divalent cations and SH-group reagents on the 2′- and 5′-O-methylating activity	116

		PAGE
18	pI values of C. americanum OMTs determined under different experimental conditions	119
19	Extent of purification of C. americanum OMTs	123
20	Tissue localization of the 5-OMT from Lupinus luteus	127
21	Purification of the 5-OMT from L. luteus	131
22	Affinity of the 5-OMT for the different isoflavones	132
23	Effect of divalent cations and SH-group reagents on the 5-OMT	138
24	Kinetic parameters of L. luteus 5-OMT	148
25	Kinetic constants of Chrysosplenium OMTs	160

LIST OF FIGURES

FIGURES		PAGE
1	The relationship between primary and secondary metabolism	12
2	Basic skeletons of naturally occurring quinones	17
3	Biosynthesis of emodin (1,6,8-trihydroxy-3-methyl-anthraquinone)	23
4	Biosynthesis of alizarin (1,2-dihydroxyanthraquinone)	24
5	Representatives of each of the six major groups of flavonoids	29
6	Common steps in flavonoid biosynthesis	31
7	Cinchona succirubra callus and cell suspension cultures	38
8	The set-up used for maintaining growth of Cinchona succirubra cells	40
9	Structural formulae of the six highly O-methylated flavonol glucosides of Chysosplenium americanum	43
10	Early and late exponential phase cells of C. succirubra cultured in B5 medium	54
11	Changes in growth parameters and anthraquinone production of C. succirubra suspension culture	56
12	HPLC profile of 12 reference anthraquinones	59
13	HPLC profile of C. succirubra callus culture extract	61
14	HPLC profile of C. succirubra cell suspension culture extract	63
15	Elution profile of glucosyltransferase activity after gel-filtration on Superose 12 HR column using emodin as substrate	74
16	Elution profile of glucosyltransferase activity after chromatofocusing on a Mono P HR 5/20 column using emodin as substrate	76

		PAGE
17	Comparison of positions 1 and 2 in an anthraquinone ring system with positions 5 and 7 in a flavonoid ring	81
18	pH optimum of the glucosylation reaction of anthraquinones using emodin as substrate	83
19	Proposed pathway for the enzymatic synthesis of polymethylated flavonol glucosides in C. americanum	88
20	Elution profile of the 7-OMT activity after gel filtration on Superose 12 HR 16/50 column	91
21	Elution profile of the 7-OMT activity after chromatofocusing on a Mono P HR 5/20 column	93
22	Elution profile of the 7-OMT after ion-exchange chromatography on Mono Q HR 5/5 column	93
23	Double-reciprocal plots of initial velocities with the flavonol, 3-methylquercetin, as the variable substrate and SAM as the changing fixed substrate	101
24	Product inhibition kinetics of the 7-OMT	104
25	Proposed kinetic mechanism for the 7-OMT of C. americanum	106
26	Elution profiles of the 2´-/5´-OMT activity after gel filtration on Superose 12 HR 10/30 column and chromatofocusing on Mono P HR 5/20 column	110
27	pH optimum of the 2´-/5´-OMT after chromatofocusing using substrates A (5,5´-dihydroxy-3,6,7,4´-tetramethoxyflavone-5´-O-glucoside) and B (5,2´-dihydroxy-3,7,4´-trimethoxy-flavone-2´-O-glucoside)	114
28	Elution profile of O-methyltransferase activity of the C. americanum OMT system, after chromatofocusing on Mono P HR 5/20, under optimum conditions	121
29	Structural formulae of 5-O-methylgenistein and 5-O-methylderrone	125
30	Elution profiles of the 5-OMT activity after gel filtration on Superose 12 HR 15/60 column, and after ion-exchange chromatography on Mono Q HR 5/5 column, using genistein as substrate	128

		PAGE
31	Autoradiogram of the chromatographed enzyme reaction products on silica plates in chloroform-acetone-ammonia (70:60:1, v/v) with genistein, 2´-hydroxygenistein, or 8-prenyl-2´-hydroxygenistein as substrates showing their respective 5-O-methyl derivatives	133
32	pH optimum of the isoflavone 5-OMT from Lupinus luteus using genistein as substrate	135
33	Elution profile of the isoflavone-5-OMT after chromatofocusing on a Mono P HR 5/20 column	139
34	Double-reciprocal plots of initial velocities with SAM as the changing, fixed substrate and genistein as the variable substrate	142
35	Product inhibition kinetics of the isoflavone-5-OMT	144
36	Proposed kinetic mechanism for the isoflavone-5-OMT of L. luteus	146
37	Proposed pathway for the biosynthesis of isoflavones in L. luteus	155
38	Proposed model of the multienzyme, membrane-associated complex of C. americanum	163

LIST OF APPENDICES

Appendix		PAGE
I	Inorganic macronutrient components of selected culture media	182
II	Inorganic micronutrient components of selected culture media	183
III	Organic constituents of selected culture media	184

ABBREVIATIONS

AQ	Anthraquinone
ATP	Adenosine 5′-triphosphate
BA	Benzyl adenine
CoA	Coenzyme A
DTE	Dithioerythreotol
2,4-D	2,4-Dichlorophenoxyacetic acid
EDTA	Ethylenediamine tetraacetic acid
FPLC	Fast protein liquid chromatography
GT	Glucosyltransferase
HPLC	High performance liquid chromatography
IAA	Indoleacetic acid
Kat	Katal
K	Kinetin
2-ME	2-Mercaptoethanol
M_r	Relative molecular mass
NAA	Naphthaleneacetic acid
OMT	O-Methyltransferase
PB	Polybuffer
PBE	Polybuffer exchanger
SAH	S-Adenosyl-L-homocysteine
SAM	S-Adenosyl-L-methionine
SH	Sulfhydryl
UDPG	Uridine 5′-diphosphoglucose
Z	Zeatin

A. INTRODUCTION

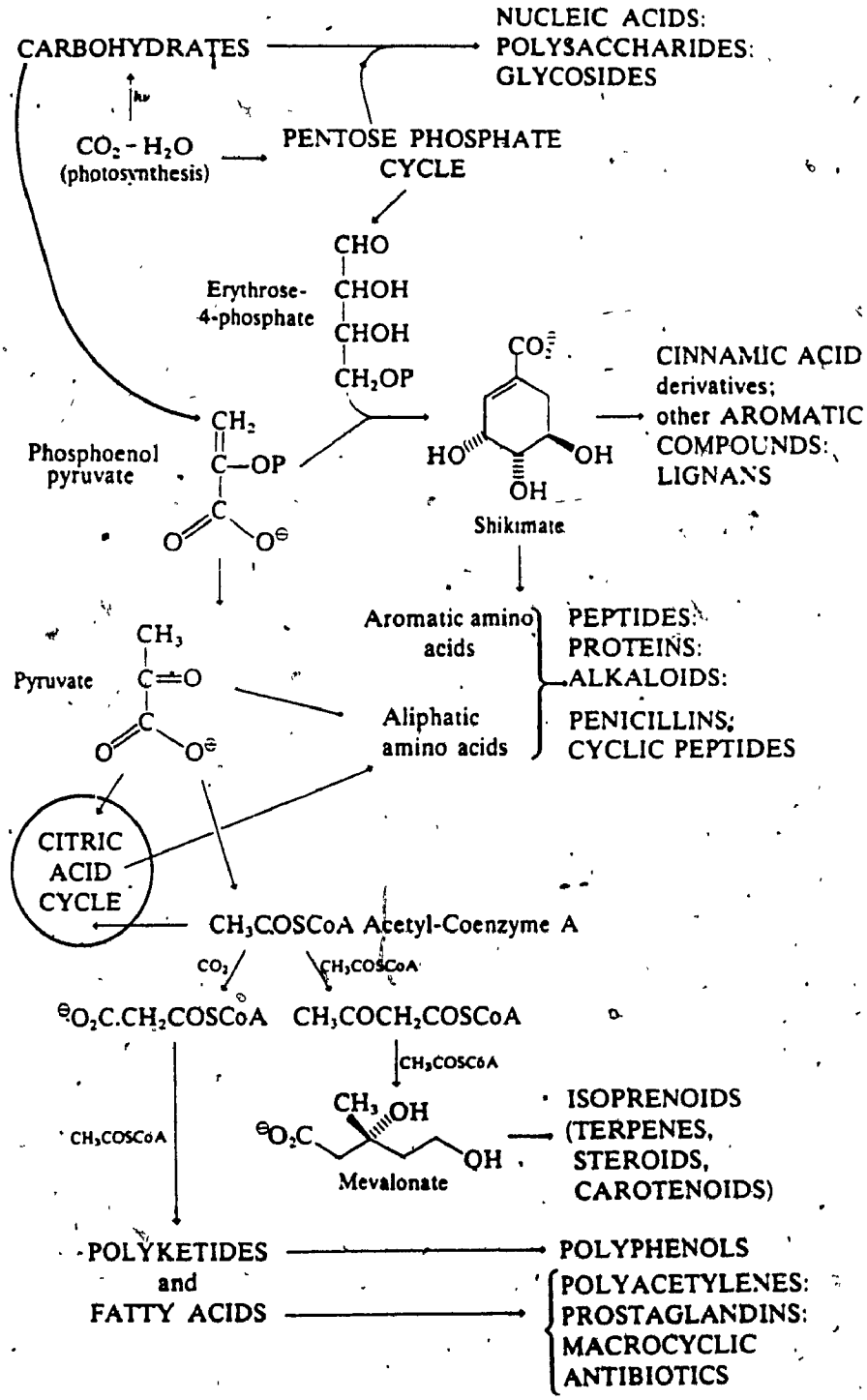
Except for photosynthesis, most organisms possess similar metabolic pathways by which they synthesize essential metabolites such as amino acids, fatty acids, nucleotides and sugars (1). These metabolites serve as starting material for the biosynthesis of proteins, lipids, nucleic acids and polysaccharides, respectively. Such compounds are involved in the biochemical processes that support growth, development and reproduction of the organism in which they occur and are, therefore, referred to as "primary metabolites" (2). On the other hand, such compounds as alkaloids, flavonoids, phenylpropanoids, quinones, steroids, tannins and volatile oils are believed to play no important role in the maintenance of fundamental life processes in most organisms and are, therefore, considered to be "secondary metabolites" (3). However, the dividing line between primary and secondary metabolism is rather blurred; since primary metabolism provides a number of molecules which are used as starting material in secondary metabolic pathways (Fig. 1). It is apparent that there are three principal chemical species which serve as precursors of secondary metabolism (4):

(a) Acetate, the precursor of polyketides, polyacetylenes, prostaglandins and isoprenoids (e.g. terpenes, steroids, and carotenoids), as well as ring A of flavonoids.

(b) Amino acids which give rise to the alkaloids and the peptide antibiotics.

(c) Shikimic acid, the precursor of many aromatic compounds including the aromatic amino acids, phenylpropanoids, and the B-ring of flavonoids.

Figure 1: The relationship between primary and secondary metabolism (2).


CARBOHYDRATES
NUCLEIC ACIDS:
POLYSACCHARIDES:
GLYCOSIDES
hν
$CO_2 - H_2O$
(photosynthesis)
PENTOSE PHOSPHATE CYCLE
CHO
CHOH
CHOH
CH_2OP
Erythrose-4-phosphate
Phosphoenol pyruvate
CINNAMIC ACID derivatives;
other AROMATIC COMPOUNDS:
LIGNANS
Shikimate
Aromatic amino acids
PEPTIDES:
PROTEINS:
ALKALOIDS:
Pyruvate
Aliphatic amino acids
PENICILLINS;
CYCLIC PEPTIDES
CITRIC ACID CYCLE
$CH_3COSCoA$ Acetyl-Coenzyme A
CO_2
$CH_3COSCoA$
$^{\ominus}O_2C.CH_2COSCoA$
$CH_3COCH_2COSCoA$
$CH_3COSCoA$
$CH_3COSCoA$
Mevalonate
ISOPRENOIDS
(TERPENES,
STEROIDS,
CAROTENOIDS)
POLYKETIDES
and
FATTY ACIDS
POLYPHENOLS
POLYACETYLENES:
PROSTAGLANDINS:
MACROCYCLIC
ANTIBIOTICS

As our knowledge of biochemical ecology has increased, we have started to realize the role and significance of some secondary metabolites (5). In plants, some compounds act as allelopathic agents or phytoalexins, others serve as deterrents to potential predators or to attract pollinators. Therefore, secondary metabolites seem to be essential for the plant's survival, although they may not play a direct role in its growth and development. Accordingly, it may be more appropriate to refer to secondary metabolites as "natural products" which seek specific functions!

Whereas a number of natural products are found in fungi (6), bacteria (7) and marine animals (8), most are of plant origin (9). Despite advances in the field of synthetic organic chemistry, plants are still the major source of natural products (10). In recent years however, there has been increasing difficulty in securing ample supply of commercially useful plants; mainly because of a disturbance of the natural environment, land exploitation, increasing labor cost, and/or technical difficulties in cultivating wild plants. Some of these difficulties can, and have been overcome by the use of cell/tissue cultures as a source of biochemicals (11). Plant cells, in culture, have the potential to produce and accumulate chemicals similar to, or identical with, those produced by the plants from which they were derived. Many natural products are important commercially and find applications as natural flavours and food colors or dyes, perfume ingredients, antiviral agents and particularly as pharmaceuticals (12). The use of cell cultures for the production of useful natural products has, therefore, gained increasing attention recently by the pharmaceutical and agri-food industries.

In order to understand the regulatory mechanisms controlling the biosynthesis of secondary metabolites and their accumulation, it is necessary to develop the detailed knowledge of their biosynthetic pathways as well as their enzymology. Among the various pathways of secondary metabolism, the biosynthesis of flavonoids (13) and quinones (14) have gained increasing attention in recent years. However, many questions remain to be answered concerning the enzymatic synthesis of these two classes of natural products.

In the present study, some aspects of the enzymatic synthesis and accumulation of naturally occurring phenolic compounds have been investigated. This work is being described in two main parts. The first part deals with the productivity and the enzymatic glucosylation of hydroxyanthraquinone glucosides in cell cultures of Cinchona succirubra. The second part involves the enzymatic methylation of the highly methylated flavonoid glucosides in Chrysosplenium americanum, and of isoflavonoids in Lupinus luteus.

B. REVIEW OF LITERATURE

B.1. Anthraquinones

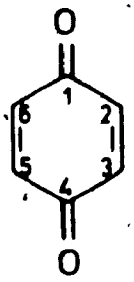
B.1.1. Occurrence and distribution

Chemically, quinones are compounds with either a 1,4-diketocyclohexa-2,5-dienoid (p-quinones) or a 1,2-diketocyclohexa-3,5-dienoid (o-quinones) moiety (15). The structure of many naturally occurring quinones is based on the benzoquinone (I, II), naphthoquinone (III, IV), or anthraquinone (V) ring systems (Fig. 2). Quinoid natural products are rather unevenly distributed in the plant kingdom. They occur in fungi, lichens, gymnosperms, and angiosperms (14). Quinones have been encountered in almost all parts of the plant (16). Thus, anthraquinones have been isolated from leaves and stems (17), pods (18), seed coats and embryos (19), roots (20), fruits (21), flowers (22), tubers (23), leaf glands (24), root bark (25) and heart wood (26). A summary of some common naturally occurring anthraquinones and the source from which they were isolated is given in Table 1.

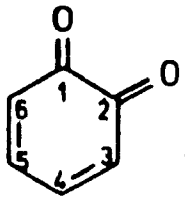
B.1.2. Productivity in tissue culture

Most species of the Rubiaceae have been shown to accumulate large amounts of anthraquinones in tissue/cell cultures (57). Many species produce even more anthraquinones on a dry weight basis in cultured tissues than does the parent plant. Table 2 summarizes the type and the source of cultures used to produce anthraquinones in vitro. Although anthraquinone production in cultured tissues seems to be regulated by nutritional and hormonal factors (57, 74), however, no generalization could be made regarding the optimum conditions for the production of

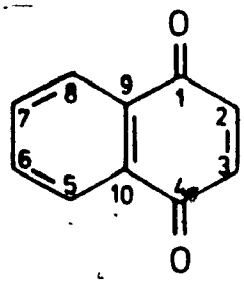
Figure 2: Basic skeletons of naturally occurring quinones and their numbering systems (16).



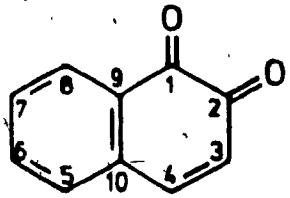
I



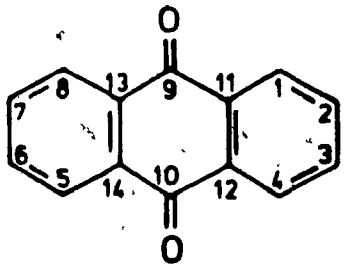
II



III



IV



V

Table 1: Some common naturally occurring anthraquinones.

Compound[1]	Source	Reference[2]
Alizarin (1,2-dihydroxy AQ)	Rubia tinctorum	26
	Galium spp.	27
	Morinda citrifolia	28
	Rheum palmatum	29
Xanthopurpurin (1,3-dihydroxy AQ)	Rubia tinctorum	30
	Rubia cordifolia	31
	Galium spp.	27
	Morinda umbellata	32
Chrysophanol (1,8-dihydroxy-3-methyl AQ)	Rheum spp.	33
	Rumex spp.	34
	Rhamnus spp.	35
	Cassia spp.	36
	Aloe spp.	37
Aloe-emodin (1,8-dihydroxy-3-hydroxymethyl AQ)	Aloe spp.	38
	Rheum spp.	39
	Rhamnus spp.	40
	Cassia spp.	41

Contd. on p. 20

Table 1 (contd.)

Rhein (1,8-dihydroxy-3-carboxy AQ)	Rheum spp.	42
	Rumex spp.	43
	Cassia spp.	44
Purpurin (1,2,4-trihydroxy AQ)	Rubia tinctorum	26
	Rubia cordifolia	45
	Galium spp.	27
Morindone (1,5,6-trihydroxy-3-methyl AQ)	Morinda citrifolia	46
	Morinda tinctoria	47
	Morinda umbellata	48
Emodin (1,6,8-trihydroxy-3-methyl AQ)	Rheum spp.	49
	Rumex spp.	50
	Polygonum spp.	51
	Rhamnus spp.	52
	Cassia spp.	53
Physcion (1,8-dihroxy-6-methoxy-3-methyl AQ)	Rheum spp.	39
	Rumex spp.	43
	Polygonum spp.	54
	Rhamnus spp.	55
	Cassia spp.	56

[1] AQ, anthraquinone.

[2] Representative references.

Table 2: The type and source of anthraquinone-producing tissue cultures

Type of culture	Source	References
Cell suspension	*Morinda citrifolia*	58-61
Cell suspension	*Galium mollugo*	62-64
Cell suspension	*Rubia cordifolia*	65-67
Cell suspension	*Cinchona ledgeriana*	68
Cell suspension	*Cinchona succirubra*	69
Callus	*Cinchona succirubra*	70,71
Callus	*Cinchona succirubra*	72-74

these metabolites.

B.1.3. Biosynthesis

Anthraquinones in higher plants are derived from a variety of precursors and by different pathways (75-77).

B.1.3.1. The acetate-polymalonate pathway

One of the most common pathways leading to anthraquinone biosynthesis is the polyacetate or acetate-polymalonate pathway. Anthraquinones often exhibit a characteristic substitution pattern reflecting their biosynthesis from acetyl-coenzyme A (CoA) and malonyl CoA. Anthraquinones which are substituted on both rings A and C are normally derived by this pathway. Emodin for example, is derived from one acetate and seven malonate units (Fig. 3).

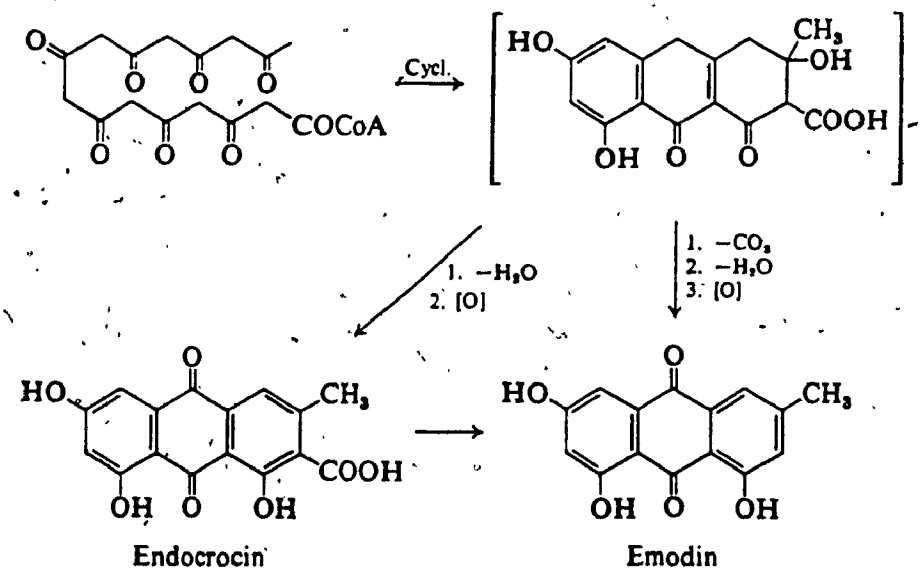
B.1.3.2. The shikimic acid-o-succinylbenzoic acid pathway

Anthraquinones which are substituted on ring A only are usually derived from o-succinylbenzoic acid . Alizarin, for example, has been shown to be derived from shikimic acid, via o-succinylbenzoic acid, and mevalonic acid (Fig. 4). Shikimic acid gives rise to o-succinylbenzoic acid which, in turn, following cyclization, prenylation and decarboxylation gives rise to alizarin (Fig. 4).

B.1.4. Enzymatic synthesis

Studies of the enzymes which catalyze the biosynthesis of anthraquinones have been hampered by the fact that cultured cells contain high concentration of anthraquinones which, on homogenization, inactivate enzymes. A systematic study of the different methods aimed at removal of anthraquinones and isolation of active enzyme preparations from Galium mollugo cells (79) led to the detection of o-succinylbenzoic

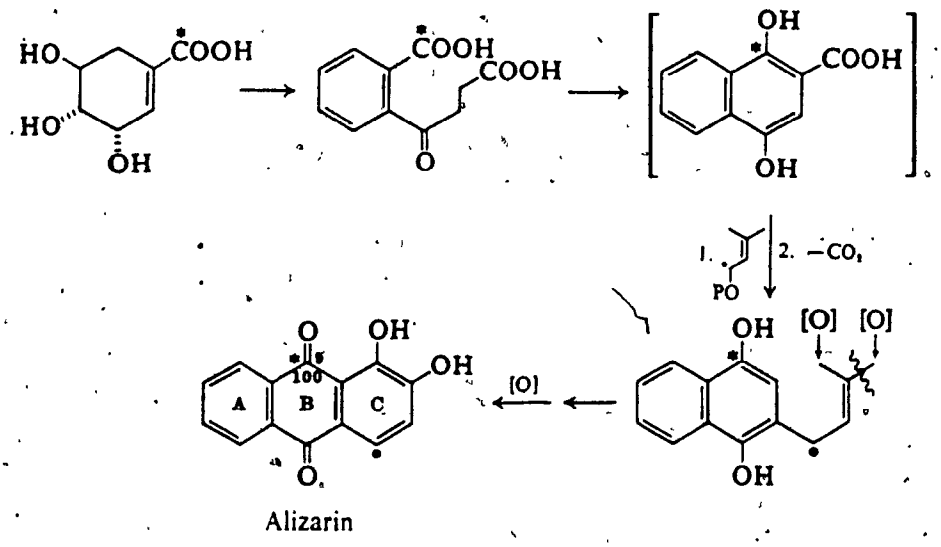
Figure 3: Biosynthesis of emodin (1,6,8-trihydroxy-3-methyl anthraquinone).



Endocrocin

Emodin

Figure 4: Biosynthesis of alizarin (1,2-dihydroxy anthraquinone).


HO
COOH
HO
OH
COOH
COOH
O
OH
COOH
OH
1.
2. −CO₂
PO
OH
[O]
[O]
O
OH
OH
100
A
B
C
O
[O]
OH

Alizarin

acid: CoA ligase (80) which mediates the formation of o-succinylbenzoic acid-CoA from o-succinylbenzoic acid. This enzyme has been purified approximately 1,200-fold from Mycobacterium phlei and has been shown to require ATP and Mg^{2+} for optimal activity (81). Very recently, o-succinylbenzoic acid synthase which catalyzes the formation of o-succinylbenzoic acid was purified from E. coli (82) and was shown to require Mn^{2+} for activity. However, little is known of the enzymes involved in the later steps of biosynthesis of anthraquinones.

B.1.5. Significance

Many anthraquinone-containing plants, such as the Rubiaceae, have been used for dyeing textiles in many parts of the world since ancient times (14). For example, Cassia (Leguminosae), Rhamnus (Rhamnaceae) and Rheum (Polygonaceae) spp. are associated with the well known purgative extracts obtained from these plants (senna, cascara and rhubarb, respectively). Anthraquinone glucosides which are derived mainly from Cassia and Rheum spp., on the other hand, are important cathartic compounds (83). They have been effectively used as purgatives and are widely used in geriatric and pediatric medicine for their unique pharmacological effects. Some anthraquinones have been used as antispasmodics, diuretics and relaxants (84). Others have been commercially used as antifungal and antibacterial agents (85). A number of anthraquinones isolated from Morinda were also shown to have antileukemic effects (86). Recently, it was suggested that anthraquinones in Cinchona may be stress compounds or phytoalexins, since they exhibited antimicrobial activity towards a range of microorganisms, and that their occurrence was only observed in infected

plants or in tissue cultures (87).

B.2. Flavonoids

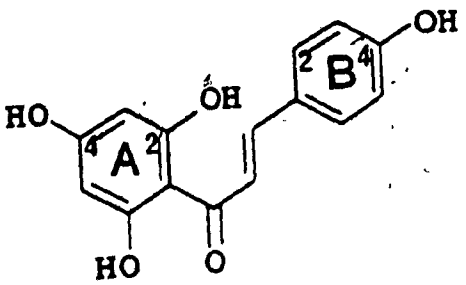
B.2.1. Occurrence and distribution

Flavonoid compounds constitute one of the most characteristic classes of natural products in higher plants. Many flavonoids are easily recognized as flower and fruit pigments (the anthocyanins) and the yellow pigments (flavones and flavonols) in all parts of the plant (88). The chemical structures of flavonoids are based on the C_{15} skeleton of flavone (Fig. 5). The various groups of flavonoids are classified according to the oxidation state of the heterocyclic ring. However, the substitution pattern of ring B further contributes to their structural variation. The major flavonoids occur mostly in higher plants, but not excluding mosses and ferns. However, their levels may vary greatly during different stages of plant development (89). Flavonoids usually exist in the plant as glycosides with different pattern of glycosylation (88).

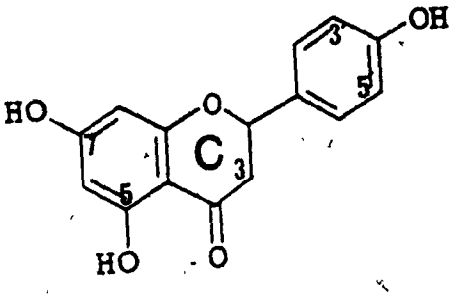
B.2.2. Biosynthesis

Earlier experiments with radioactively labeled precursors established that the carbon skeleton of the flavonoid ring system is derived from acetate and phenylalanine (90). Ring A is formed from three acetate (malonate) units, whereas phenylalanine gives rise to ring B and C-2, C-3, and C-4 of the heterocyclic ring C (Fig. 6). A common carbon intermediate in the formation of all flavonoids is the chalcone or the isomeric flavanone.

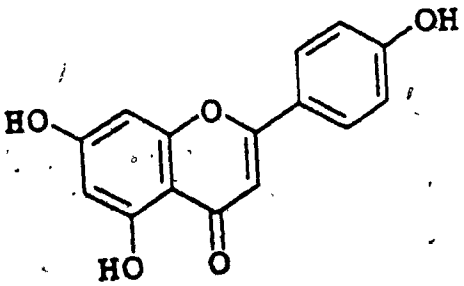
Figure 5: Representatives of each of six major subgroups of flavonoids (88).



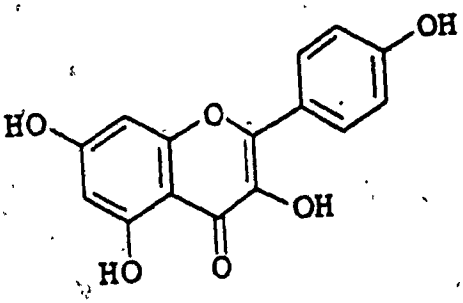
Chalcone
Naringenin chalcone



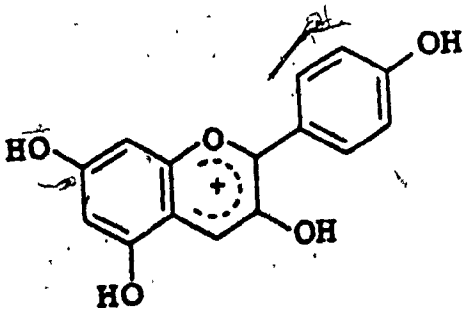
Flavanone
Naringenin



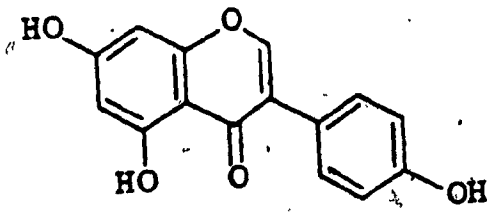
Flavone
Apigenin



Flavonol
Kaempferol

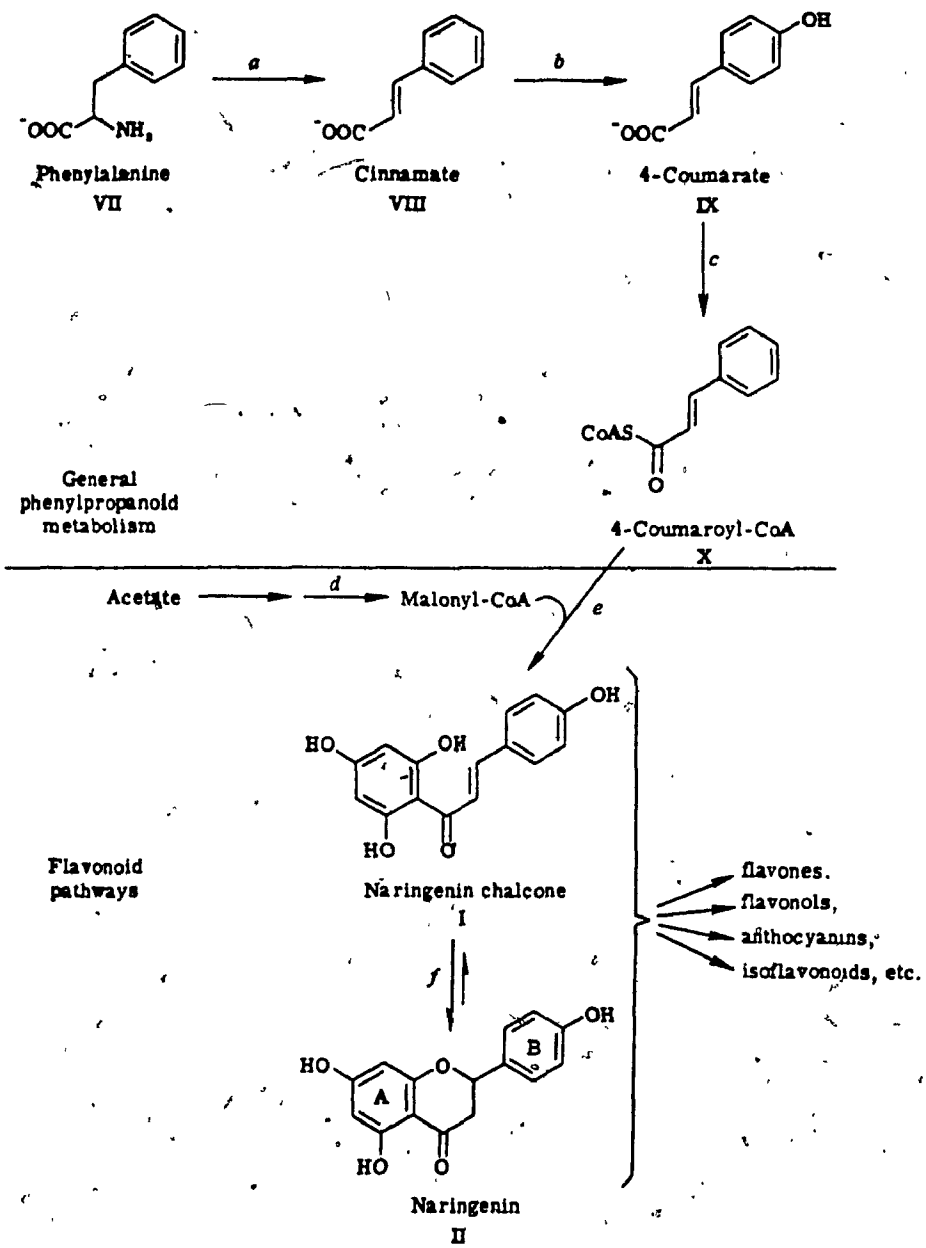


Anthocyanidin
Pelargonidin



Isoflavone
Genistein

Figure 6: Common steps of biosynthesis of flavonoids.
Enzymes: (a) phenylalanine ammonia-lyase; (b) cinnamate 4-hydroxylase; (c) 4-coumarate: CoA ligase; (d) acetyl-CoA carboxylase; (e) chalcone synthase and (f) chalcone isomerase.


a
b
c
d
e
f
Phenylalanine
VII
Cinnamate
VIII
4-Coumarate
IX
4-Coumaroyl-CoA
X
General
phenylpropanoid
metabolism
Acetate
Malonyl-CoA
Flavonoid
pathways
Naringenin chalcone
I
Naringenin
II
flavones,
flavonols,
anthocyanins,
isoflavonoids, etc.

B.2.3. Enzymatic synthesis

Recent work at the enzymatic level has confirmed earlier isotopic studies of the incorporation of acetate and phenylalanine into flavonoids, where cinnamoyl-CoA ester condenses with 3 molecules of malonyl-CoA.

B.2.3.1. Enzymes of general phenylpropanoid metabolism

The first two enzymes of the general phenypropanoid metabolism, phenylalanine ammonia-lyase and cinnamate 4-hydroxylase (Fig. 6) provide precursors, not only for flavonoid biosynthesis, but also for most other phenylpropanoid compounds such as the lignin building blocks and cinnamoyl esters (88). The third enzyme of this sequence, 4-coumarate:CoA ligase catalyzes the conversion of trans-4-coumarate to its CoA ester. The latter plays a central role as an intermediate at the branching point between the general phenylpropanoid metabolism and the various subsequent specific pathways (Fig. 6). Another enzyme, acetyl-CoA carboxylase, catalyzes the formation of malonyl-CoA. The latter is not only an important building block for the carbon skeleton of flavonoids, but also a substrate for malonylation of flavonoid glycosides in a number of tissues (91).

B.2.3.2. Chalcone synthase

Chalcone synthase, the key enzyme in the formation of the flavonoid skeleton (Fig. 6), mediates the stepwise condensation of 4-coumaroyl-CoA with three molecules of malonyl CoA (92,93). This enzyme has been purified from parsley (Petroselinum hortense) (94) and Haplopappus gracilis (95) cell cultures. Substituted coumaroyl-CoA esters did not serve as good substrates for this enzyme, thus suggesting that further hydroxylation as well as methylation may take place at a later stage of

flavonoid biosynthesis.

B.2.3.3. Chalcone isomerase

This enzyme, which catalyzes the formation of flavanones from chalcones (Fig. 6), has been isolated from a variety of plants including parsley (96). Chalcone isomerase has no cofactor requirement and often occurs in multiple forms which are isoenzymes. Usually, the substrate specificity of the isomerases corresponds with the substitution pattern of the flavonoids found in plant tissues from which the enzymes were isolated (90).

B.2.3.4. Hydroxylases

The hydroxyl groups located at the 5-, 7- and 4´-positions are usually derived from the assembly of flavonoid rings A and B (Fig. 6). Recently, however, a number of flavonoid-specific enzymes have been reported to introduce hydroxyl groups at the flavonoid ring system. The 3´-hydroxylases of Matthiola incana (97) and of Antirrhinum majus flowers (98) and of parsley cell culture (99) were shown to catlyze the 3´-hydroxylation of the B-ring of flavanones and dihydroflavonols. However, the enzyme from Verbena hybrida (100) mediated the hydroxylation of positions 3´ as well as 5´ of flavanones and dihydroflavonols. These enzymes were found to be associated with the microsomal fraction and required NADPH as cofactor. Two distinct enzymes, an NADH-dependent monooxygenase (98) and a 2-oxoglutarate-dependent dioxygenase (99) were reported to catalyze the oxidation of flavanones to flavonols. However, nothing is known of the introduction of hydroxyl groups at positions 6, 8, and/or 2´ of the flavonoid ring systems. Very recently, however, the cytochrome P450-dependant 2´-hydroxylation of isoflavone has been reported from Cicer arietinum cell culture (101).

B.2.3.5. O-Methyltransferases

Until recently, our knowledge of O-methylation of flavonoids has been restricted to the formation of monomethyl ether derivatives (102). A number of flavonoid-specific O-methyltransferases (OMTs) have been reported to catalyze single methylation steps of positions 3′ of flavones or flavonols (103-105), 3′/5′ of anthocyanins (106), 4′ of isoflavones (107) and flavones (108), 7 of flavonols (109, 110) and C-glycoflavones (111) and 8 of flavonols (112). None of these enzymes was reported to accept partially methylated substrates for further O-methylation. However, recent work in this laboratory demonstrated the step-wise O-methylation of a number of flavonoid substrates to various methylated derivatives by partially purified preparations of Calamondin orange (113,114). These enzyme preparations accepted partially methylated flavonoid substrates for further methylation, although they did not lend themselves for further purification.

Very recently, a number of OMTs were isolated from Chrysosplenium americanum and were shown to mediate the step-wise methylation of flavonoids (115). These enzymes catalyzed the methylation of quercetin ------> 3-methylquercetin ------> 3,7-dimethylquercetin ------> 3,7,4′-trimethylquercetin and of 3,7,4′-trimethylquercetagetin to its 6-methyl derivative. These OMTs were partially purified (116) and their kinetic properties determined (117). However, the 7-O-methyltransferase was unstable during purification and, therefore, its characterization was not achieved (116). Furthermore, nothing was known, at the time, of the methylation of flavonoids at positions 2′ and 5′. The characterization of the latter O-methyltransferases will be the subject of a part of this study.

B.2.3.6. O-Glucosyltransferases

Whereas glucosyltransferases (GTs) were believed to possess a broad substrate specificity, however, recent reports tend to indicate their specificity towards the different classes of flavonoids (118, 119). A number of enzymes have been reported to catalyze the glucosylation of positions 3 or 7 of flavonols (120), and 3 or 5 of anthocyanins (121). Furthermore, a flavonol 3-glucosylating system from Pisum sativum seedlings was resolved into three distinct enzymes (122); one of which accepted the flavonol aglycone, whereas the other two, accepted the mono- and diglucosides as substrates, respectively.

Previous studies in our laboratory (123) indicated the presence in Chrysosplenium americanum of a ring B-specific O-glucosyltransferase which attacked the 2′- or 5′- positions of partially methylated flavonols. The fact that the 2′- and 5′-glucosylating activities could not be separated by conventional chromatography (123), as well as their similarities in the kinetic constants and kinetic mechanism (124), suggested that both glucosylation steps may be catalyzed by a single enzyme. Very recently, however, the separation of these two O-glucosylatransferases was achieved using affinity chromatography (161).

B.2.4 Significance

Due to their bright colours, flavonoids in higher plants may serve to attract insects involved in the pollination of flowers (88). Other important functions have been attributed to flavonoids such as protection of plant tissues against UV radiation (125) or infection by pathogens (126). Some flavonoids have been shown to be antiviral (127), anti-inflammatory (128, 129) or anti-histaminic (130, 131) agents.

C. MATERIALS AND METHODS

C.1. Plant material

C.1.1. Cinchona succirubra

This plant is known to accumulate a variety of quinoline alkaloids (132). However, it was brought to our attention that during the growth of cell suspension cultures of Cinchona succirubra (Rubiaceae) both medium and cells turned yellow-orange in color (Fig. 7) which was suspected to be due to the production of phenolic/flavonoid compounds. These constituents turned out to be anthraquinone glucosides which prompted us to investigate their composition and production by this tissue culture.

An inoculum was obtained from Dr. M. Rideau (Univ. of Tours, France) and was subcultured at 4-weekly intervals on B5 medium (133) containing glucose (20 g/l); 2,4-D (1mg/l); kinetin (0.1 mg/l); myo-inositol (0.1 g/l); coconut water (50 ml/l) and agar (7 g/l). A suspension culture was initiated by transferring the callus tissues to one-liter nippled flasks, each containing 100 ml of liquid medium. The latter consisted of the same nutrient composition as the solid medium with the omission of agar. The flasks were allowed to rotate centripetally around an axle connected by a universal joint to a variable-gear motor at 2 rpm (Fig. 8). Free cells and small cell aggregates were separated from the large clumps by filtering the suspension through a sterile nylon mesh. A 20% inoculum (ca. 1.5 g fresh weight) of fine cell suspension was transferred into fresh medium at 15-day intervals. Culture growth was determined by measuring fresh and dry weights as well as protein content of cells.

Figure 7: Cinchona succirubra (1) cell suspension and (2) callus cultures.

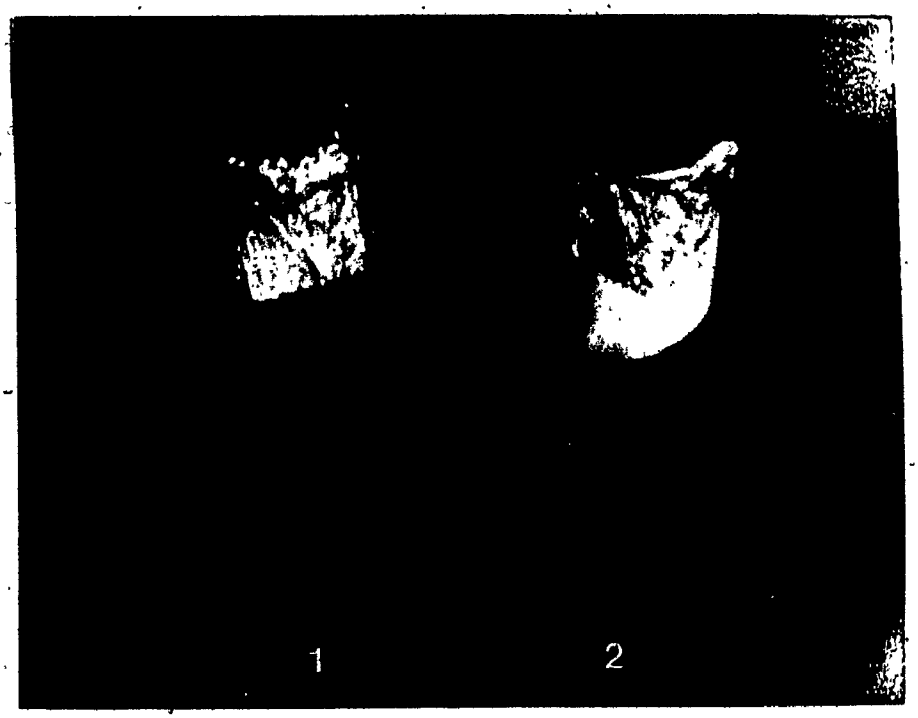
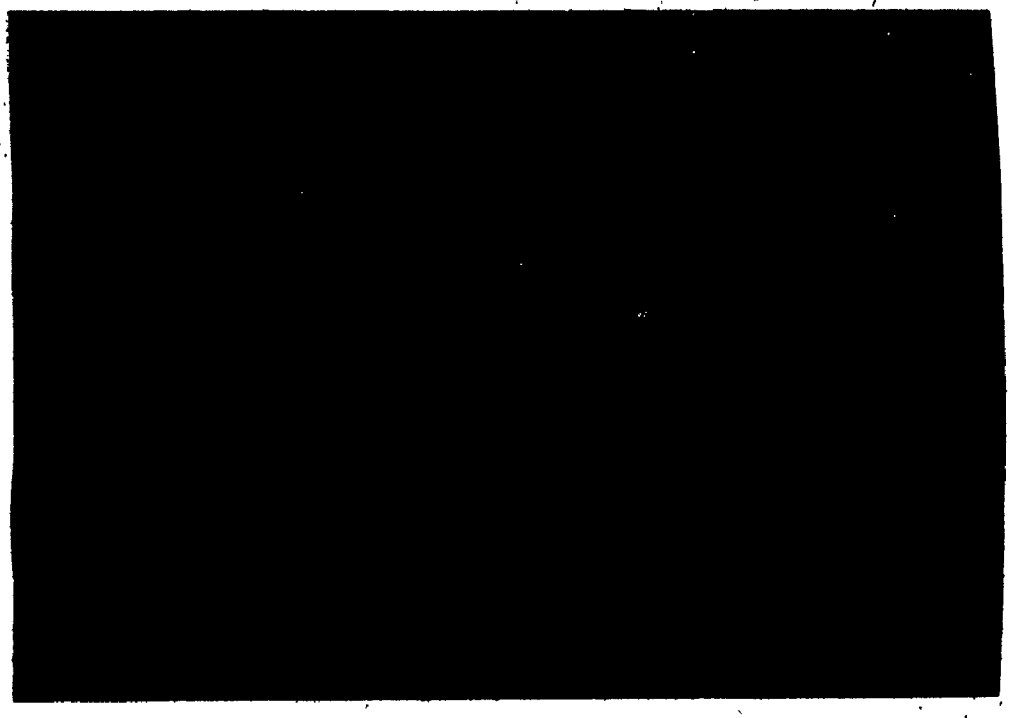

1
2

Figure 8: The set-up used for growth of Cinchona succirubra cells.



C.1.2. Chrysosplenium americanum

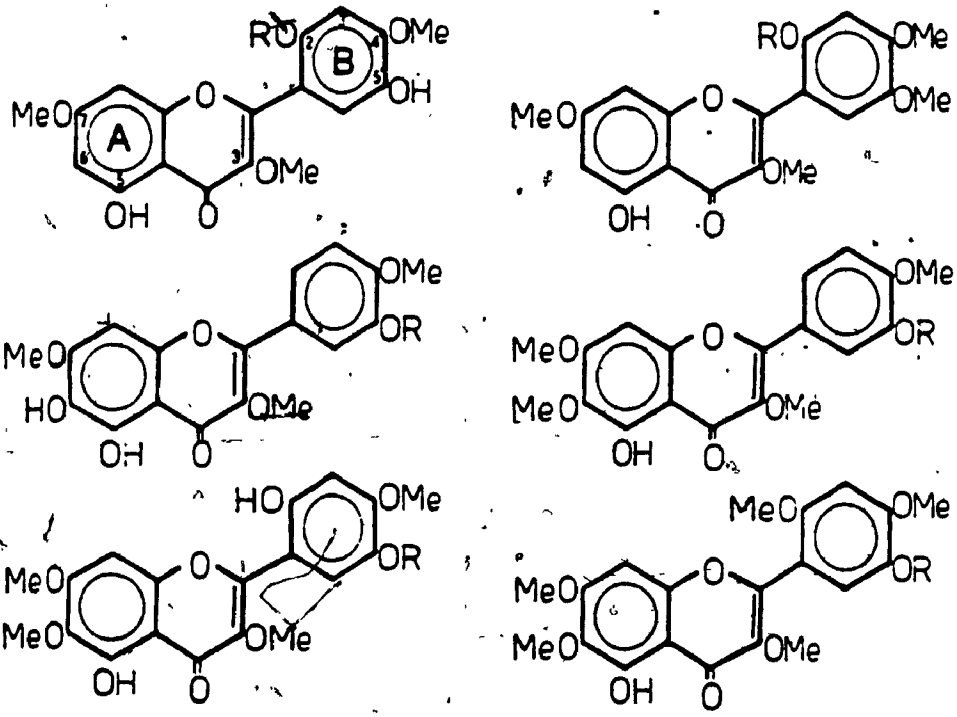
C. americanum Schwein ex Hooker (Saxifragaceae) accumulates a variety of tri-to-penta-O-methylated flavonol glucosides (134) which are derived from 2'-hydroxyquercetin and its 6-hydroxy derivative, 2'-hydroxyquercetagetin (Fig. 9). This plant material has been the subject of previous enzymatic studies, and was used as a source of the enzymes reported in this work. It was collected from Valleyfield, Province of Quebec, and its growth was maintained in the greenhouse under conditions simulating its natural habitat with respect to temperature, light and humidity. The plants were installed in a separate compartment of the greenhouse, equipped with a netting ceiling in order to decrease the light intensity. The temperature was controlled by a refrigeration unit and was maintained at 15-18 °C. The plants were grown in flat plastic containers (25 x 36 cm) with a running water stream to near flooding at all times. Shoot tips of this tissue were used as enzyme source in this study.

C.1.3. Lupinus luteus

Roots of L. luteus L. cv. Barpine (Leguminosae) have been shown to accumulate a number of 5-O-methylisoflavones (135). Since the 5-hydroxyl group of flavonoid compounds is considered to be the least reactive, due to its chelation with the carbonyl group of the heterocyclic ring (Fig. 5), it was considered important, therefore, to investigate the enzymatic 5-O-methylation of isoflavones in this tissue. L. luteus seeds were purchased from Barenbrug Holland B. V. (Netherlands). They were germinated in vermiculite and subsequently maintained in a mixture of potting soil under greenhouse conditions. Roots of 4 to 6 weeks old seedlings were used as a source of the 5-O-methyltransferase.

Figure 9: Structural formalae of the six highly O-methylated flavonol glucosides of Chrysosplenium americanum (134).

R = glucose.


RO
OMe
B
OH
MeO
A
OMe
OH
O
RO
OMe
OMe
MeO
OMe
OH
O
OMe
OR
MeO
HO
OMe
OH
O
OMe
OR
MeO
MeO
OMe
OH
O
HO
OMe
OR
MeO
MeO
OMe
OH
O
MeO
OMe
OR
MeO
MeO
OMe
OH
O

C.2. Chemicals

S-Adenosyl-L-[methyl-^{14}C]methionine (SAM) (42 Ci/mol) was purchased from ICN (Irvine, CA, U.S.A.). Uridine 5'-diphospho-[U-^{14}C]glucose (278 Ci/mol) was purchased from Amersham (Arlington Heights, IL, U.S.A.). Sephadex G-25, Superose 12 (prep grade), prepacked Superose 12 HR 10/30, Mono P HR 5/20, Mono Q HR 5/5 columns; polybuffer (PB-74) and Polybuffer exchanger (PBE-94) as well as the FPLC system (section C.8.) were from Pharmacia (Uppsala, Sweden). Unlabelled SAM and S-adenosyl-homocysteine (SAH) were obtained from Boehringer, Mannheim (Germany). Most flavonoid compounds were from our laboratory collection. The isoflavone substrates and reference compounds were generous gifts from Dr. S. Tahara (Hokkaido Univ., Sappora, Japan). Anthraquinones were purchased from Aldrich (Milwaukee, WI, U.S.A.), Pfalz and Bauer (Waterbury, CT, U.S.A.) and Sarsynthese (Bordeaux, France). Dowex 1X2 and Bio-Rad protein reagent were purchased from Bio-Rad Labs (Richmond, CA, U.S.A.). Commercial Polyamide 6-MN TLC plates were from Brinkmann Intruments (Mississauga, ONT). Imidazole was recrystalized from ethylacetate. All other chemicals were of analytical grade.

C.3. Extraction of anthraquinones

The cells from several culture flasks (ca. 20 g) were filtered through a fritted glass funnel using suction. The cake of cells was extracted twice with hot 75% methanol (v/v). The combined alcoholic extracts were filtered and reduced *in vacuo* to an aqueous residue which contained the anthraquinone glucosides and trace amounts of the aglycones. An aliquot of this extract was saved for analysis of the anthraquinone glucosides. The remaining portion of the aqueous extract

was hydrolyzed with 2N HCl for 15 min at 80 °C and the hydrolyzate was extracted with chloroform. After evaporation of the organic layer, the remaining residue was dissolved in ether and partitioned against 1M NaOH. The aqueous layer was acidified with HCl and extracted with diethyl ether. The latter contained the anthraquinone aglycones.

C.4. Estimation of anthraquinone content

The absorbance of the methanolic extracts of either anthraquinone glucosides or their aglycones was measured using an LKB (Ultrospec 4050) spectrophotometer at 435 nm. The anthraquinone content, relative to alizarin (1,2-dihydroxyanthraquinone), was calculated using a molar extinction coefficient of 5500, considering that the differences in the molar extinction coefficient of different anthraquinones did not exceed 5% (60).

C.5. Characterization of anthraquinones

The major anthraquinones were characterized by co-chromatography with authentic samples on silica plates and by HPLC, as well as by their color in visible and UV-light before and after exposure to ammonia vapor. The TLC solvent systems used for the glucosides were: (A) benzene-MeOH-ethyl formate-formic acid (15:5:5:1) and (B) ethylacetate-MeOH-water (77:13:10); whereas those for the aglycones were: (C) chloroform-MeOH-ammonia (85:14:1); (D) toluene-diethyl ether-EtOH (70:25:5) and (E) toluene-MeOH (9:1). HPLC of the aglycones was performed on a Waters-Millipore solvent delivery system using a μ-Bondapack C_{18} column (3.9 mm x 30 cm), 436 nm filter and 1% AcOH in 70% aqueous MeOH as solvent system at a flow rate of 1 ml/min.

C.6. Protein extraction

All extraction steps were carried out at 4 °C. Tissues (ca 15 g) were frozen in liquid nitrogen, mixed with Polyclar AT (1:5, w/w) and ground to a fine powder. The latter was homogenized with 0.2 M Tris/HCl (1:4, w/v) buffer (pH 7.6) containing 14 mM 2-mercaptoethanol, 5 mM EDTA and 10 mM diethylammonium diethyldithiocarbamate. The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 10,000 xg for 10 min. The supernatant was stirred with Dowex 1x2 (10%, w/v) which had previously been equilibrated with the same buffer, then filtered through glass wool. The filtrate was fractionated with solid ammonium sulfate, and the protein that precipitated between 35 and 80 % salt saturation was collected by centrifugation.

C.7. Enzyme purification

The enzyme protein was purified using the Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The protein (35 - 80%) pellet was suspended in the minimal amount of buffer A and chromatographed on Superose 12 HR 10/30 (300 x 10 mm I.D.) column which had previously been equilibrated with the same buffer. The column was developed with buffer A at a flow rate of 0.5 ml/min (2.5 MPa) and 1 ml fractions were collected and assayed for enzyme activity. The active fractions were pooled and subjected to further purification on either a Mono P HR 5/20 (chromatofocusing) or Mono Q HR 5/5 (anion-exchange), columns.

C.7.1. Buffers

The following buffers were used: (A) 25 mM Tris/HCl (pH 7.5) containing 14 mM 2-mercaptoethanol and 10% glycerol; (B) 25 mM bis Tris/iminodiacetic acid (pH 7.1) containing 14 mM 2-mercaptoethanol and

10% glycerol; (C) 25 mM imidazole (pH 6.4) containing 14 mM 2-mercaptoethanol; (D) same as buffer C containing 10% glycerol and (E) 0.2 M KCL in 25 mM imidazole (pH 8.0) containing 14 mM 2-mercaptoethanol and 10% glycerol.

C.7.2. Ion exchange chromatography

Further purification of the enzyme protein was achieved by ion exchange chromatography on Mono Q HR 5/5 column (50 x 5 mm I.D.). The protein was applied to the column which had previously been equilibrated with buffer A. The column was washed with 5 bed volumes of the same buffer and the bound proteins were eluted using a linear salt gradient (0 - 0.5 M KCl in buffer A) at a flow rate of 1 ml/min (2.5 MPa). One-ml fractions were collected and assayed for enzyme activity.

C.7.3. Chromatofocusing

Chromatofocusing of the enzyme protein was performed by chromatography on a Mono P 5/20 column (200 x 5 mm I.D.). The Superose 12 column was developed using buffer B and the active fractions were pooled and applied to the Mono P column which had previously been equilibrated with the same buffer. The bound proteins were eluted with 50 ml of Polybuffer-94:water (1:10, v/v) (pH4.0) containing 14 mM 2-mercaptoethanol and 10% glycerol at a flow rate of 0.7 ml/min (3.0 MPa). This generated a linaer gradient of pH 7-4. One-ml fractions were collected in tubes containing 0.25 ml of 0.2 M Tris/HCl buffer (pH 8.5) and were assayed for enzyme activity.

C.8. Fast protein liquid chromatography system

The Pharmacia FPLC system consisted of two P-500 pumps, a GP-250 gradient programmer, a V-7 injection valve, a 10 ml superloop, a single

path UV-1 monitor with HR flow cell, a pH monitor with flow-through electrode, a REC-482 two pen chart recorder and a Frac 100 fraction collector.

C.9. O-Glucosyltransferase assay

The standard assay mixture consisted of 10 µM of the phenolic substrate (in 10 µl of 50% dimethylsulfoxide, v/v), 3 µM of [U-^{14}C]UDPglucose (50 nCi), 25 mM Tris/HCl buffer (pH 7.5), 14 mM 2-mercaptoethanol and the enzyme protein in a total volume of 100 µl. The reaction was started by the addition of protein, and the mixture was incubated for 30 min at 30 °C. The reaction was stopped by the addition of 10 µl of 6N HCl and the glucosylated products were extracted using 250 µl of ethylacetate. The mixture was shaken in an Eppendorf rotary shaker for 2 min and the organic phase containing the glucosylated products was separated from the aqueous layer containing UDPglucose by a 2 min centrifugation using an Eppendorf centrifuge. An aliquot (ca 100 µl) of the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid (5g 2,5-diphenyloxazole/l of toluene).

C.10. O-Methyltransferase assay

The assay for O-methyltransferase activity was performed as described previously (115, 116) using S-adenosyl-L-[methyl-^{14}C]methionine (SAM) as methyl donor. The standard assay mixture consisted of 10 µM of the phenolic substrate (in 10 µl of 50% dimethylsulfoxide, v/v), 6 µM of SAM (containing 25 nCi), 25 mM Tris/HCl buffer (pH 7.5), 14 mM 2-mercaptoethanol and the enzyme protein in a total volume of 100 µl. The reaction was started by the addition of enzyme protein and the mixture

was incubated at 30 °C for 30 min. The reaction was terminated by the addition of 10 µl of 6N HCl and the methylated products were separated from unreacted SAM by extraction with 250 µl of benzene-ethylacetate (1:1, v/v). The mixture was shaken in an Eppendorf shaker for 2 min and the organic phase containing the methylated products was separated from the aqueous layer by a 2 min centrifugation. An aliquot (ca 100 µl) of the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid (5g 2,5-diphenyloxazole/l of toluene).

C.11. Identification of reaction products

The reaction products of the enzymes studied were subjected to similar procedures for identification purposes. The organic phase from several assays was pooled, evaporated to dryness, and subsequently dissolved in methanol. The latter was then chromatographed on commercial Polyamide-6 MN TLC plates in different solvent systems (as described in the Figure legends). The identity of the products was confirmed by co-chromatography with reference compounds, visualization in UV-light and by autoradiography on X-ray film.

C.12. Molecular weight determination

An estimate of the molecular weight of the partially purified enzymes was obtained by determining their elution volumes on gel fitration using molecular weight standards.

C.13. Determination of pI values

The pI values of the enzymes were determined from their elution pattern following chromatofocusing on a Mono P HR 5/20 column.

C.14. Analysis of kinetic data

Substrate interaction data was fitted to the following equations:

$$v = \frac{Vmax\,[A][B]}{K_{iA}K_B + [A]K_B + [B]K_A + [A][B]} \qquad [1]$$

or

$$v = \frac{Vmax\,[A][B]}{[A]K_B + [B]K_A + [A][B]} \qquad [2]$$

Where A and B are the varied substrates, K_A and K_B are the respective Michaelis constants, Vmax is the maximum velocity and $K_{iA}K_B$ is an interaction term (135-138). All data from inhibition studies was fitted to the equation for competitive (Eq. [3]), noncompetitive (Eq. [4]) or uncompetitive inhibition (Eq. [5]) as determined by inspection of the double-reciprocal plots:

$$v = \frac{Vmax}{1 + K_A/[A] + K_A[I]/K_I[A]} \qquad [3]$$

$$v = \frac{Vmax}{1 + K_A/[A] + K_A[I]/K_I[A] + [I]/K_I} \qquad [4]$$

$$v = \frac{Vmax}{1 + K_A/[A] + [I]/K_I} \qquad [5]$$

Where A is the variable substrate, I is the inhibitor (product); K_A and Vmax are the Michaelis constant and maximum velocity, respectively, in the presence of inhibitor and K_I is the inhibition constant (136-139). The data is presented as double-reciprocal plots which were fitted by linear-regression analysis (method of least squares), and the appropriate equation chosen. The kinetic constants K_A, K_B, K_{iP}, K_{iQ}, K_{iA} and Vmax were calculated from intercept and slope replots of the generated data (140).

C.15. Protein estimation

The protein was determined according to Bradford (141) using the Bio-Rad protein reagents and bovine serum albumin as standard protein.

C.16. Definition of enzyme units

Enzyme units are expressed in katals (kat) as recommended by the International Union of Biochemists (142). One katal is defined as the amount of activity which converts one mole of substrate per second under the assay conditions.

D. RESULTS

D.1. Anthraquinones

D.1.1. Growth of Cinchona succirubra cell culture

Culture growth did not respond to conventional gyrotary shaking and required a large air-to-liquid ratio, as well as slow rotation (ca 2 rpm) of flasks. These conditions were satisfied by growing the cell suspension cultures in nippled flasks using the set up described in the Methods section (section C.1.1.) (Fig. 8).

C. succirubra cells in culture grew as a homogeneous suspension, yellow-orange in color (Fig. 7). Early exponential-phase cells appeared isodiametric (Fig. 10A) which, on continued growth, became mostly elongated and tandemly joined at their narrow ends (Fig 10B).

Growth parameters (fresh and dry weights as well as protein content of cells) and anthraquinone production of cells were determined at daily intervals. The formation of anthraquinone paralleled cell growth and reached its maximum after approximately 11 days in culture (Fig. 11). Furthermore, anthraquinone production was concomitant with maximum protein content of cells.

D.1.2. Characterization of anthraquinone glucosides

Acid hydrolysis of the anthraquinone glucosides revealed the presence of alizarin (1,2-dihydroxyanthraquinone) and emodin (1,6,8-trihydroxy-3-methylanthraquinone) as the major constituents of C. succirubra; both of which amounted to 80% of the total anthraquinones. The remaining 20% consisted of a number of di- to tetrahydroxyanthraquinones. Hydrolysis of the anthraquinone glucoside

Figure 10: Early (A) and late (B) exponential phase cells of Cinchona succirubra suspension cultured in B5 medium (Appendix I).

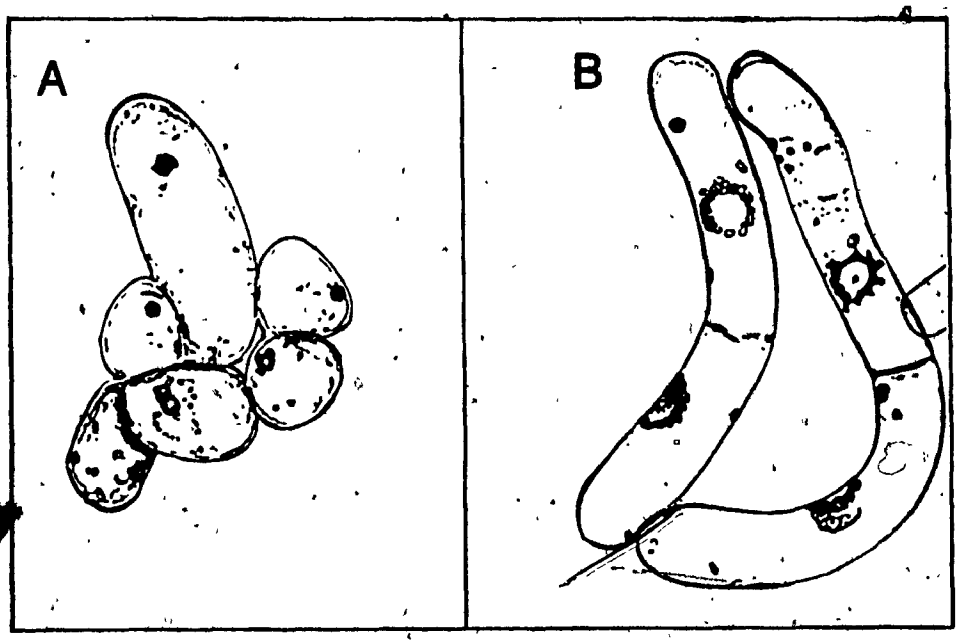
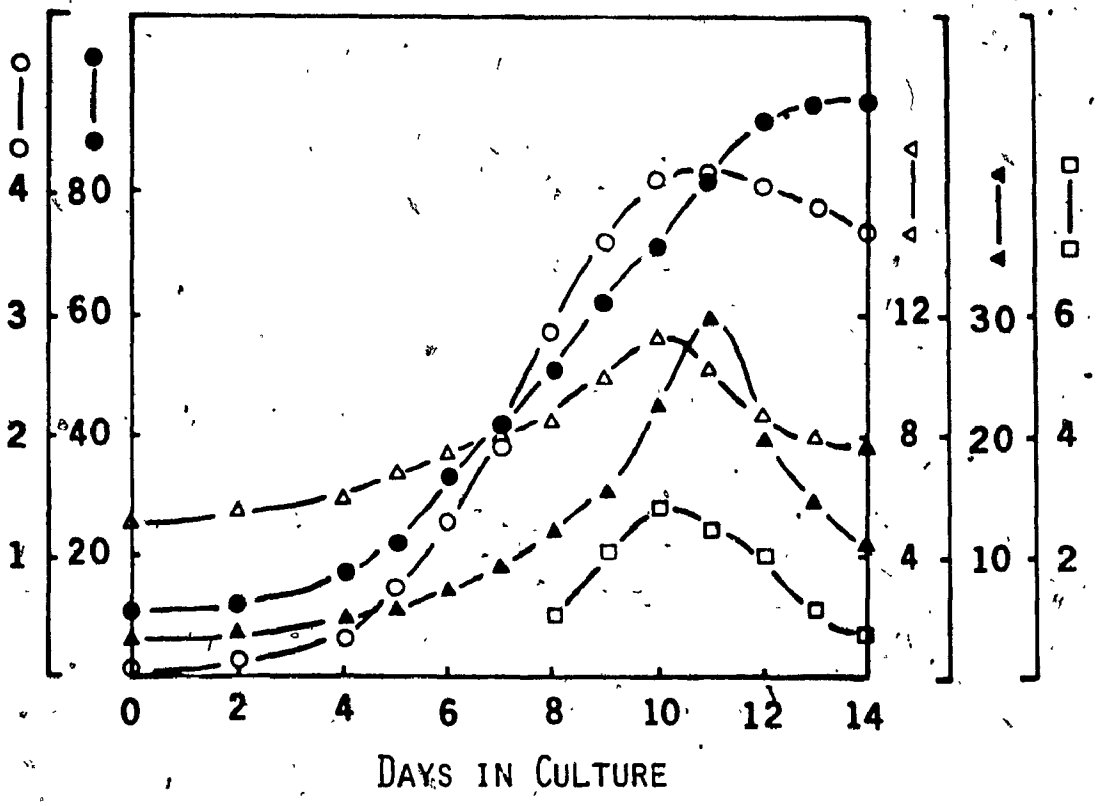

A
B

Figure 11: Changes in growth parameters and anthraquinone production of Cinchona succirubra suspension culture. ●—● , fresh weight (g/l); ○—○ , dry weight (g/l); △—△, protein content (mg/l); ▲—▲ , total anthraquinone content (µmol/l); and □—□ , glucosyltransferase activity (pkat/mg).


4
3
2
1
80
60
40
20
12
8
4
30
20
10
6
4
2
0
2
4
6
8
10
12
14
DAYS IN CULTURE

fraction with β-glucosidase, however, resulted in the release of the sugar residue which was identified by TLC as D-glucose (143). All of the anthraquinone constituents of this culture were found to be bound as their corresponding O-glucosides.

D.1.3. Separation of anthraquinone aglycones by HPLC

The separation of anthraquinone aglycones by HPLC was optimized in order to identify the anthraquinone constituents of C. succirubra. Figure 12 shows the HPLC profile of 12 reference anthraquinones which were separated on a μ-Bondapack C18 column using 1% AcOH in aqueous MeOH as solvent system, at a flow rate of 1 ml/min (2000 psi). This system was used to compare the anthraquinone constituents of callus tissues and cell suspension cultures and to determine any qualitative differences that occur under different culture conditions .

D.1.4. Anthraquinone profiles of callus and cell suspenion cultures

Figure 13 shows an HPLC profile of the anthraquinone aglycones of C. succirubra callus tissues. The major anthraquinones were identified as alizarin (8% of total); 1,2,7-trihydroxyanthraquinone (14%); emodin (5%) and 1,4-dihydroxyanthraquinone (12%). Those of the cell suspension culture were identified as 2,6-dihydroxyanthraquinone (4%); alizarin (31%); 1,2,7-trihydroxyanthraquinone (9%); rhein (16%); 1,4-dihydroxyanthraquinone (11) and emodin (24%) (Fig. 14).

D.1.5. Anthraquinone profile as a function of day in culture

The anthraquinone constituents of C. succirubra cell suspension cultures were determined as a function of growth in culture. Table 3 shows that by day 11, 2,6-dihydroxyanthraquinone; 1,2,7-trihydroxy-

Figure 12: HPLC profile of 12 reference anthraquinones. This was performed on a Waters-Millipore solvent delivery system using a μ-Bondapack C18 column (3.9 mm x 30 cm), 436 nm filter and 1% acetic acid in 70% aqueous methanol as solvent at a flow rate of 1 ml/min.

1: 2,6-dihydroxyanthraquinone;

2: anthrapurpurin (1,2,7-trihydroxyanthraquinone);

3: alizarin (1,2-dihydroxyanthraquinone);

4: aloe-emodin (1,8-dihydroxy-3-hydroxymethylanthraquinone);

5: rhein (1,8-dihydroxy-3-carboxyanthraquinone);

6: purpurin (1,2,4-trihydroxyanthraquinone)
or quinalizarin (1,2,5,8-tetrahydroxyanthraquinone);

7: anthrarufin (1,5-dihydroxyanthraquinone)
or 1,8-dihydroxyanthraquinone;

8: quinizarin (1,4-dihydroxyanthraquinone);

9: emodin (1,6,8-trihydroxy-3-methylanthraquinone);

10: chrysophanol (1,8-dihydroxy-3-methylanthraquinone).

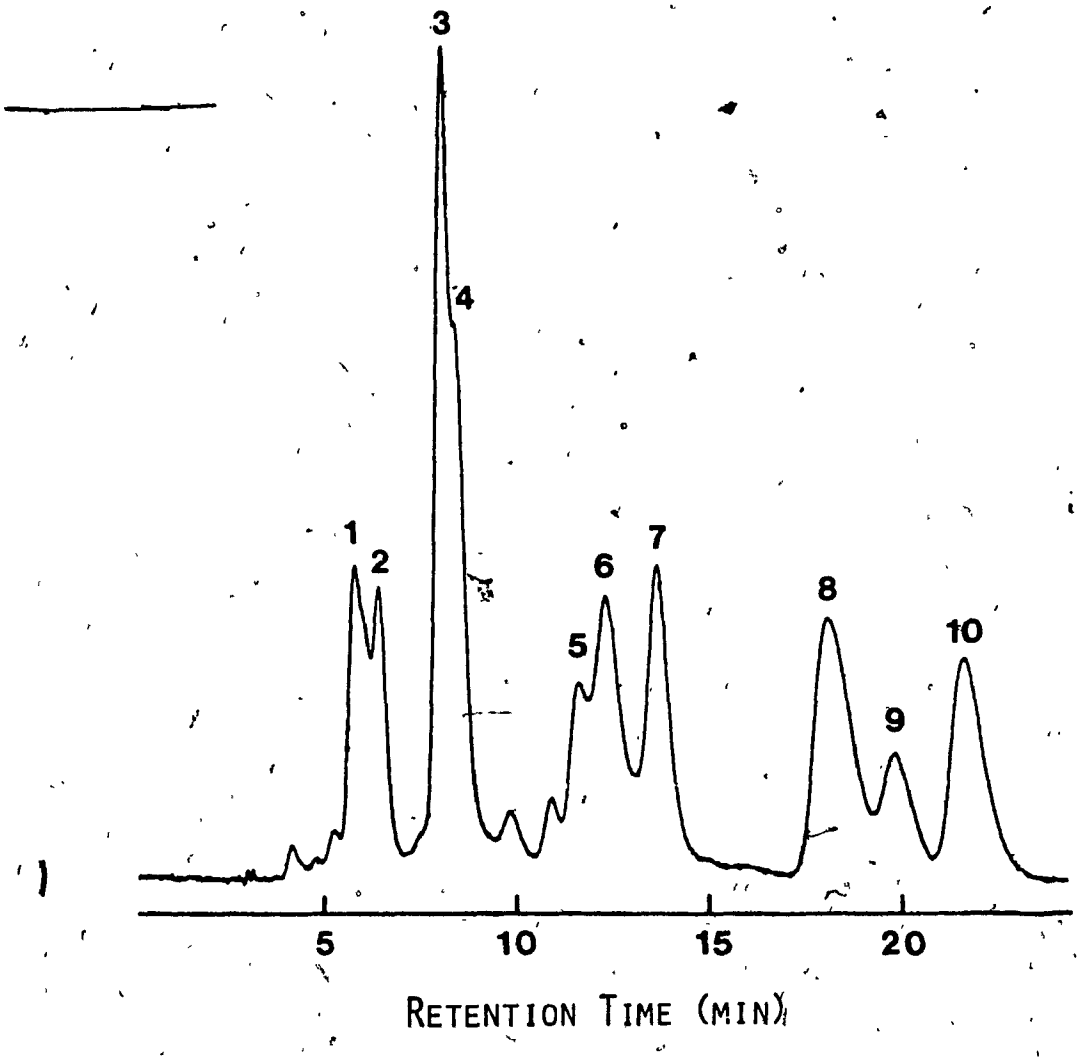

3
4
1
2
6
7
5
8
10
9
5
10
15
20
RETENTION TIME (MIN)

Figure 13: HPLC profile of Cinchona succirubra callus culture.
Conditions are the same as in the legend of Figure 12.
1: anthrapurpurin (1,2,7-trihydroxyanthraquinone);
2: alizarin (1,2-dihydroxyanthraquinone);
3: aloe-emodin (1,8-dihydroxy-3-carboxyanthraquinone);
4: purpurin (1,2,4-trihydroxyanthraquinone);
5: anthrarufin (1,5-dihydroxyanthraquinone);
6: quinizarin (1,4-dihydroxyanthraquinone);
7: emodin (1,6,8-trihydroxy-3-methylanthraquinone).

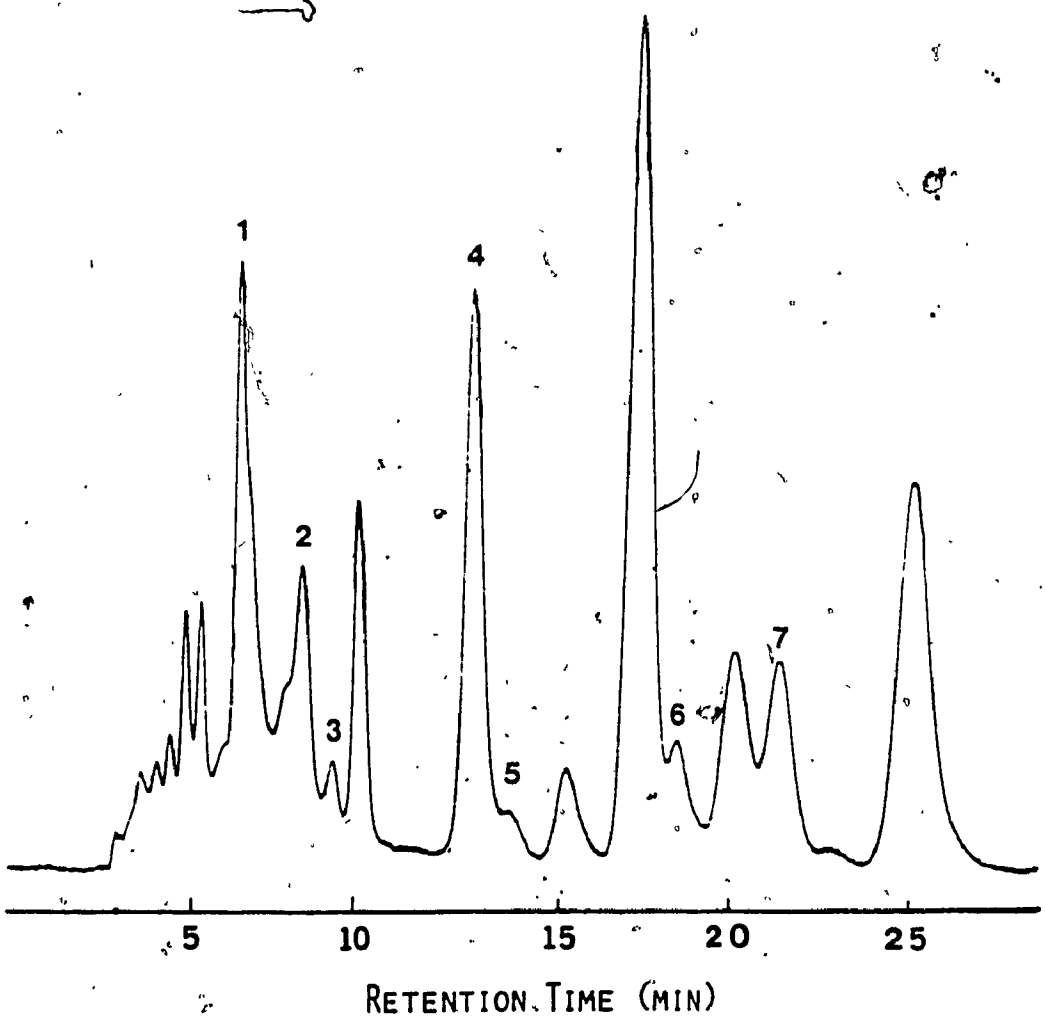

1
2
3
4
5
6
7
5
10
15
20
25
RETENTION TIME (MIN)

Figure 14: HPLC profile of Cinchona succirubra cell suspension culture. Conditions are the same as in the legend of Figure 12.

1: 2,6-dihydroxyanthraquinone;

2: anthrapurpurin (1,2,7-trihydroxyanthraquinone);

3: alizarin (1,2-dihydroxyanthraquinone);

4: aloe-emodin (1,8-dihydroxy-3-hydroxymethylanthraquinone);

5: rhein (1,8-dihydroxy-3-carboxyanthraquinone);

6: purpurin (1,2,4-trihydroxyanthraquinone);

7: 1,8-dihydroxyanthraquinone;

8: quinizarin (1,4-dihydroxyanthraquinone);

9: emodin (1,6,8-trihydroxy-3-methylanthraquinone).

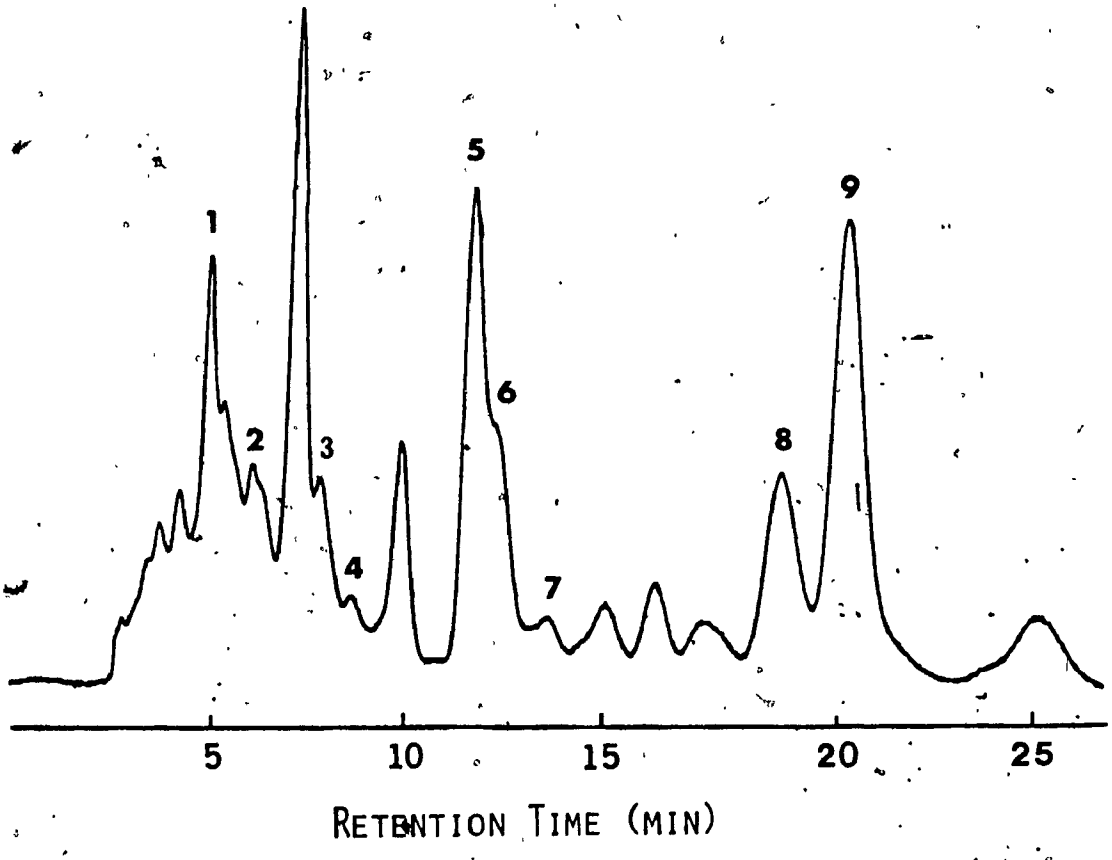

1
2
3
4
5
6
7
8
9
5
10
15
20
25
RETENTION TIME (MIN)

Table 3: The anthraquinone content of C. succirubra cell suspension as a function of growth in culture [1].

	Anthraquinones[2]									
Day	2,6-OH	1,2,7-OH	AE	1,2,4-OH	1,8-OH	UI	1,4-OH	E	UI	UI
1	5	3	2	2	1	2	3	4	1	1
3	5	3	2	2	1	2	3	4	1	1
5	9	4	8	6	6	9	7	16	4	4
7	8	4	9	7	6	9	7	17	4	4
9	9	4	9	2	7	9	6	22	4	5
11	11	7	3	2	7	8	6	26	5	7
13	14	8	3	2	8	8	7	26	3	4
17	14	8	2	2	8	5	7	24	3	5
19	14	9	2	4	8	5	8	35	3	5
21	15	9	2	5	9	5	8	26	3	5
24	15	9	2	5	9	5	8	34	5	6

[1] As percent of total.

[2] AE, aloe-emodin; E, emodin; UI, unidentified; OH, hydroxy-.

Values are average of 3 determinations, S.E. < 10%.

anthraquinone; 1,4-dihydroxyanthraquinone; 1,8-dihydroxyanthraquinone and emodin made up more than 60% of the total anthraquinone constituents.

D.1.6. Effect of nutritional factors

The effect of nutritional factors on the growth and anthraquinone content of C. succirubra cells in culture were investigated.

D.1.6.1. Effect of sucrose

Various concentrations of sucrose (2 to 8 %, w/v) were used to test their effect on growth and anthraquinone production of C. succirubra cells cultured in B5 medium. Optimum growth was observed with 2% sucrose (Table 4). Increasing sugar concentration, however, resulted in a progressive decrease in cell yield, although anthraquinone production was increased. The latter was more pronounced when anthraquinone productivity was calculated on cell dry weight basis (200 %) than per liter of culture medium (33 %); since an increase in sugar concentration above 2 % resulted in decreased cell growth. However, when sucrose concentration was raised above 8 % both parameters were severly inhibited.

D.1.6.2. Effect of different media

The effect of the different nutrient media (B5, MS, White's and Heller's), supplemented with 2% sucrose, 1 ppm 2,4-D and 0.1 ppm kinetin, was studied on growth and anthraquinone production (see Appendix for media composition). Cells grown in MS or B5 media showed similar growth rates as well as anthraquinone content (Table 5). On the other hand, those grown in White's or Heller's media exhibited a decline in cell growth but an increase in anthraquinone production, whether the

Table 4: Effect of sucrose concentration on growth and anthraquinone production.

Sucrose Concentration	F.W. g/l	D.W. g/l	Anthraquinone content µmol/l	Anthraquinone content µmol/g D.W.
2%	96.7	4.8	8.6	1.8
4%	79.2	4.2	11.4	2.7
6%	68.9	3.8	11.4	3.0
8%	64.9	3.1	11.2	3.6

Cells were grown in B5 medium as described in the Methods section.

F.W., fresh weight; D.W., dry weight.

Values are average of 3 determinations; S. E. < 10%.

Table 5: Effect of different media on growth and anthraquinone production[1].

Medium	F.W.	D.W.	Anthraquinone content	
	g/l	g/l	µmol/l	µmol/g D.W.
B5	95.0	4.2	8.4	2.0
1/2 B5	83.5	3.7	10.4	2.8
1/4 B5	67.5	3.0	11.2	3.7
MS	104.0	4.4	7.6	1.7
White	97.0	3.6	11.6	3.2
Heller	103.2	3.7	12.3	3.3

[1] See Appendix for composition of different media.

F.W., fresh weight; D.W., dry weight.

Values are averages of 3 determinations; S. E. < 10%.

latter was determined per cell dry weight or per liter of medium.

When the macro- and microelements of the B5 medium were diluted 2- and 4-fold, anthraquinone production was increased by 40% and 85%; although cell yield was decreased by 23% and 38%, respectively (Table 5).

D.1.7. Effect of growth regulators

The effect of different concentrations of growth regulators, both individually and in combination, was tested on cell growth and anthraquinone production in B5 medium. The cytokinins used were kinetin (K), benzyladenine (BA) and zeatin (Z); whereas the auxins used were 2,4-dichlorophenoxyacetic acid (2,4-D), α-indoleacetic acid (IAA) and α-naphthaleneacetic acid (NAA).

In the absence of auxin, either benzyladenine or zeatin was more effective than kinetin for the production of anthraquinones; although zeatin slightly inhibited cell growth as compared with the two other cytokinins (Table 6). However, in the absence of cytokinins, either IAA or NAA increased the production of anthraquinones as compared with 2,4-D, although IAA slightly inhibited cell growth.

Increasing concentrations of cytokinin, at a constant level of 2,4-D (1.0 ppm) showed no differences in either cell growth or production of anthraquinones (Table 7). On the other hand, increasing concentrations of NAA (from 1.25 to 12.5 ppm) or IAA (from 2.5 to 25 ppm), in the presence of constant concentrations of kinetin (1 ppm), enhanced both cell growth and anthraquinone production; whereas increasing concentrations of 2,4-D (from 0.25 to 2.5 ppm) inhibited both parameters (Table 8). These results show that in the presence of 1 ppm kinetin,

Table 6: Effect of growth regulators on cell growth and anthraquinone production of C. succirubra cultures.

Addition	Concentration	F.W.	D.W.	Anthraquinone content	
	ppm	g/l	g/l	µmol/l	µmol/g D.W.
none	-	130	5.7	12.0	2.11
K	0.1	174	6.2	7.8	1.25
BA	0.1	161	6.5	12.7	1.95
Z	0.1	115	5.6	17.6	3.15
2,4-D	1	119	6.0	6.8	1.14
IAA	5	92	4.1	22.6	5.53
NAA	25	124	5.7	12.0	2.11

F.W., fresh weight; D.W., dry weight; K, kinetin; BA, benzyladenine; Z, zeatin; 2,4-D, 2,4-dichlorophenoxy acetic acid; IAA, α-indoleacetic acid; NAA, α-naphthalene acetic acid.

Values are the mean of 3 determinations; S. E. < 10%.

Table 7: Effect of increasing concentrations of cytokinins on growth and anthraquinone production (at 1 ppm 2,4-D).

Addition	Concentration	F.W.	D.W.	Anthraquinone content	
	ppm	g/l	g/l	µmol/l	µmol/g D.W.
K	0.025	130	4.5	5.5	1.22
	0.10	124	4.4	5.6	1.27
	0.25	124	4.4	5.5	1.25
BA	0.025	103	4.2	8,9	2.12
	0.10	115	4.6	9.1	1.98
	0.25	111	4.5	9.0	2.00
Z	0.025	83	4.0	8.5	2.12
	0.10	82	4.0	8.6	2.15
	0.25	82	4.1	8.5	2.07

F.W., fresh weight; D.W., dry weight; K, kinetin; BA, benzyladenine; Z, zeatin.

Values are the mean of 3 determinations; S. E. < 10%.

Table 8: Effect of increasing concentrations of auxins on cell growth and anthraquinone production of C. succirubra cultures (at 0.1 ppm kinetin).

Addition	Concentration	F.W.	D.W.	Anthraquinone content	
	ppm	g/l	g/l	µmol/l	µmol/g D.W.
2,4-D	0.25	131	4.5	8.1	1.80
	1.0	85	4.3	4.9	1.14
	2.5	57	4.0	3.4	0.85
NAA	1.25	82	4.0	11.9	3.00
	5.0	88	4.1	12.6	3.10
	12.5	124	4.3	12.4	2.88
IAA	2.5	39	2.6	15.2	5.85
	10.0	65	2.9	16.2	5.59
	25.0	64	2.8	19.7	7.04

F.W., fresh weight; D.W., dry weight; 2,4-D, 2,4-dichlorophenoxy acetic acid; NAA, α-naphthalene acetic acid; IAA, α-indole acetic acid.
Values are the mean of 3 determinations, S.E. < 10%.

cell growth was stimulated by 2,4-D, whereas anthraquinone production was enhanced by the presence of IAA, and to a lesser extent, by NAA.

D.1.8. Changes in glucosyltransferase activity during culture growth

In order to study the enzymatic glucosylation of anthraquinones, it was essential to determine the peak of glucosyltransferase (GT) activity in relation to culture growth and the production of anthraquinones by C. succirubra cells. Figure 11 shows that the enzymatic glucosylation of anthraquinones paralleled cell growth and was concomitant with maximum protein content as well as anthraquinone production in Cinchona cells.

D.1.9. Purification of GT activity

The GT activity of 10-day old cells of C. succirubra was purified by fractional precipitation with ammonium sulfate, gel filtration on Superose 12 HR 10/30 column (Fig. 15), followed by chromatofocusing on Mono P HR 5/20 column (Fig. 16). The latter purification step resulted in the resolution of five peaks of GT activity with apparent pI values of 5.3, 4.8, 4.5, 4.3 and 4.1. These activities exhibited distinct specificity towards different substituted anthraquinones. The combined purification steps resulted in an increase in specific activity of 202-, 337-, 228-, 417- and 823-fold for peaks I, II, III, IV and V, respectively, as compared with the crude extract (Table 9).

D.1.10. Characterization of GT activities

D.1.10.1. Substrate specificity

The different activity peaks (Fig. 16) exhibited preference for different substrates, so that peak I accepted emodin (1,6,8-trihydroxy-3-methylanthraquinone); peak II, anthrapurpurin (1,2,7-

Figure 15: Elution profile of glucosyltransferase activity after gel filtration on Superose 12 HR 10/30 column using emodin as substrate. The ammonium sulfate pellet was suspended in the minimal amount of 25 mM Tris/HCl buffer (pH 7.2) containing 14 mM 2-mercaptoethanol (ME) and 10% glycerol. The protein was loaded on the column which had previously been equilibrated with the same buffer. The column was developed using the same buffer at a flow-rate of 0.5 ml/min (2.5 MPa) and 2-ml fractions were collected and assayed for glucosyltransferase activity.

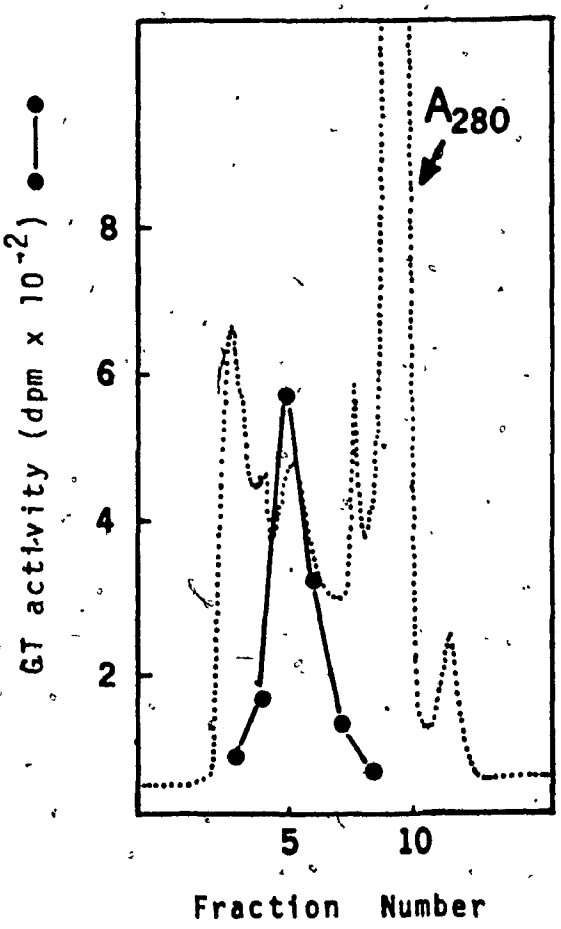

A280
GT activity (dpm x 10⁻²)
8
6
4
2
5
10
Fraction Number

Figure 16: Elution profile of glucosyltransferase activity after chromatofocusing on a Mono P HR 5/20 column using emodin as substrate. The active fractions from gel filtration were pooled and applied on the column which had previously been equilibrated with 25 mM Tris/HCl buffer (pH 7.2) containing 14 mM 2-mercaptoethanol (ME) and 10% glycerol. Elution of the bound proteins was performed with 40 ml of Polybuffer (74): water (1:10, v/v) (pH 4.0) containing 14 mM 2-ME and 10% glycerol, at a flow-rate of 1.0 ml/min (2.0 MPa). One-ml fractions were collected into 0.2 M Tris buffer (pH 8.0) containing 14 mM 2-ME and were assayed for glucosyltransferase activity.

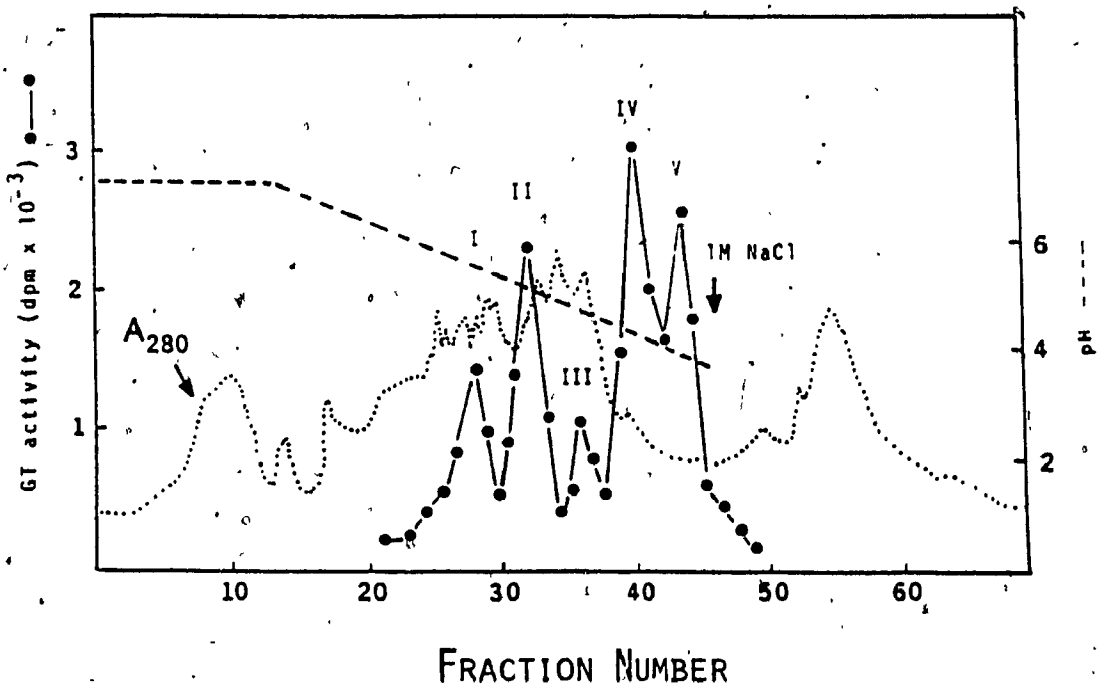

GT activity (dpm x 10^{-3})
3
2
1
A_{280}
I
II
III
IV
V
1M NaCl
6
4
2
pH
10
20
30
40
50
60
FRACTION NUMBER

Table 9: Purification of C. succirubra glucosyltransferase activity.

Purification step	Total protein (mg)	Specific activity (pkat/mg)	purification (-fold)	Recovery (%)
Crude extract[1]	101	1.07	-	100
Ammonium Sulfate[2]	92.7	1.21	1.1	100
Gel Filtration[3]	7.4	12.0	11.2	82
Chromatofocusing[4]				
Peak I	0.138	216	202	28
Peak II	0.102	361	337	34
Peak III	0.087	244	228	20
Peak IV	0.078	446	417	32
Peak V	0.054	881	823	44

[1] Dowex 1 X 2

[2] Desalted on Sephadex G-25

[3] On Superose 12 HR 10/30 column

[4] On Mono P HR 5/20 column

trihydroxyanthraquinone); peak III, quinizarin (1,4-dihydroxyanthraquinone); whereas peaks IV and V accepted 2,6-dihydroxyanthraquinone and 1,8-dihydroxyanthrhaquinone as the best substrates, respectively (Table 10). Due to the difficulty in the separation of the anthraquinone glucosides (144), the individual reaction products could not be identified. However, based on the substitution pattern of the different substrates used (Table 10), it seems that these GTs attack C-1 and C-2 of anthraquinones, or their equivalent positions. Furthermore, all five enzymes accepted quercetin, as well as 3,7,3´,4´-tetramethylquercetin as substrates to various extent (Table 10). This may be due to the fact that positions 5 and 7 of a flavone are stucturally equivalent to positions 1 and 2 of an anthraquinone, respectively (Fig 17). However, it is interesting to note that the least reactive 5-hydroxyl of quercetin could be subjected to enzymatic glucosylation, despite the fact that it is chelated with the carbonyl group, as is evident from the reactivity of 3,7,3´,4´-tetramethylquercetin (Table 10). Other phenolic compounds tested were poor glucosyl acceptors.

D.1.10.2. Effect of pH

The pH optimum for the glucosylation reaction as determined in different buffers, was found to be pH 7. Maximum GT activity was observed in histidine/HCl buffer. A change of one pH unit resulted in a loss of 60 % of enzyme activity (Fig. 18).

D.1.10.3. Linearity of the glucosylation reaction

At optimum pH and 10 µM of substrate, the glucosylation of anthraquinones was linear with time up to 60 min and with protein concentration up to 50 µg.

Table 10: Substrate specificity of C. succirubra glucosyltransferases.

Substrate	Relative activity (%)[1]				
	I	II	III	IV	V
Alizarin (1,2-diOH-AQ)	44	50	46	44	59
Quinalizarin (1,2,5,8-tetra-OH-AQ)	34	22	14	21	55
Chrysophanol (1,8-diOH-3Me-AQ)	41	50	86	85	80
Emodin (1,6,8-triOH-3-Me-AQ)	100	80	87	87	67
2,6-diOH-AQ	73	49	35	100	54
Purpurin (1,2,4-triOH-AQ)	32	29	25	30	48
1,8-diOH-AQ	39	42	85	87	100
Quinizarin (1,4-diOH-AQ)	33	50	100	77	64
Anthrarufin (1,5-diOH-AQ)	11	28	43	32	34
Anthrapurpurin (1,2,7-triOH-AQ)	48	100	43	44	54
3,7,3´,4´-tetra-O-Me-Quercetin	29	72	82	22	71
Apigenin	35	32	29	34	27
Kaempferol	58	37	48	42	36
Quercetin	60	52	77	76	71
p-Coumaric acid	35	30	32	29	30
Caffeic acid	40	45	35	37	41

[1] 100% activity represents 216, 361, 244, 446, and 881 pkat/mg protein for peaks I, II, III, IV and V, respectively using the standard enzyme assay described in the Methods section (section C.8.).

Figure 17: Formula of an anthraquinone ring system showing similarity of positions 1 and 2 to those of 5 and 7 in a flavonoid ring.

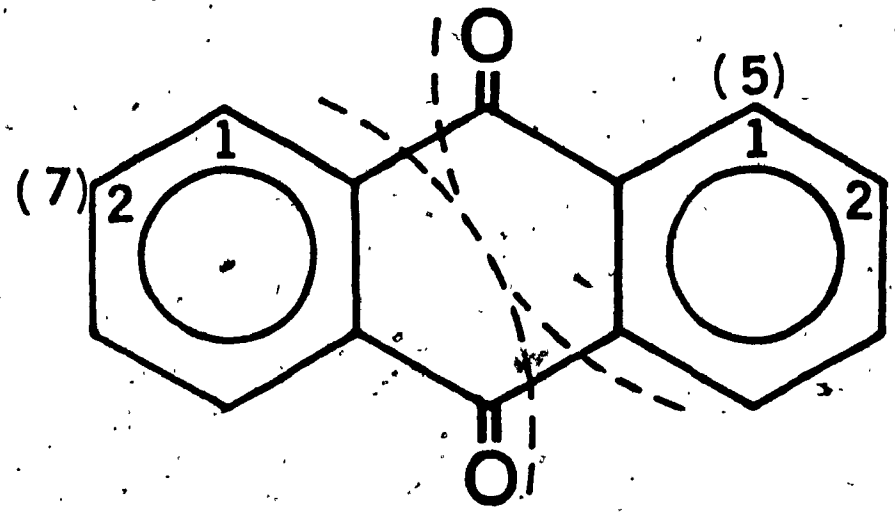
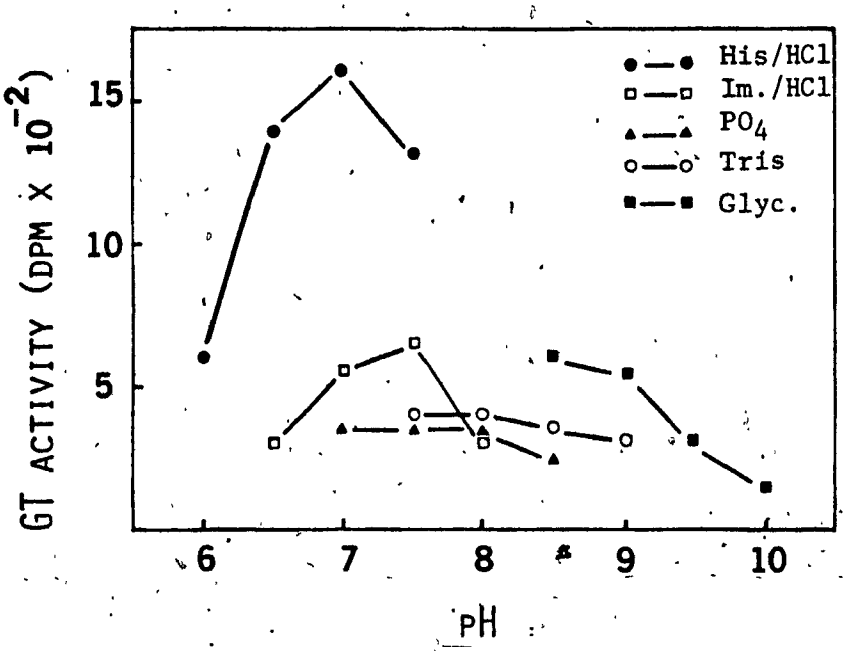

O
(5)
1
1
(7)
2
2
O

Figure 18: pH optimum of the glucosylation reaction of anthraquinones using emodin as substrate. The enzyme protein was used after gel filtration.


His/HCl
Im./HCl
PO4
Tris
Glyc.
GT ACTIVITY (DPM X 10^-2)
15
10
5
6
7
8
9
10
pH

D.1.10.4. Enzyme stability

In the absence of sulfhydryl group protectors, the partially purified enzymes lost more than 50 % of their activity within 48 hr. However, the addition of 14 mM 2-mercaptoethanol to either of the enzymes resulted in 50 % loss of activity after two weeks. The partially purified GTs were stored in 25 mM histidine/HCl buffer pH 7.0 containing 10 % glycerol and 10 mM DTE at -20 ^{o}C and were fairly stable for two months.

D.1.10.5. Effect of divalent cations and sulfhydryl group reagents

The effect of cations and sulfhydryl group inhibitors on the glucosylation reaction was studied (Table 11). The fact that EDTA did not inhibit the enzyme activity seems to indicate that the glucosylation of anthraquinones has no requirement for divalent cations. However, low concentrations of Co^{+2}, Cu^{+2}, and Zn^{+2} inhibited the glucosylation reaction by 50-75%. Furthermore, GT activity was inhibited by various SH-group reagents, but the addition of DTE resulted in 100% recovery from inhibition (Table 11).

D.1.10.6. Other properties

The five GTs had an apparent M_r of 50,000 and an apparent K_m value for their respective anthraquinone substrates of 10 µM. It is interesting to note that the activity of peaks III, IV and V following chromatofocusing was recovered only when 1 ml fractions were collected into 0.25 ml of 0.2 M Tris/HCl buffer (pH 8.5). This is probably due to their low pI values (Fig. 16) and their instability at that pH.

Table 11: Effect of divalent cations and SH-group reagents on *C. succirubra* glucosyltransferase activity [1].

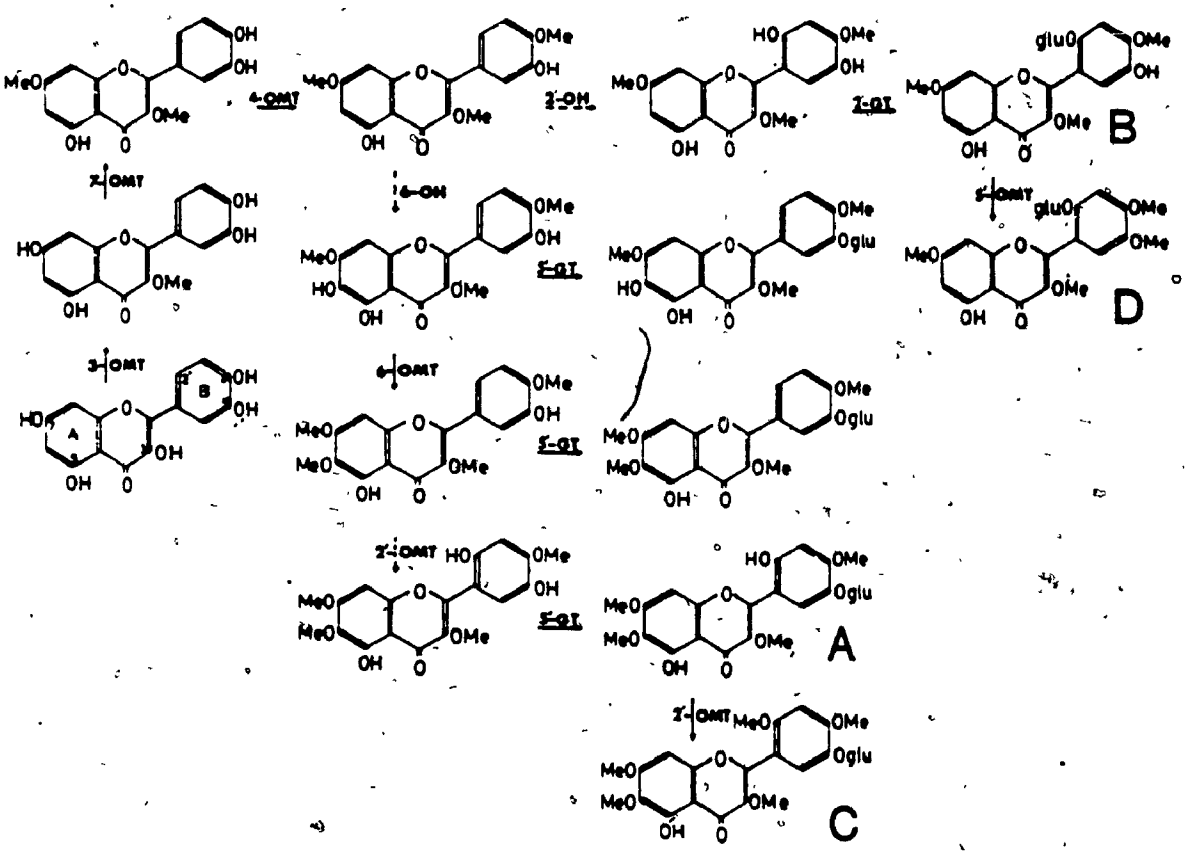
Additions	Concentration (mM)	Relative activity (%)
None	-	100
Mg^{+2}	1	110
Mg^{+2}	10	105
Mn^{+2}	1	105
Mn^{+2}	10	55
Ca^{+2}	1	110
Co^{+2}	1	40
Cu^{+2}	1	50
Zn^{+2}	1	23
EDTA	1	130
EDTA	10	125
DTE	10	163
Iodoacetamide	1	40
Iodoacetate	1	70
N-Ethylmaleimide	1	8
Phenyl mercuriacetate	1	20
Phenyl mercuriacetate + DTE	1,10	116
p-Chloromercuribenzoate	1	53
p-Chloromercuribenzoate + DTE	1,10	131

[1] Enzyme preparation was used after gel filtration with emodin as substrate.

D.2. Flavonoid O-methyltransferases

Chrysosplenium americanum accumulates a variety of tri- to penta-O-methylated flavonol glucosides (134) which are derived from 2′-hydroxyquercetin and its 6-hydroxy derivative, 2′-hydroxyquercetagetin (Fig 19). Previous studies on the O-methyltransferase system of Chrysosplenium indicated the presence of four distinct, position-specific OMTs which catalyzed the coordinated sequence of methylation of quercetin ----> 3-methylquercetin ----> 3,7-dimethylquercetin ----> 3,7,4′-trimethylquercetin; and of 3,7,4′-trimethylquercetagetin to 3,6,7,4′-tetramethylquercetagetin (115, 116). However, the instability of the 7-OMT during the previous purification scheme did not allow its purification or its characterization (116). This enzyme catalyzes the methylation of 3-methylquercetin to 3,7-dimethylquercetin (Fig. 19), an essential early step in the enzymatic sequence of polymethylated flavonol glucosides synthesis in C. americanum (Fig. 19). The later steps of methyl transfer in the biosynthesis of Chrysosplenium flavonoids, on the other hand, are catalyzed by yet another enzyme, namely the 2′-O-methyltransferase. In contrast with the four previously reported OMTs (115,116) which accepted aglycones as substrates, this enzyme accepts only its natural glucosylated substrate, 5,2′-dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-O-glucoside (Fig. 19). Earlier studies with the 2′-OMT preparation indicated that it catalyzed the O-methylation of 5,5′-dihydroxy-3,7,4′-trimethoxyflavone-2′-O-glucoside as well, with the formation of its 5′-O-methyl derivative. Our interest in the characterization of the Chrysosplenium system made it imperative to purify and characterize the 7-OMT with the aim to compare its properties and its kinetic mechanism to those of the 3-, 6- and 4′-OMTs of this

Figure 19: Proposed pathway for the enzymatic synthesis of polymethylated flavonol glucosides in Chrysosplenium americanum. GT, O-glucosyltransferase; OH, hydroxylase; OMT, O-methyltransferase. Dashed arrows are hypothetical steps.


4-OMT
2-OH
2-GT
B
2-OMT
6-OH
5-GT
5'-OMT
D
3-OMT
6-OMT
5-GT
2'-OMT
5-GT
A
2'-OMT
C

tissue (115, 116). Furthermore, it was considered important to investigate whether both the 2'- and the 5'-methylation steps are catalyzed by one or two distinct enzymes. This section will deal with the purification and characterization of the 7- and 2'-/5'-OMTs of C. americanum.

D.2.1. Purification of the 7-OMT

The enzyme was purified by fractional precipitation with ammonium sulfate (35 to 70 % saturation), followed by successive gel filtration on a Superose 12 HR 16/50 column (Fig. 20), chromatofocusing on a Mono P HR 5/20 column (Fig. 21) and ion-exchange chromatography on a Mono Q HR 5/5 column (Fig. 22) using an FPLC system. The combined purification steps resulted in an increase in specific activity of 700-fold of the partially purified enzyme as compared with the crude extract with a 37% yield (Table 12). The use of a linear pH gradient between 7 and 4, in chromatofocusing, resulted in complete loss of enzyme activity during earlier studies (116). However, by resorting to a shallower gradient (pH 7-5), the 7-OMT eluted in an active state at pH of 6.0 (Fig. 21). Furthermore, enzyme activity was recovered only when eluted fractions were collected into 0.25 ml of 0.2 M Tris/HCl buffer (pH 8.5). This was attributed to the instability of the 7-OMT at a pH near its pI value (pH 6.0).

D.2.2. Properties of the purified 7-OMT

D.2.2.1. Substrate specificty

The partially purified enzyme preparation accepted 3-methylquercetin as the only substrate and gave rise to 3,7-dimethylquercetin as the sole product (115). It did not accept any of

Figure 20: Elution profile of the 7-OMT activity after gel filtration on Superose 12 HR 16/50 column. The ammonium sulfate pellet was suspended in the minimal amount of 25 mM bis Tris/ imonodiacetic acid buffer (pH 7.1) containing 14 mM 2-mercaptoethanol (ME) and 10% glycerol. The protein was loaded on the column which had previously been equilibrated with the same buffer. The column was developed with the same buffer and 3-ml fractions were collected and assayed for OMT activity.

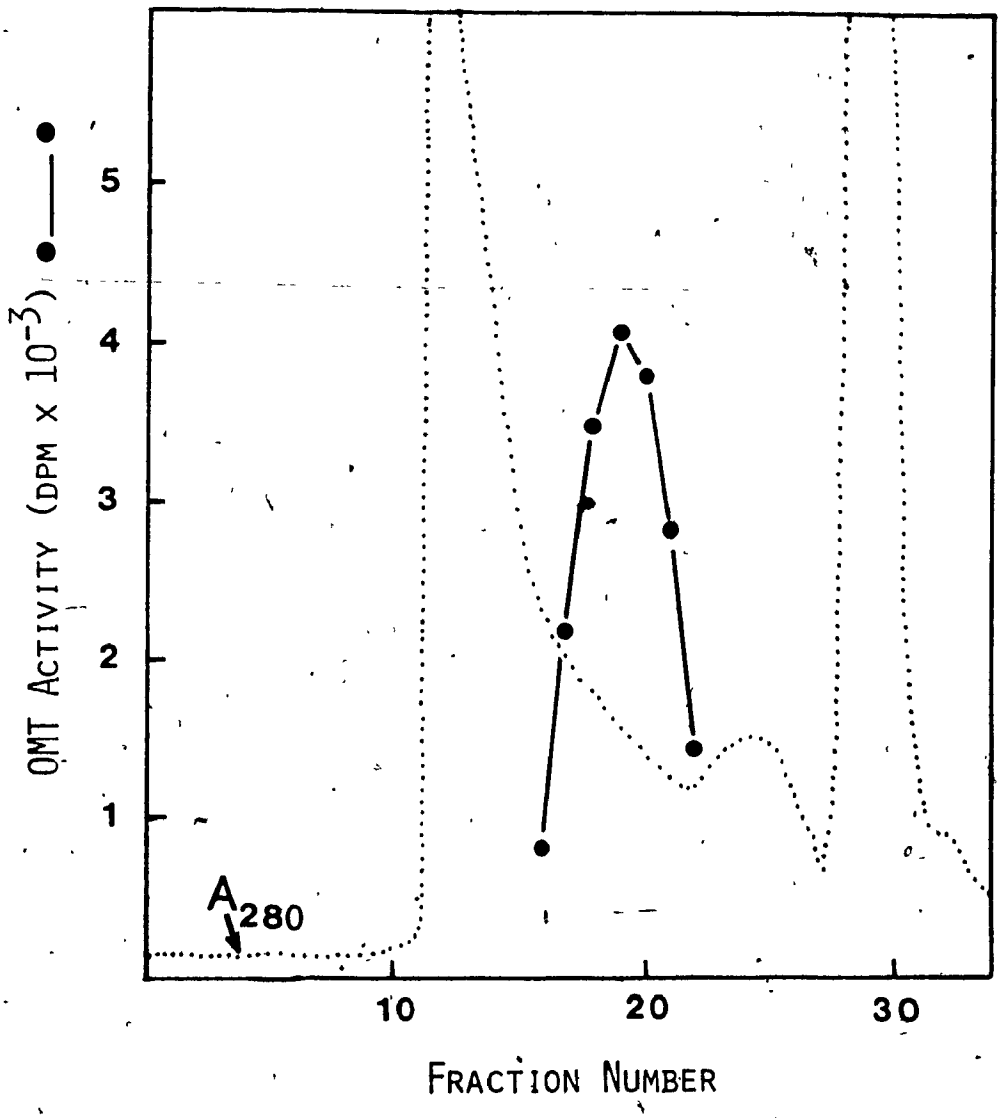

OMT Activity (DPM x 10^{-3})
5
4
3
2
1
A_{280}
10
20
30
Fraction Number

Figure 21: Elution profile of the 7-OMT activity after chromatofocusing on a Mono P HR 5/20 column. The active fractions from gel filtration were pooled and loaded on the column which had previously been equilibrated with 25 mM bis Tris/HCl buffer (pH 7.1) contaning 14 mM 2-mercaptoethanol (ME) and 10% glycerol. The bound proteins were eluted with 30 ml of Polybuffer (74): water (1:10, v/v) (pH 5.0) containing 14 mM 2-ME and 10% glycerol, at a flow-rate of 1.0 ml/min (2.0 MPa). Fractions (1 ml) were collected into 0.25 ml of 0.2 M Tris buffer (pH 8.5) and were assayed for OMT activity.

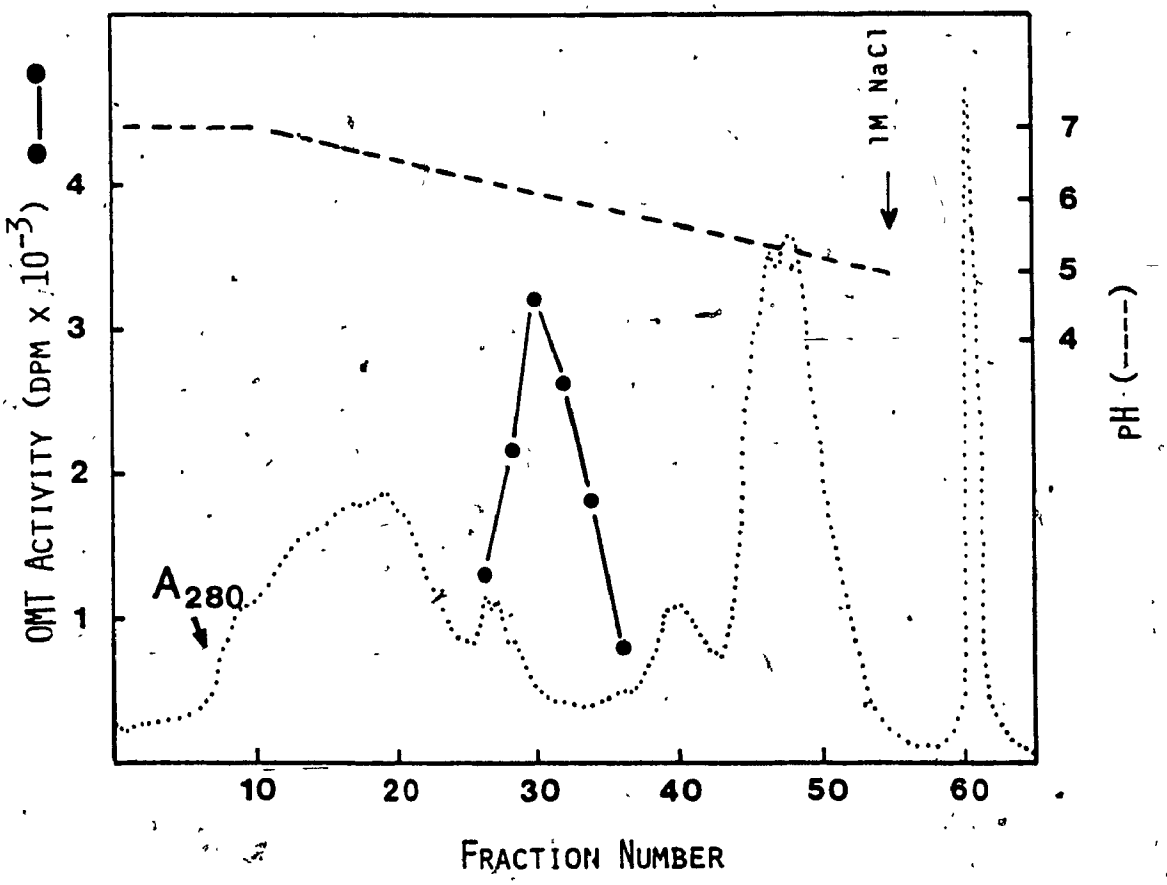

OMT Activity (DPM x 10^{-3})
4
3
2
1
A_{280}
1M NaCl
7
6
5
4
pH (----)
10
20
30
40
50
60
Fraction Number

Figure 22: Elution profile of the 7-OMT activity after ion-exchange chromatography on Mono Q HR 5/5 column. The active fractions from chromatofocusing were pooled and applied to the column which had previously been equilibrated with 25 mM Tris/HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol (ME) and 10% glycerol. The column was washed with the same buffer and the bound proteins were eluted using a linear salt gradient of 0-0.5 M KCl in 25 mM Tris/HCl (pH 7.5) buffer containing 14 mM 2-ME and 10% glycerol. Fractions (1 ml) were collected and assayed for OMT activity.

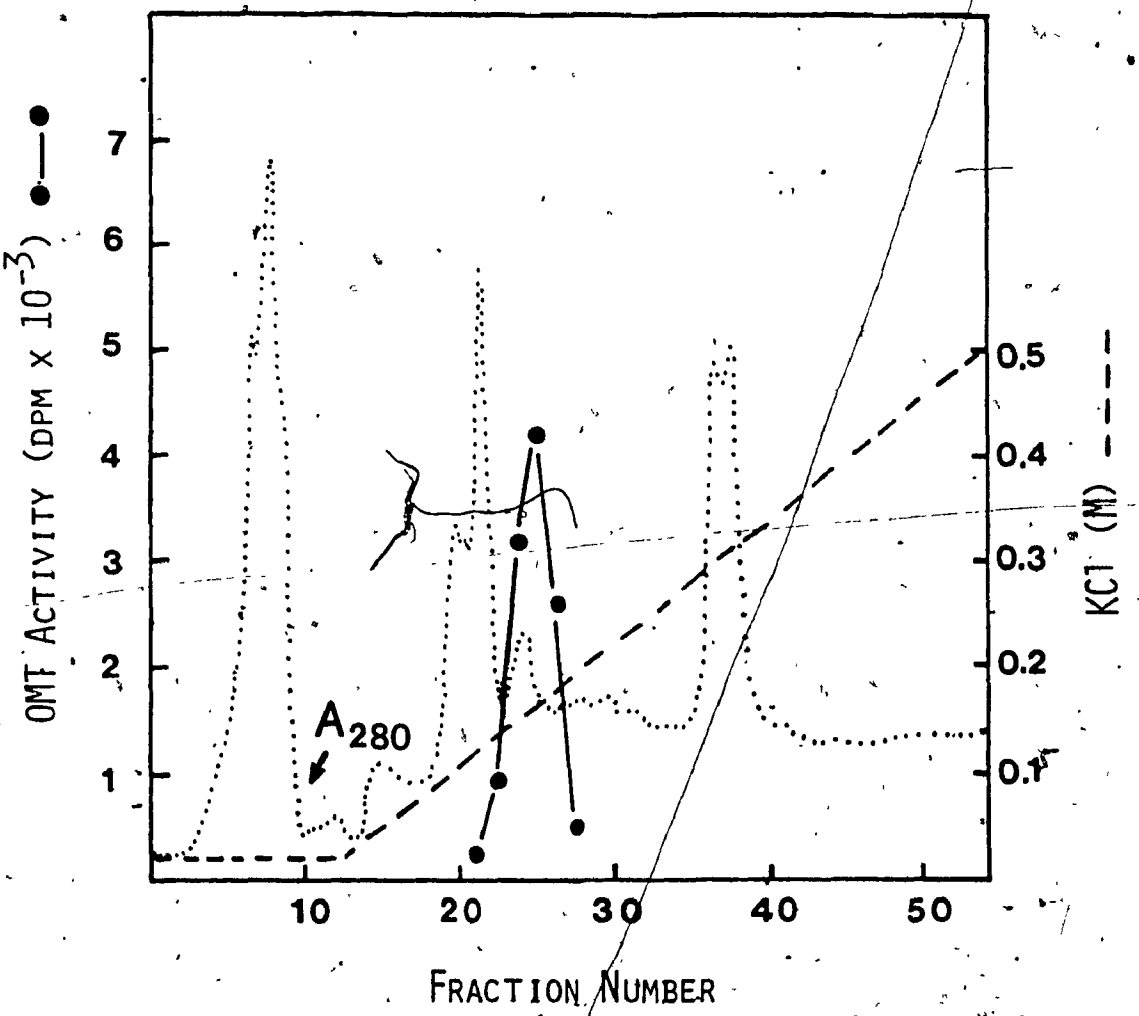

OMT Activity (DPM x 10^{-3})
7
6
5
4
3
2
1
A_{280}
0.5
0.4
0.3
0.2
0.1
KCl (M)
10
20
30
40
50
Fraction Number

Table 12 : Purification data of the 7-OMT of *C. americanum*[1].

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
Crude[2]	38	0.27	-	100
Ammonium sulfate[3]	30	0.31	1.1	90
Gel filtration[4]	2	4.52	17	88
Chromatofocusing[5]	0.25	31	115	75
Ion exchange[6]	0.02	189	700	37

[1] Purification procedure was conducted with 20 g fresh tissue and the standard enzyme assay was used as described in Section D.10.

[2] Dowex 1x2.

[3] Desalted on Sephadex G-25.

[4] On Superose 12 HR 15/60 column.

[5] On Mono P HR 5/20 column.

[6] On Mono Q HR 5/5 column.

the other mono-, di- or trimethyl derivatives tested (Table 13). Furthermore, the 7-OMT exhibited no activity with phenylpropanoids, flavones, dihydroflavonols or any of their glucosides; thus indicating its specificity for 3-methylquercetin, the product of the first enzyme in the methylation sequence (Fig. 19).

D.2.2.2. Effect of divalent cations and SH-group reagents

The effect of divalent cations and SH-group reagents on the 7-O-methylation was studied. The fact that EDTA did not inhibit the enzyme activity (Table 14) indicated that the methylation reaction has no requirement for divalent cations; although it was slightly stimulated by Mg^{2+} by approx. 25% above control. However, the 7-OMT activity was inhibited by Ca^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} (between 20 and 55 %). SH-group reagents were found to inhibit O-methylation. In contrast with other reagents used (Table 14), the inhibition with p-chloromercuribenzoate was partially prevented by the addition of 10 mM DTE.

D.2.3. Kinetic analysis of the 7-OMT

D.2.3.1. Substrate interaction kinetics

Double-reciprocal plots with 3-methylquercetin as the variable substrate and several, fixed concentrations of SAM gave intersecting lines (Fig. 23). Intercept and slope replots versus reciprocal fixed substrate concentrations (insert of Fig. 23) generated straight lines. On the basis of the initial velocity data thus obtained, the mechanism that best fit was that of sequential binding. The experimental data was therefore represented by Eq. [1] (Section C.14.), the rate equation for a sequential bireactant binding mechanism (136).

Table 13: Substrate specificity of the 7-OMT from *C. americanum*[1,2]

Substrate	Relative activity (%)
Quercetin (Ø)	0
Rhamnetin (7-methyl-Q)	0
3-Methyl-Q	100
Quercetagetin (Qg)	0
3,7-Dimethyl-Q	0
3,7-Dimethyl-Qg	0
3,7,4´-Trimethyl-Q	0
3,7,3´-Trimethyl-Qg	0

[1] Enzyme preparation was used after chromatofocusing.

[2] The standard enzyme assay was used as described in Section C.10.

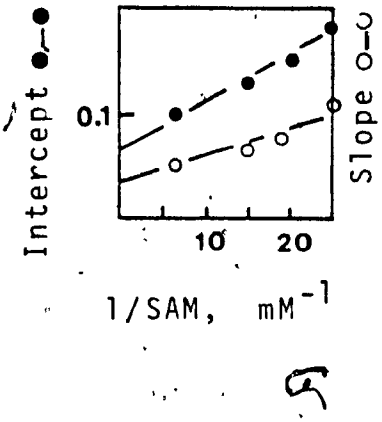
[3] 100% activity amounted to 189 pkat/mg protein.

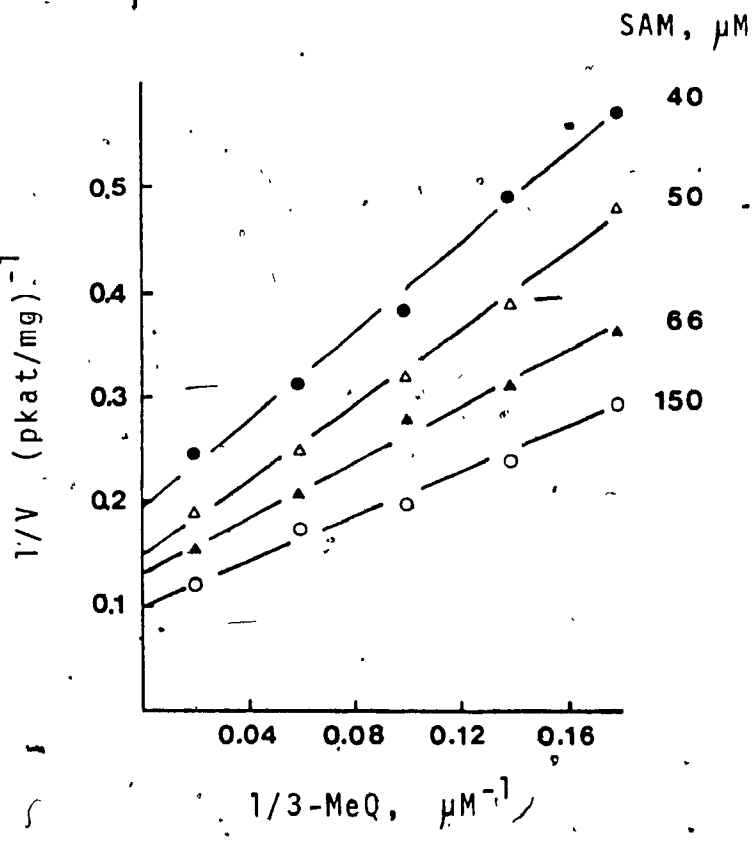
Table 14: Effect of divalent cations and SH-group reagents on the 7-OMT activity[1].

Addition	Concentration (mM)	Activity (%)
None	-	100
Mg^{2+}	1	122
Mg^{2+}	10	127
Mn^{2+}	1	125
Mn^{2+}	10	129
Ca^{2+}	1	81
Ca^{2+}	10	80
Cu^{2+}	1	84
Co^{2+}	1	67
Zn^{2+}	1	46
EDTA	1	107
EDTA	10	96
Iodoacetate	1	5
Iodoacetamide	1	14
N-Ethylmaleimide	1	25
p-Chloromercuribenzoate	1	22
Phenylmercuriacetate	1	6
DTE	10	94
p-Chloromercuribenzoate + DTE	1,10	76

[1] Enzyme preparation was used after chromatofocusing.

Figure 23: Double-reciprocal plots of initial velocities with the flavonol, 3-methylquercetin, as the variable substrate and SAM as the changing fixed substrate at concentrations of 150 µM (o—o), 66 µM (▲—▲), 50 µM (△—△) and 40 µM (●—●). 23.8 µM of [^{14}C-methyl]SAM (0.1 µCi) in addition to varying amounts of unlabelled SAM were used. The velocities are expressed in pkat/mg. Insert: slope and intercept replots.


Intercept
Slope
0.1
10
20
1/SAM, mM⁻¹


SAM, μM
40
50
66
150
0.5
0.4
0.3
0.2
0.1
1/V (pkat/mg)⁻¹
0.04
0.08
0.12
0.16
1/3-MeQ, μM⁻¹

D.2.3.2. Product inhibition studies

The order of substrate binding and product release was obtained from product inhibition studies. The rate equations derived from a bi bi sequential mechanism (136-139), predict that each product is a competitive inhibitor with respect to the substrate that binds to the same enzyme form. It also predicts a non-competitive pattern with respect to the other substrate. The results obtained are in agreement with the theoretical predictions previously mentioned. SAH was found to be a competitive inhibitor with respect to SAM (Fig. 24A) and a non-competitive inhibitor with respect to 3-methylquercetin (Fig. 24B). On the other hand, 3,7-dimethylquercetin was a non-competitive inhibitor with respect to either the co-substrate, SAM (Fig. 24C) or the flavonol substrate, 3-methylquercetin (Fig. 24D). These kinetic patterns are consistent with the predicted expectations of an ordered bi bi mechanism where SAM is the first substrate to bind to the enzyme, followed by 3-methylquercetin (Fig. 25). The first product released is 3,7-dimethylquercetin, followed by SAH. However, these results do not rule out the possibility of a random binding mechanism involving the formation of a dead-end complex (140).

The kinetic constants K_A, K_B, K_{iP}, K_{iQ}, K_{iA} and V_{max} were calculated from slope and intercept replots of the generated data and are listed in Table 15 (140).

Figure 24: Product inhibition kinetics of the 7-OMT. The velocities are expressed in pkat/mg.

(A) Inhibition by SAH with SAM as the variable substrate at constant concentration of 3-methylquercetin (1 μM). 23.8 μM of [^{14}C-methyl]SAM (0.1 μCi) in addition to varying amounts of unlabelled SAM were used at SAH concentrations of 40 μM (■——■), 20 μM (△——△), 10 μM (●——●) or 0 μM (○——○).

(B) Inhibition by SAH with 3-methylquerectin as the variable substrate at constant concentrations (23.8 μM) of SAM (0.1 μCi). The concentration of SAH was varied at 50 μM (■——■), 30 μM (△——△), 20 μM (●——●) or 0 μM (○——○).

(C) Inhibition by 3,7-dimethylquerectin with SAM as the variable substrate at constant concentrations of 3-methylquercetin (1 μM). 23.8 μM of [^{14}C-methyl]SAM (0.1 μCi) in addition to varying amounts of unlabelled SAM were used at 3,7-dimethylquercetin concentration of 70 μM (■——■), 50 μM (△——△), 20 μM (●——●) or 0 μM (○——○).

(D) Inhibition by 3,7-dimethylquercetin with 3-methylquercetin as the variable substrate at constant concentrations (23.8 μM) of SAM (0.1 μCi). The concentration of SAH was varied at 70 μM (■——■), 50 μM (△——△), 20 μM (●——●) or 0 μM (○——○).

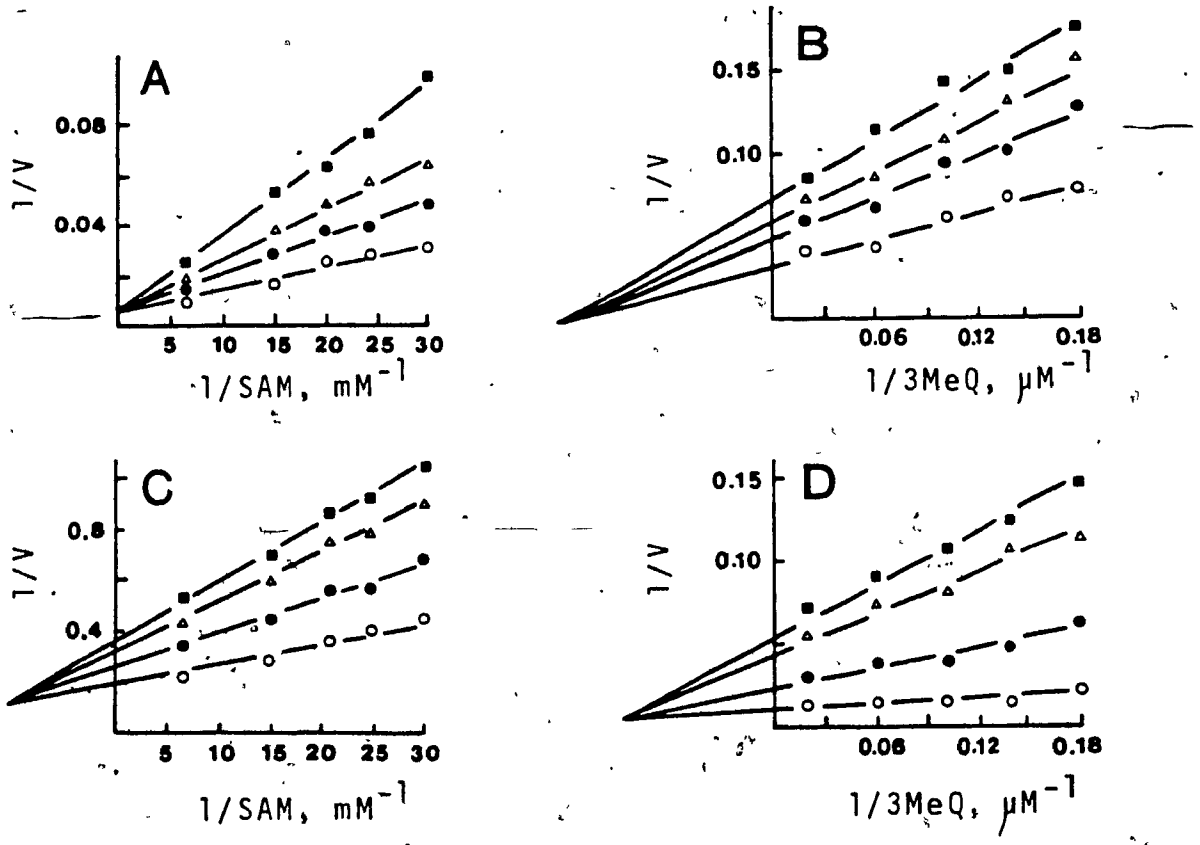

A
0.08
0.04
1/V
5 10 15 20 25 30
1/SAM, mM⁻¹
B
0.15
0.10
1/V
0.06 0.12 0.18
1/3MeQ, μM⁻¹
C
0.8
0.4
1/V
5 10 15 20 25 30
1/SAM, mM⁻¹
D
0.15
0.10
1/V
0.06 0.12 0.18
1/3MeQ, μM⁻¹

Figure 25: Proposed kinetic mechanism for the 7-O-methyltransferase of Chrysosplenium americanum. A, S-adenosyl-L-methionine; B, 3-methylquerectin; P, 3,7-dimethylquercetin; Q, S-adenosyl-L-homocysteine and E, flavonol-7-O-methyltransferase.

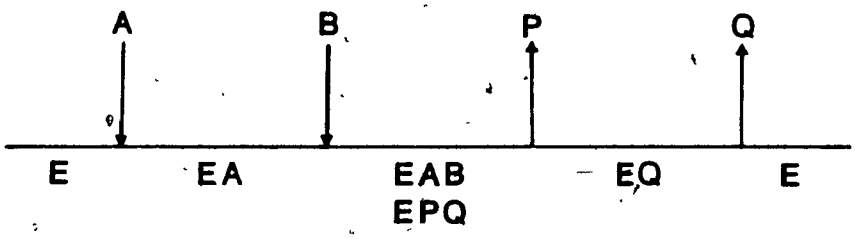

A
B
P
Q
E
EA
EAB
EPQ
EQ
E

Table 15: Kinetic constants of the 7-OMT from *C. americanum*.

Parameter	Value
K_A (μM)	65
K_B (μM)	7
K_{iA} (μM)	60
K_{iQ} (μM)	10
K_{iP} (μM)	15
V_{max} (pkat/mg)	10

D.2.4. Purification of the 2′-/5′- OMT from C. americanum

The 2′-/5′-OMT which catalyzes the terminal steps of methyl transfer in the biosynthesis of C. americanum flavonoids (Fig. 19) was purified by fractional precipitation with ammonium sulfate, gel filtration on Superose 12 column (Fig. 26A), and chromatofocusing on Mono P column (Fig. 26B). The purified enzyme preparation was equally active against both 5,2′-dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-O-glucoside (A) and 5,5′-dihydroxy-3,7,4′-trimethoxyflavone-2′-O-glucoside (B); thus demonstrating the methylation at the 2′- and 5′-positions of the partially methylated flavonol glucosides A and B (Fig. 19). The combined purification steps described above resulted in an increase in specific activity of 420-fold of the enzyme preparation as compared with the crude extract (Table 16). In contrast with the enzyme purification by conventional gel filtration and chromatofocusing techniques, the use of FPLC almost quadrupled the extent of purification of this enzyme (Table 16). However, the recovery of total activity was comparable with those of the OMTs which were purified by conventional methods from the same tissue (115) (Table 16). Using a linear pH gradient between 7 and 4 in earlier attempts resulted in complete loss of both the 2′- and 5′-O-methylating activities. However, the use of a shallower gradient (pH 7-5) resulted in recovery of both enzyme activities in the high salt (Fig. 26B).

D.2.5. Characterization of the 2′-/5′-OMT

D.2.5.1. Substrate specificty

The enzyme preparation accepted partially O-methylated flavonol glucosides as substrates, namely A or B, and gave rise to their

Figure 26: Elution profile of the 2´-/5´-OMT activity after gel filtration on Superose 12 HR 10/30 column (A) and chromatofocusing on Mono P HR 5/20 column (B). Substrates A and B gave similar activity peaks. The protein pellet was suspended in the minimal amount of 25 mM imidazole (pH 7.2) buffer containing 14 2-mercaptoethanol (ME) and 10% glycerol; and was chromatographed on a Superose column which had previously been equilibrated with the same buffer. The column was developed with the same buffer at a flow-rate of 0.5 ml/min (2.5 MPa) and 1-ml fractions were collected and assayed for the 2´- and 5´-O-methylating activity using substrate A (5,5´-dihydroxy-3,6,7,4´-tetramethoxyflavone-5´O-glucoside) and B (5,2´-dihydroxy-3,7,4´-trimethoxyflavone-2´-O-glucoside), respectively. The active fractions were pooled and subjected to chromatofocusing on a Mono P column which had previously been equilibrated with 25 mM imidazole (pH 7.2) buffer containing 14 mM 2-ME and 10% glycerol. Elution of the bound proteins was first performed with 50 ml of Polybuffer (74):water (1:10, v/v) (pH 5.0) containing 14 mM 2-ME and 10% glycerol which generated a linaer gradient between pH 7 and 5; and subsequently with 10 ml of 1 M NaCl in 25 mM imidazole (pH 7.2) containing 14 mM 2-ME and 10% glycerol. The flow-rate was 0.4 ml/min (3.0 MPa) and one-ml fractions were collected and assayed for OMT activity using substrates A and B.

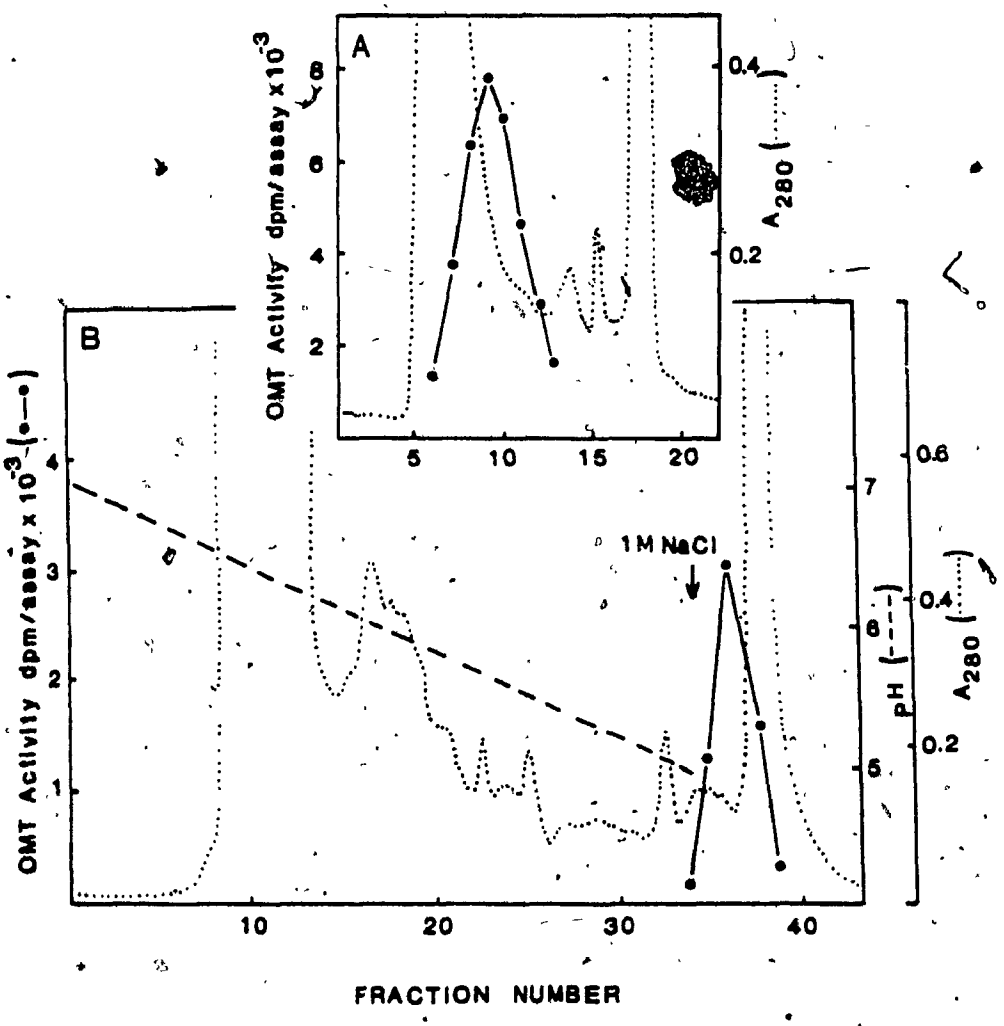

A
B
OMT Activity dpm/assay x10-3
A280
pH
1M NaCl
FRACTION NUMBER

Table 16: Purification of Chrysosplenium 2´-/5´-O-methyltransferase[1].

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)	Purification[2] (-fold)
Crude[3]	105	0.53	-	100	-
Ammonium sulfate[4]	88	0.61	1.2	96	-
Gel filtration[5]	2.5	11.3	21.3	51	18.7
Chromatofocusing[6]	0.025	222	419	20	123

[1] Purification procedure was conducted with 15 g fresh tissue.

[2] By conventional chromatography.

[3] Dowex 1x2

[4] Desalted on Sephadex G-25.

[5] On Superose 12 HR 10/30 column.

[6] On Mono P HR 5/20 column.

respective 2′- or 5′-methyl derivatives, C and D (Fig. 19). On the other hand, neither of the corresponding aglycones of compounds A or B nor any of the hydroxylated or partially methylated flavonols tested acted as methyl acceptor.

D.2.5.2. Linearity of the methylation reaction

The 2′-/5′-O-methylation reaction was linear with time up to 90 min and with protein concentration up to 30 ug, at 10 uM of either substrate A or B.

D.2.5.3. pH optimum

The pH optimum of both methylations was found to be pH 7.0 in Pi buffer of both the 2′- and 5′-methylation reactions (Fig. 27). A change of one unit resulted in a loss of 50% of enzyme activity.

D.2.5.4. Effect of metal ions and SH-group reagents

The effect of divalent cations and SH-group inhibitors on the 2′-/5′-O-methylation was studied. The results (Table 17) indicated that the methylation reaction had no requirement for Mg^{2+} with either substrate. Furthermore, there were no significant differences observed for the effect of other divalent cations on the methylation of either substrate, except for variable inhibition by Ca^{2+}, Co^{2+} and Cu^{2+} (Table 17). SH-group reagents, such as N-ethylmaleimide and phenylmercuriacetate, were found to inhibit O-methylation of both substrates to the same extent. However, in contrast with other reagents used, inhibition with NEM was partially prevented by the addition of 14 mM 2-mercaptoethanol. Neither -mercaptoethanol nor DTE, when added to the reaction mixture, had any effect on the methylation of either substrate (Table 17).

Figure 27: pH optimum of the 2′-/5′-OMT after chromatofocusing using substrates A (5,5′-dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-O-glucoside) and B (5,2′-dihydroxy-3,7,4′-trimethoxyflavone-2′-O-glucoside). Substrates A and B gave similar activities.

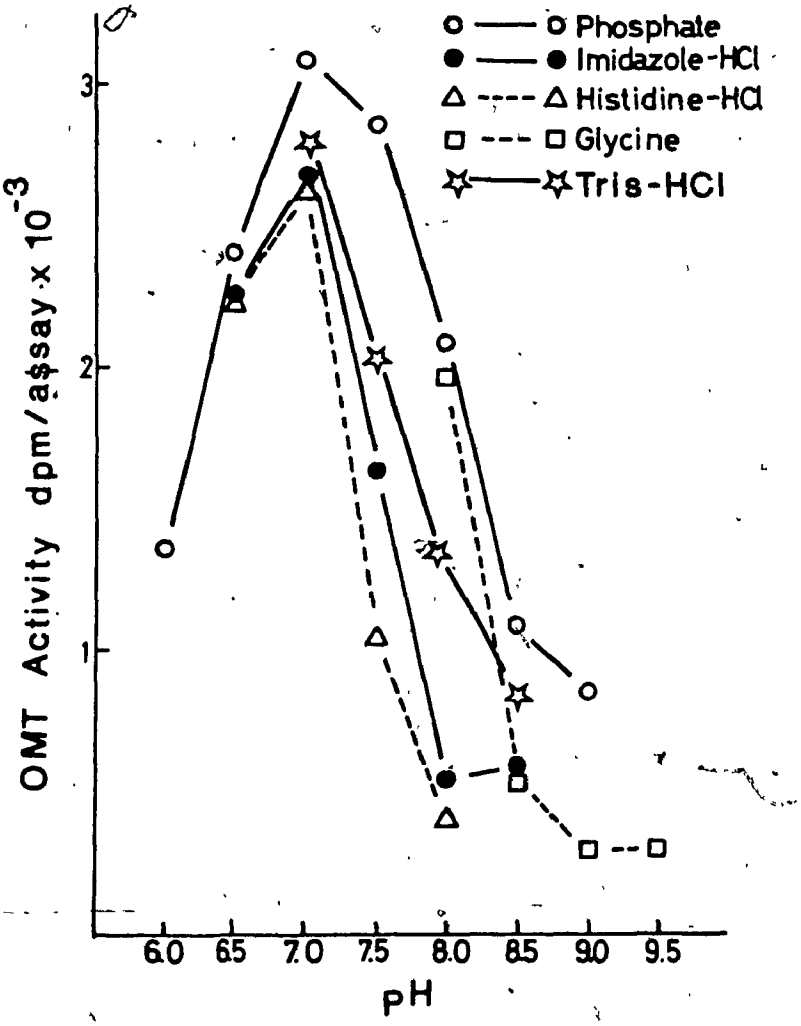

Phosphate
Imidazole-HCl
Histidine-HCl
Glycine
Tris-HCl
OMT Activity dpm/assay x 10^-3
3
2
1
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH

Table 17: Effect of divalent cations and SH-group reagents on the 2′- and 5′-O-methylating activity.

Additions	Concentration (mM)	Relative activity (%) 2′-OMT	5′-OMT
None	-	100	100
Mg^{2+}	1	100	92
Mg^{2+}	10	76	50
Mn^{2+}	1	73	62
Mn^{2+}	10	14	41
Ca^{2+}	1	110	88
Ca^{2+}	10	84	48
Co^{2+}	1	27	87
Cu^{2+}	1	77	42
Zn^{2+}	1	14	7
EDTA	1	99	87
EDTA	10	87	90
2-Mercaptoethanol (2-ME)	14	95	89
DTE	10	102	92
Iodoacetamide	1	59	39
Iodoacetate	1	95	86
N-Ethylmaleimide	1	7	4
N-Ethylmaleimide + 2-ME	1,14	82	59
Phenylmercuriacetate	1	31	3
p-Chloromercuribenzoate	1	100	84

D.2.5.5. Other properties

The M_r of the 2′-/5′-OMT was estimated to be 57,000 by its elution profile on gel filtration using molecular weight standards, being similar in size to those of the 3-, 7-, 4′- and 6-OMTs.

D.2.6. Resolution of the Chrysosplenium OMT system.

Chromatofocusing is an ion-exchange technique used for the separation of proteins according to differences in their isoelectric points (pIs) (145). Negatively charged proteins are adsorbed on the ion exchanger and are subsequently eluted by a descending pH gradient which is generated in the column as the Polybuffer titrates the ion exchanger (146-148). Proteins, therefore, elute at a pH near their pIs.

Of the enyzmes involved in the sequential methylation of flavonols in C. americanum, only three OMTs, namely the 3-, 6- and 4′-, could be recovered by conventional, two-step chromatofocusing, although with substantial loss of activity (116). The inability to recover the 7- and 2′-/5′-OMT activities is attributed to the exposure of the enzyme proteins to low pH during the lengthy chromatofocusing process (ca. 8-10 h). Parameters which affect the resolution of chromatography include the nature and pH of the starting buffer; dilution of the Polybuffer which determines the length of the migration; the presence of counter- and co-ions of salts; as well as the presence of zwitterions that reduce the aggregation caused by charge interactions (149).

The effect of the parameters mentioned above was investigated on the recovery of activity and the resolution of the OMTs of C. americanum using a one-step chromatofocusing process.

D.2.6.1. Nature and pH of starting buffer

Preliminary experiments indicated that all the OMTs examined focused at a range between pH 4.2 and 5.5 (115). Optimum resolution of these enzymes was achieved when a gradient between pH 6 and 4 was used, regardless of the nature of the starting buffer. However, a substantial loss of activity (ca. 75%) was observed with the 6-OMT, whereas the 7- and 2′-/5′-OMT activities could not be detected when chromatofocused on a conventional Polybuffer exchanger (PBE) (115). This was attributed to long exposure of these enzymes to low pH of the starting buffer. Optimum resolution and recovery of activity of all the OMTs was achieved on Mono P using bis Tris buffer and a gradient between pH 6 and 4 (Table 18). The pI values of the OMTs differed with the different buffers used, suggesting that these were apparent values.

D.2.6.2. Dilution and flow-rate of Polybuffer

Investigation of both parameters indicated that slow flow-rate (0.3 as compared with 1 ml/min), as well as the increasing dilution of Polybuffer (1:20 instead of 1:10) resulted in an improved resolution of the different OMTs. However, the latter conditions gave rise to concomitant broadening of the peaks and low recovery of enzyme activities. These drawbacks were attributed to the low ionic strength of the Polybuffer and the lengthy chromatographic process, and were overcome by the addition of 10 mM potasssium chloride to the eluting buffer. A compromise in flow-rate (0.5 ml/min) and dilution of Polubuffer (1:15), together with the addition of 10 mM potassium chloride to the eluting buffer, improved the resolution and enzyme activity, except for the 7 and 4′-OMTs which did not separate from each other as indicated by their pI values (Table 18).

Table 18: pI values of *C. americanum* OMTs determined under different experimental conditions.

OMT	PBE[1]	PBE[2]	PBE[3]	Mono P[2]	Mono P[3]
3-	4.8	4.2	4.4	4.2	4.0
7-	NA	4.6	4.9	5.4	4.8
4′-	5.4	4.6	4.9	5.4	5.0
6-	5.8	5.0	5.2	5.6	5.2
2′-/5′-	NA	NA	4.6	4.8	4.6

[1] Using 25 mM imidazole, pH 7.3 as the starting buffer.

[2] Using 25 mM bis Tris, pH 7.1 as the starting buffer.

[3] Using bis Tris, pH 6.3 containing 5% betaine (w/v) as the starting buffer.

PBE, Polybuffer exchanger (conventional chromatofocusing); NA, no activity.

D.2.6.3. Presence of zwitterions

Zwitterions, such as betaine, are known to reduce the molecular interactions which result in aggregation of protein (150). The effect of a range of concentrations of betaine (0-10%, w/v) on the resolution of the OMTs of this sytem was studied. Optimum resolution was achieved when 5% betaine was added to both the starting buffer and Polybuffer (Table 18 and Fig. 28). The effect of betaine on the resolution of different proteins may be attributed to its ability to minimize the formation of charge aggregates. Betaine is believed to disperse the charge densities of charged proteins and minimize the microenvironmental pH effects close to the surface of the matrix, thus resulting in decreased association of proteins with one another. Therefore, the presence of betaine allows the different proteins to be eluted from the column closer to their true pI values as determined by isoelectric focusing (149).

Since proteins are eluted during chromatofocusing at pH values near their pIs, it is not unexpected that a loss of activity is encountered with this technique. No activity for the 7- and 2′-/5′-OMTs was recovered unless the fractions eluted from Mono P were collected into 0.25 ml of 0.2 M Tris buffer pH (8.5) in order to increase the pH of the fractions. Furthermore, the speed and high resolving power of chromatofocusing on Mono P resulted in high recovery of OMT activity as compared with conventional chromatofocusing (Table 19).

Figure 28: Profile of O-methyltransferase activity of the Chrysoslepnium americnum OMT system, after chromatofocusing on Mono P HR 5/20 , under optimum conditions. The protein was loaded on a Mono P column which had previously been equilibrated with bis Tris/iminodiacetic acid (pH 6.3), containing 14 mM 2-mercaptoethanol (ME), 10% glycerol and 5% betaine. The bound proteins were eluted with 50 ml of Polybuffer (74)-water (1:15, v/v) (pH 4.0), containing 10% glycerol, 14 mM 2-ME and 5% betaine. The flowrate was 0.5 ml/min and 1-ml fractions were collected into 0.25 ml of 0.2 M Tris (pH 8.0), and assayed for OMT activity. Quercetin was used as a substrate for the 3-OMT; 3-methylquercetin for the 7-OMT; 3,7-dimethylquercetin for the 4´-OMT; 3,7,4´-trimethyl-querecetagetin for the 6-OMT; 5,5´-dihydroxy-3,6,7,4´-tetramethoxyflavone-5´-O-glucoside for the 2´-OMT and 5,2´-dihydroxy-3,7,4´-trimethoxyflavone-2´-O-glucoside for the 5´-OMT (see Fig. 19 for structures).

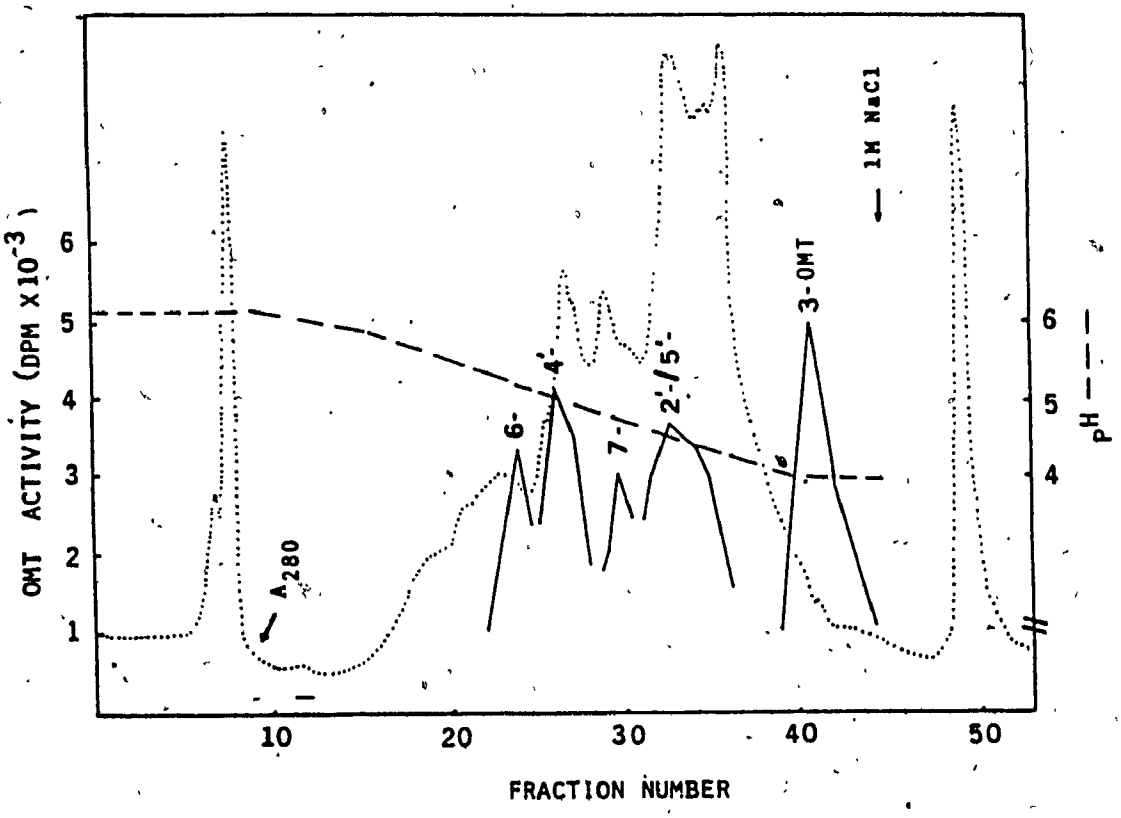

OMT ACTIVITY (DPM X10^{-3})
pH
FRACTION NUMBER
A_{280}
1M NaCl
6-
4'
7-
2'-/5'-
3-OMT
1
2
3
4
5
6
10
20
30
40
50

Table 19: Extent of purification of C. americanum OMTs.

OMT	Purification (-fold)	
	Conventional	FPLC[a]
3-	85[b]	650
7-	6[c]	460
4'-	164[b]	640
6-	92[b]	400
2'-/5'-	123[b]	420

[a] By successive chromatography on Superose 12 and Mono P.

[b] By successive chromatography on Sephacryl S-200, hydroxyapatite and PBE-94 (115).

[c] By successive chromatography on Sephacryl S-200 and hydroxyapatite (115).

D.2.7. The Lupinus luteus system

Roots of the yellow lupin (Lupinus luteus) accumulate a number of 5-O-methylisoflavone derivatives. These have been identified (135) as 5-O-methylgenistein, 5-O-methyllupiwighteone (8-prenylgenistein), 5-O-methylderrone, barpisoflavone A (5-O-methyl-2´-hydroxygenistein), barpisoflavone B (5-O-methyl-8-prenyl-2´-hydroxygenistein) and barpisoflavone C (5-O-methyl-2´-hydroxyderrone) (Fig. 29). Recent reviews (150, 151) indicated the presence of more than 20 naturally occurring 5-O-methylisoflavones, most of which possess methoxy or methylene dioxy groups elsewhere in the molecule. In L. luteus, O-methylation of isoflavones occurs specifically at position 5, whereas other hydroxyl groups on the flavonoid skeleton are unaffected (135). Since the 5-hydroxyl group of flavonoid compounds is considered to be the least reactive, due to its chelation with the carbonyl group of the heterocyclic ring, it was considered important therefore, to investigate the enzymatic 5-O-methylation of isoflavones in yellow lupin.

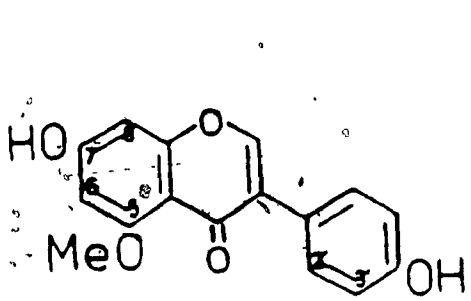
D.2.8. Tissue localization of the 5-OMT from Lupinus luteus

The 5-OMT activity was found to be restricted to the roots of Lupinus luteus (yellow lupin) seedlings, with little or no activity in the shoot or flower tissues (Table 20). Maximal 5-OMT activity was obtained with roots of young plants just prior to flowering, ca. 4-6 weeks old.

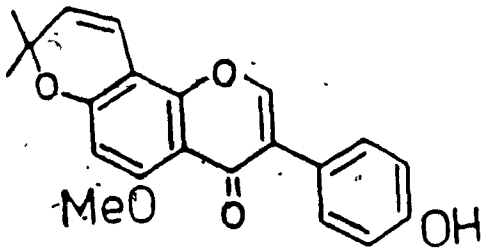
D.2.9. Purification of the 5-OMT

The enzyme was purified by fractional precipitation with ammonium sulfate, gel filtration on Superose 12 HR 10/30 column (Fig. 30A) and ion exchange chromatography on a Mono Q HR 5/5 column (Fig. 30B) using

Figure 29: Structural formalae of 5-O-methylgenistein (A) and 5-O-methylderrone (B).



A



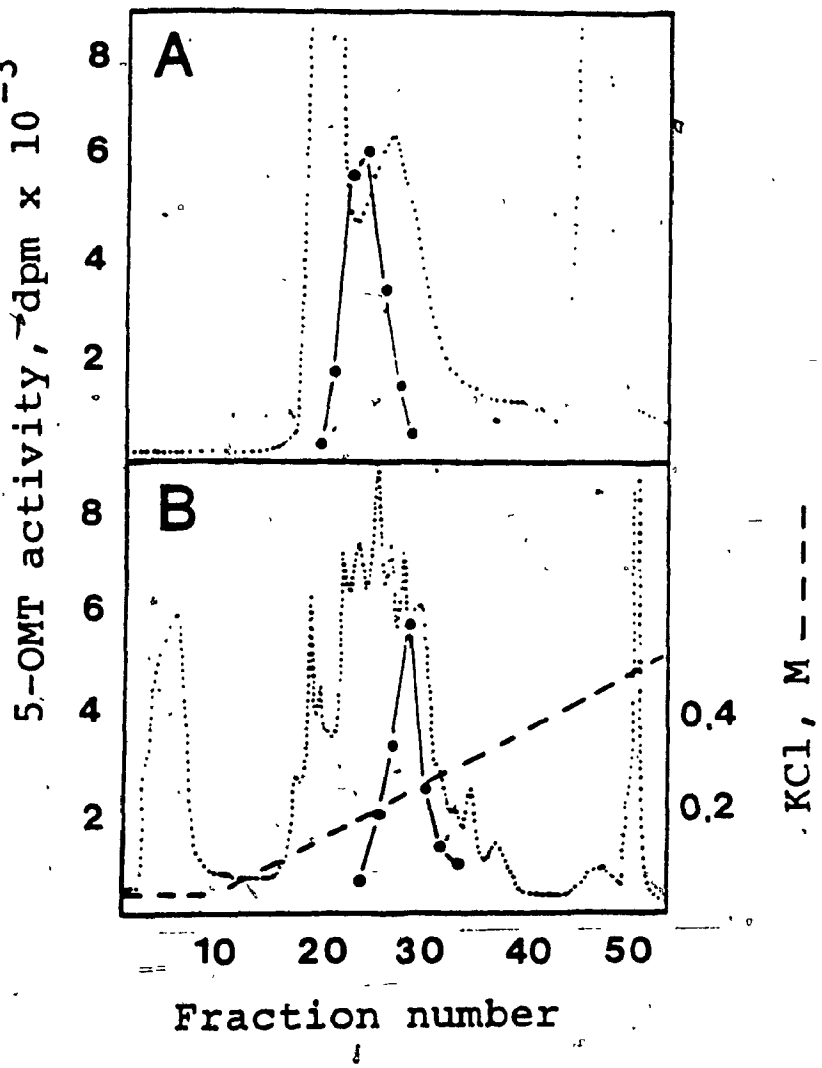
B

Table 20: Tissue localization of the 5-OMT[1].

Tissue	Specific activity (pkat/mg)
Flower	0.034
Shoot	0.038
Root	0.308

[1] Enzyme was used after Dowex 1x2.

Figure 30: (A) Elution profile of the 5-OMT activity after gel filtration on a Superose 12 HR 15/60 column using genistein as substrate. The column was preequilibrated and developed (at a flow-rate of 1 ml/min) with 25 mM Tris/HCl buffer (pH 7.5) containing 14 mM 2-mercaptethanol (ME) and 10% glycerol; and 2-ml fractions were collected and assayed for OMT activity. (B) Elution profile of the 5-OMT activity after ion-exchange chromatography on a Mono Q HR 5/5 column using genistein as substrate. The column was preequilibrated with 25 mM Tris/HCl (pH 7.5) buffer containing 14 mM 2-ME and 10% glycerol. The column was washed with the same buffer and the bound proteins were eluted using a linear salt gradient of 0-0.5 M KCl in 25 mM Tris/HCl buffer (pH 7.5) containing 14 mM 2-ME and 10% glycerol. One-ml fractions were collected and assayed for OMT activity.


A
B
8
6
4
2
8
6
4
2
0.4
0.2
10
20
30
40
50
5-OMT activity, dpm x 10^{-3}
KCl, M
Fraction number

an FPLC system. This resulted in an 810-fold increase in specific activity of the enzyme preparation, with a 36% recovery, as compared with the crude extract (Table 21).

D.2.10. Enzyme properties

2.10.1. Substrate specificity

The partially purified enzyme preparation was assayed against a number of methyl acceptors. The 5-OMT accepted several isoflavones, but showed little or no activity with flavones, flavonols, coumarins or phenylpropanoids (Table 22). The best isoflavone substrates were found to be 2,3-dehydrokievitone (8-prenyl-2´-hydroxygenistein) > 2´-hydroxygenistein > genistein (5,7,4´-trihydroxyisoflavone) (Fig. 31) as demonstrated by their low K_m values and high V_{max}/K_m ratios. The enzyme exhibited an expressed specificity for the 5-position of the latter compounds as shown by chromatography and autoradiography of the reaction products (Fig. 31). This was further confirmed by the fact that neither 5-hydroxy-3,7,3´4´-tetramethoxyflavone nor 5-hydroxyflavone was accepted for methylation (Table 22).

D.2.10.2 Linearity of the methylation reaction

The 5-O-methylation reaction was linear with time up to 90 min and with protein concentration up to 50 µg, at 1 µM of the isoflavone substrate and optimum pH.

D.2.10.3. pH optimum

The pH optimum of the methylation reaction was studied in several buffers between pH 6 and 9.5. Optimum activity was found to be at pH 7 in Pi buffer. A change of one pH unit resulted in 50% loss of enzyme activity (Fig. 32).

Table 21: Purification of the 5-OMT from L. luteus[1].

Purification step	Total protein (mg)	Specific activity (pkat/mg)	Purification (-fold)	Recovery (%)
Crude[2]	44.8	0.093	-	100
Gel filtration[3]	1.8	1.768	20	76
Ion exchange[4]	0.02	75.76	810	36

[1] Using genistein as substrate and 15 g fresh tissue.

[2] Dowex 1x2.

[3] On Superose 12 HR 10/30 column.

[4] On Mono Q HR 5/5 column.

Table 22: Affinity of the 5-OMT for the different isoflavones.

Substrate[1]	Activity (%)	Km (μM)	Vmax (pkat/mg)	Vmax/Km (pkat/mg/μM)
2,3-Dehydrokievitone (8-prenyl-2´-hydroxygenistein)	100	1	10.0	10.0
2´-Hydroxygenistein	62	1	9.2	10.0
Genistein	50	1	6.4	6.4
Lupiwighteone (8-prenylgenistein)	26	10	4.5	0.45
Derrone	24	10	4.2	0.42

The standard assay was used at substrate concentrations of 1 μM as desribed in the methods section (Section C.10.).

[1] The following substrates were also used as methyl acceptors with activities relative to the control (100%): caffeic acid (15%); 3´-prenyl-2´-hydroxygenistein (14%); 3,7,3´,4´-tetramethylquecetin (8%); 6-prenylgenistein (7%); 5-hydroxyflavone (7%); 6-prenyl-2´-hydroxygenistein (6%); 7,2´,4´-tri-O-methyl-6-prenylgenistein (5%); quercetin (3%); kaempferol (2%); umbelliferone (2%); scopoletin (2%) and ferulic acid (2%).

For structures of the isoflavones see Fig. 29.

Figure 31: Autoradiogram of the chromatographed 5-OMT reaction products on silica plates in chloroform-acetone-ammonia (70:60:1, v/v/v) with genistein (A); 2´-hydroxygenistein (B); or 8-prenyl-2´-hydroxygenistein (C) as substrates showing their respective 5-O-methyl derivatives. Arrow indicates origin.

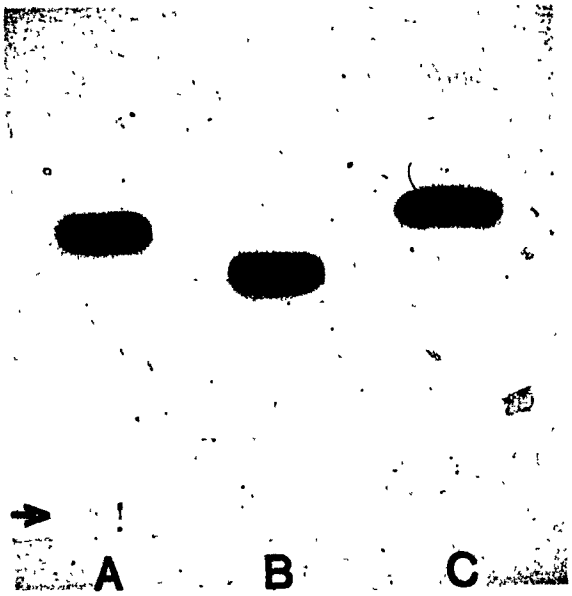
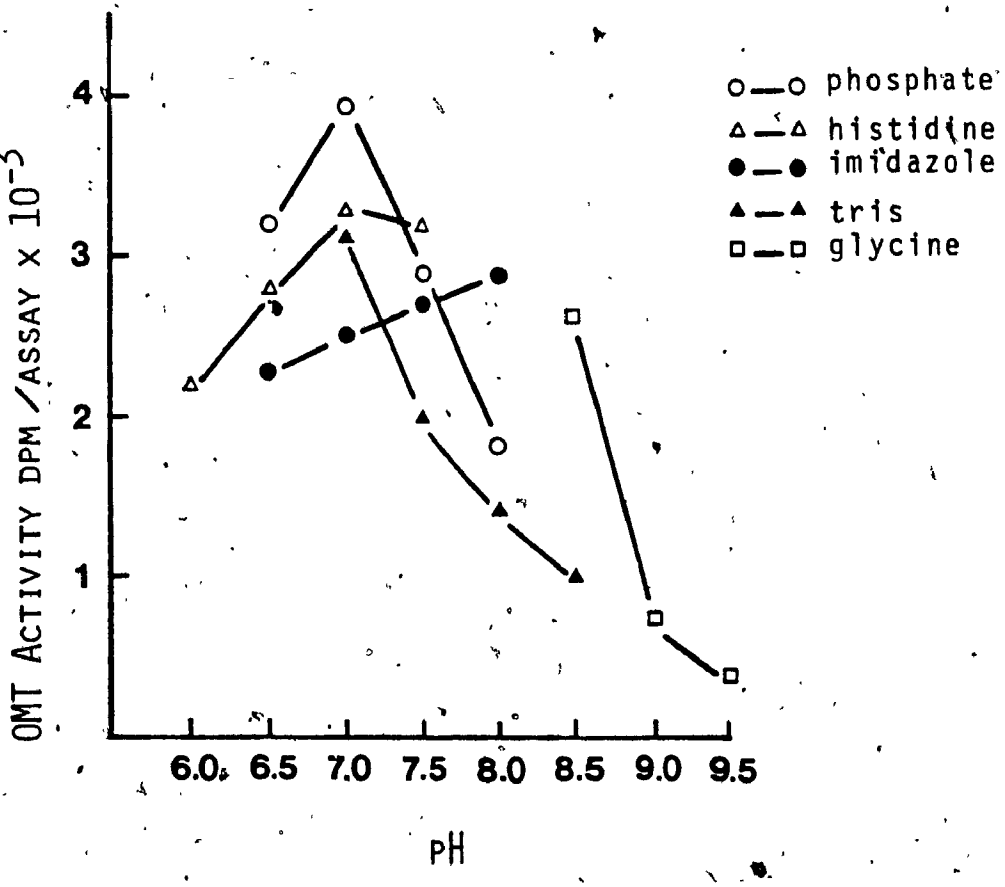

A
B
C

Figure 32: pH optimum of the isoflavone 5-OMT from Lupinus luteus using genistein as substrate.


OMT Activity DPM/Assay x 10^{-3}
4
3
2
1
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH
phosphate
histidine
imidazole
tris
glycine

D.2.10.4. Stability of the enzyme

In the absence of SH-group protectors, the partially purified enzyme lost 70% of its activity within 48 hr. However, the addition of 14 mM 2-mercaptoethanol resulted in 50% loss of activity after one week. The partially purified OMT was stored in 20 mM Tris Buffer (pH 7) containing 10% (v/v) glycerol and 10 mM DTE at -20 ^{o}C and was stable for two months.

D.2.10.5. Effect of divalent cations and SH-group reagents

The effect of cations and SH-group inhibitors on the methylation of genistein wes studied. The fact that EDTA did not inhibit the enzyme activity indicated that the methylation reaction had no requirement for divalent cations (Table 23). However, Co^{2+}, Cu^{2+} and Zn^{2+} (at 1mM) inhibited the 5-OMT activity by 70-90%. Furthermore, the enzyme activity was inhibited (80 to 90%) by the SH-group reagents tested, N-ethylmaleimide, iodoacetate or iodoacetamide (at 1mM). However, inhibition by iodoacetate or iodoacetamide was partially prevented by the addition of DTE.

D.2.10.6. Other properties

The molecular weight of the 5-OMT was 55,000 as estimated from its elution volume following gel filtration. The apparent pI of the isoflavone-OMT, as estimated from its elution pattern on chromatofocusing, was 5.2 (Fig. 33).

D.2.11. Kinetic analysis

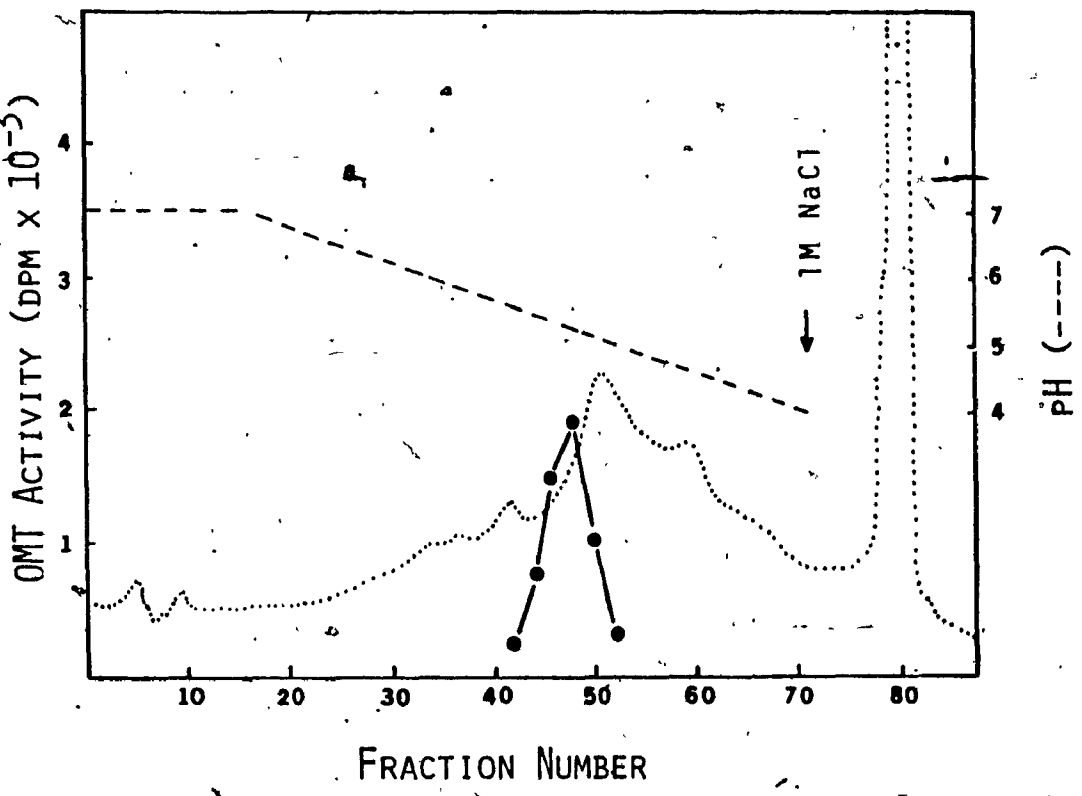
Since genistein was commercially available, it was used as the isoflavone substrate in all the kinetic analyses performed.

Table 23: Effect of divalent cations and SH-group reagents on the 5-OMT[1].

Additions	Concentration (mM)	Activity (%)
None	-	100
Mg^{2+}	1	107
Mg^{2+}	10	91
Mn^{2+}	1	90
Mn^{2+}	10	65
Ca^{2+}	1	98
Ca^{2+}	10	82
Co^{2+}	1	31
Cu^{2+}	1	11
Zn^{2+}	1	13
EDTA	1	122
EDTA	10	112
DTE	10	113
N-Ethylmaleimide	1	16
N-Ethylmaleimide + DTE	1,10	15
Iodoacetate	1	6
Iodoacetate + DTE	1,10	53
Iodoacetamide	1	22
Iodoacetamide + DTE	1,10	67

[1] Enzyme preparation was used after ion-exchange chromatography.

Figure 33: Elution profile of the isoflavone-5-OMT after chromatofocusing on a Mono P HR 5/20 column. The active fractions from gel filtration were loaded on a column which had previously been equilibrated with bis Tris/iminodiacetic acid (pH 7.1) containing 14 mM 2-mercaptoethanol (ME) and 10% glycerol. The bound proteins were eluted with 40 ml of Polybuffer (74)-water (1:10, v/v) (pH 4.0) containing 14 mM 2-ME and 10% glycerol. The flow-rate was 1.0 ml/min (2.0 MPa) and 1-ml fractions were collected into 0.25 ml of 0.2 M Tris/HCl buffer (pH 8.0) and were assayed for OMT activity using genistein as substrate.


OMT Activity (DPM x 10^{-3})
4
3
2
1
1M NaCl
7
6
5
4
pH (----)
10
20
30
40
50
60
70
80
Fraction Number

D.2.11.1.Substrate interaction kinetics

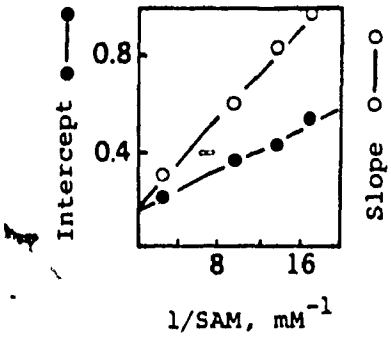
Double-reciprocal plots with genistein as the variable substrate and several, fixed concentrations of SAM gave intersecting lines (Fig. 34). Intercept and slope replots versus reciprocal fixed substrate concentrations (insert of Fig. 34) generated straight lines. On the basis of the initial velocity data thus obtained, the mechanism that fit was that of sequential binding. The experimental data was therefore represented by Eq. [1], the rate equation for a sequential bireactant mechanism (Section C.14.) (136).

D.2.11.2. Product inhibition studies

The order of substrate binding and product release was obtained from product inhibition studies. SAH was found to be a competitive inhibitor with respect to SAM (Fig 35A) and non-competitive with respect to genistein (Fig. 35C). 5-O-Methylgenistein was a non-competitive inhibitor with respect to either the co-substrate, SAM (Fig. 35B) or the isoflavone, genistein (Fig. 35D). These kinetic patterns are consistent with the predicted expectations of an ordered bi bi mechanism where SAM is the first substrate to bind to the enzyme, followed by the isoflavone (Fig. 34). The first product released is the 5-O-methylisoflavone, followed by SAH. However, these results do not rule out the possibility of a random binding mechanism involving the formation of a dead-end complex (140).

The kinetic constants K_A, K_B, K_{iP}, K_{iQ} and K_{iA} (136-139) were calculated from intercept and slope replots of the generated data and are listed in Table 24.

Figure 34: Double-reciprocal plots of initial velocities with SAM as the changing, fixed substrate at 60 μM (■——■), 74 μM (△——△), 107 μM (●——●) and 274 μM (○——○); and genistein as the variable substrate. 23.8 μM of [^{14}C-methyl]SAM (0.1 μCi) in addition to varying amounts of unlabelled SAM were used. The velocities are expressed in pkat/mg. Insert: slope and intercept replots.


Intercept
Slope
0.8
0.4
8
16
1/SAM, mM^{-1}

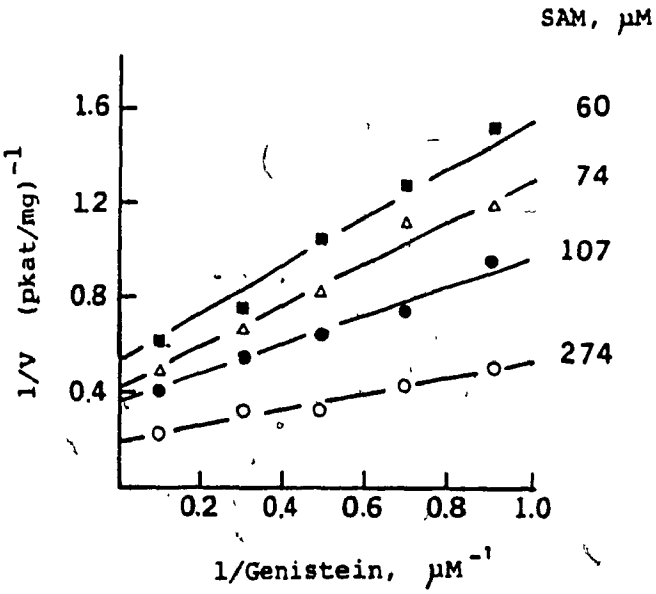

SAM, μM
60
74
107
274
1.6
1.2
0.8
0.4
1/V (pkat/mg)$^{-1}$
0.2
0.4
0.6
0.8
1.0
1/Genistein, μM^{-1}

Figure 35: Product inhibition kinetics of the isoflavone 5-OMT. The velocities are expressed in pkat/mg. (A) Inhibition by SAH with SAM as the variable substrate at constant concentration of genistein (1 μM). 23.8 uM of [^{14}C-methyl]SAM (0.1 μCi) in addition to varying amounts of unlabelled SAM were used at SAH concentrations of 40 μM (■——■), 25 μM (△——△), 10 μM (●——●) or 0 μM (○——○). (B) Inhibition by 5-O-methylgenistein with SAM as the variable substrate at constant concentrations of genistein (1 μM). 23.8 μM of [^{14}C-methyl]SAM (0.1 μCi) in addition to varying amounts of unlabelled SAM were used at 5-O-methylgenistein concentrations of 80 μM (■——■), 50 μM (△——△), 20 μM (●——●) or 0 μM (○——○). (C) Inhibition by SAH with genistein as the variable substrate at constant concentrations (23.8 μM) of SAM (0.1 μCi). The concentration of SAH was varied at 50 μM (■——■), 20 μM (△——△), 10 μM (●——●) or 0 μM (○——○). (D) Inhibition by 5-O-methylgenistein with geinstein as the variable substrate at constant concentration (23.8 μM) of SAM (0.1 μCi). The concentration of 5-O-methylgenistein was varied at 80 μM (■——■), 50 μM (△——△), 20 μM (●——●) or 0 μM (○——○).

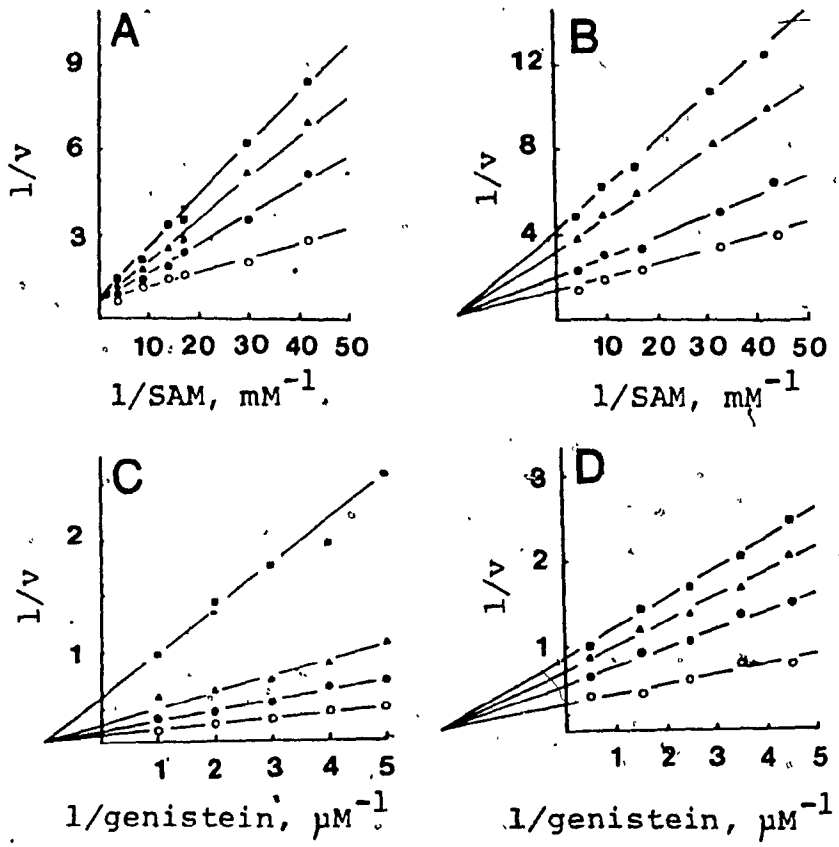

A
B
C
D
1/v
9
6
3
12
8
4
2
1
3
10 20 30 40 50
1/SAM, mM^{-1}
1 2 3 4 5
1/genistein, μM^{-1}

Figure 36: Proposed kinetic mechanism for the isoflavone 5-O-methyltransferase of Lupinus luteus. A, S-adenosyl-L-methionine; B, isoflavone substrate; P, 5-O-methylisoflavone product; Q, S-adenosyl-L-homocysteine; E, isoflavone-5-O-methyltransferase.

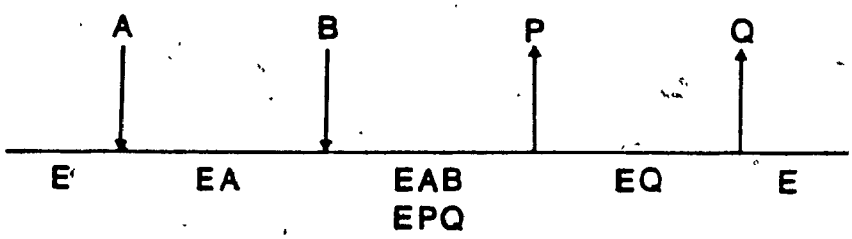

A
B
P
Q
E'
EA
EAB
EPQ
EQ
E

Table 24: Kinetic parameters of the 5-OMT of L. luteus.

Parameter	Value (μM)
K_A	150
K_B	1
K_{iA}	350
K_{iP}	30
K_{iQ}	15

E. DISCUSSION

E.1. Anthraquinones

Cell suspension cultures of Cinchona succirubra produce a number of hydroxyanthraquinone glucosides. This is in contrast with previous reports of Cinchona spp. (69-73) where anthraquinones were reported to be produced as aglycones. These contradictory results may be due to the extraction procedure previously used, in which the anthraquinone aglycones were preferentially isolated and the glucosides were, therefore, overlooked (69-73).

The study of growth parameters (fresh and dry weights, protein content) of this culture, indicated a lag phase and an exponential phase of 3 days each, followed by a linear growth phase of 6 days before entering the stationary phase. The formation of anthraquinones by C. succirubra cells in culture paralleled cell growth and was concomitant with maximum protein content (Fig. 11). This parallelism between cell growth and metabolite production in C. succirubra is similar to that of other cultured tissues (11). The production of anthraquinones is initiated at the end of the exponential phase and increases exponentially during the linear phase of the growth cycle (Fig. 11). This pattern indicates that the biosynthesis of anthraquinones is maximally induced during part of the growth cycle in which the rate of cell division has slowed down. It is desirable, therefore, to shorten the lag phase prior to the initiation of product synthesis. This may be achieved by decreasing the concentration of auxin in the culture medium as was shown for anthocyanin (152) and shikonin (153) production. This appears to be the case with C. succirubra cells, where anthraquinone

production seems to be regulated by the type and concentration of auxin used (Tables 6-8). Studies of the rates of cell growth and product formation are essential in order to obtain a basic understanding of metabolite production. However, little is known about the relationship between the rate of product formation and the age of individual cells in plant cell culture, since the latter is formed of cells at different stages of development.

The effect of various nutritional factors, such as sucrose as well as macro- and microelements, on growth and anthraquinone production was studied. Increasing sucrose concentration was found to increase metabolite production, although it resulted in decreased cell growth (Table 4). These results compare well with those reported for Cinchona ledgeriana (74), Morinda citrifolia (60) and Rubia cordifolia (66) where medium (4%) and high (8%) sucrose concentrations were reported to stimulate anthraquinone production. The use of nutrient-limited media (Heller's or White's), or dilution of the macro- and microelements of nutrient-rich media (MS or B5) resulted in stimulation of anthraquinone production (Table 5). Most studies have shown that decreasing nitrogen and/or phosphorus increased the production of phenolic metabolites (for review see ref. 154). This is in contrast with the nutrient-rich media which supported prolific cell growth and protein synthesis (Table 5).

The results obtained for the effect of growth regulators show that whereas cell growth was stimulated by 2,4-D, anthraquinone production was enhanced in the presence of IAA and, to a lesser extent, by NAA (Table 6). It seems possible that both processes may respond to different forms of auxin and may explain their dual role in the regulation of cell growth and productivity of secondary metabolites.

These results are in agreement with those reported for other cultured tissues where 2,4-D was shown to stimulate cell growth and NAA to enhance anthraquinone formation (60,62,72,74). However, it is interesting to note that further addition of a cytokinin to the auxin did not affect either cell growth or anthraquinone production (Table 8). This seems to indicate that anthraquinone production is regulated mainly by the type and concentration of auxin, rather than the cytokinin used.

Stress, due to nutritional, hormonal or other environmental factors, including microbial insult is known to cause decreased growth rates. It is suggested, however, that as growth rates decline, amino acids, nucleotides and precursors for phytochemicals accumulate. The reduced metabolic flow is believed to impose a stress on the cells which results in declined growth and specific enzymatic perturbations (155). It was suggested that secondary metabolite synthesis is greatest when the growth rates, carbohydrate, mineral and oxygen uptake decline. Recently, it was suggested that anthraquinone production in intact tissue of Cinchona may act as phytoalexins, since they exhibited anti-microbial activity towards a number of microorganisms and their occurrence in Cinchona spp. was only observed in infected tissues (87). Furthermore, their formation in cultured tissues may be the result of changes in tissue metabolism due to in vivo culture. Very recently, it was shown that the addition of heat-sterilized mycelia of the fungi Phytophthora cinnamomi and Aspergillus niger elicited the production of anthraquinones in Cinchona ledgeriana suspension cultures (87). It would be interesting, however, to determine the effect of fungal elicitors on the enzymes involved in the biosynthesis of anthraquinones in Cinchona

spp..

In order to study the enzyme catalyzed glucosylation of anthraquinones in C. succirubra it was essential to determine the stage at which glucosyltransferase (GT) activity was maximal with respect to culture growth. Maximum GT activity was found to parallel cell growth (Fig. 11) and was concomitant with maximum protein content as well as anthraquinone production (day 10 in culture). Therefore, 10-day cells were used in the purification and characterization of GT activity. The latter was resolved by chromatofocusing into five distinct glucosylating activities; peaks I, II, III, IV and V (Fig. 16). These enzymes expressed preference for different anthraquinones indicating specificity towards their phenolic substrates (Table 10). The fact that the best substrate for these GTs were emodin, anthrapurpurin, quinizarin, 1,8-dihydroxyanthraquinone and 2,6-dihydroxyanthraquinone is consistent with the accumulation of their glucosides in C. succirubra cell suspension cultures (Fig. 14). The properties of these enzymes appear to be similar to those reported for other phenol GTs with respect to pH optimum, molecular weight, requirement for SH-group and inhibition by divalent cations (118,119). Whereas it may be difficult to suggest a physiological role for five enzymes which catalyze the same reaction, however, it is possible that these GTs are merely charged isomers of the same enzyme.

E.2. Flavonoid O-methyltransferases

The results presented in Section D.2, demonstrate the existence of two novel O-methyltransferases (OMTs), namely the 2′-/5′-OMT of Chrysosplenium americanum and the 5-OMT of Lupinus lutues. In addition,

the previously reported 7-OMT of C. americanum (115,116) was further purified using fast protein chromatofocusing which utilized a shallower gradient (pH 7-5). Furthermore, the recovery of enzyme activity was improved by collecting the eluted fractions into Tris buffer pH 8.5 which protected the enzyme from denaturation by increasing the pH.

The 7- and 2´-/5´-OMTs of Chrysosplenium and the 5-OMT of Lupinus had pH optima similar to those reported for other flavonoid-specific enzymes (103-107, 109-116, 156), whereas their molecular weights were intermediate between those of the flavone/flavonol (103-105) and isoflavone OMTs (107). In contrast with the 6-OMT of C. americanum, the 8-OMT of Lotus corniculatus (112) and the 3´-OMT of parsley cell suspension cultures (105), the 5-, 7- and 2´-/5´-OMTs showed no requirement for Mg^{2+}.

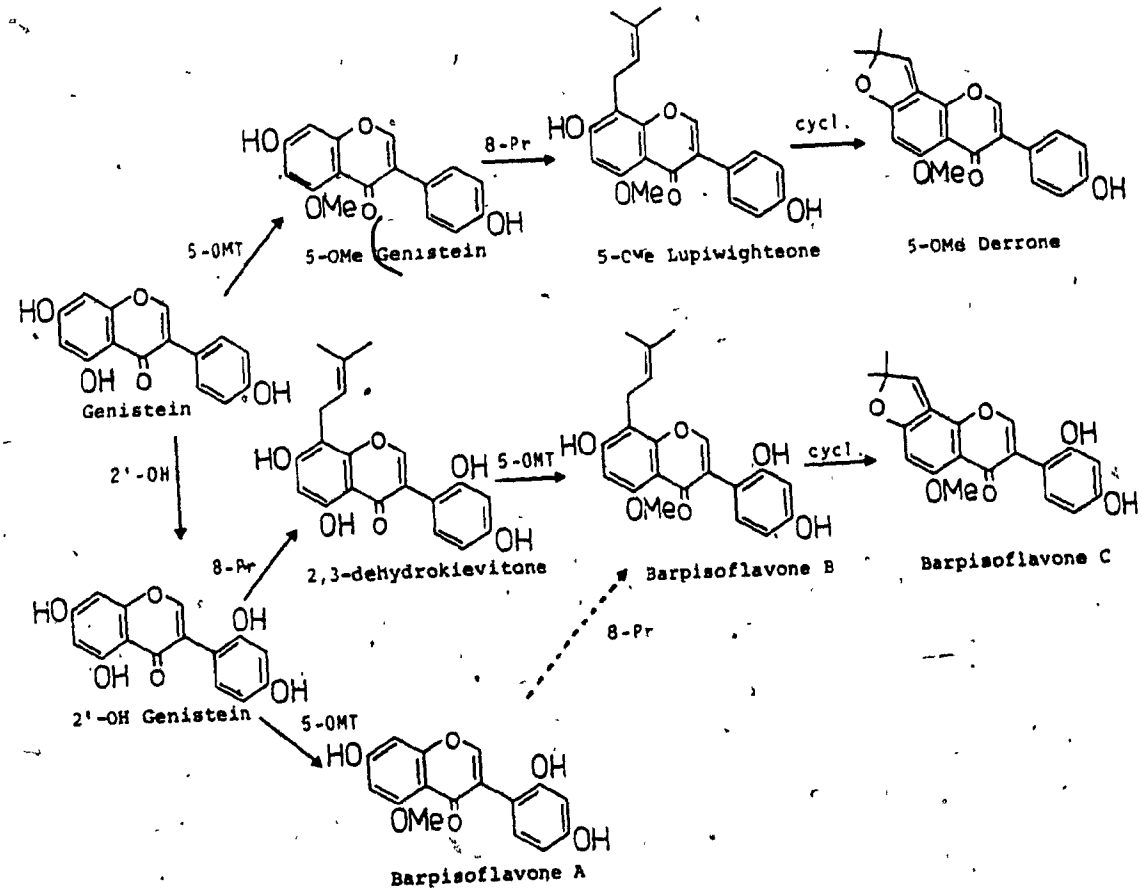
The isoflavone OMT, which was purified 810-fold from yellow lupin roots, exhibited an expressed specificity for the 5-position of 2,3-dehydrokievitone > 2´-hydroxygenistein > genistein. It showed no appreciable activity with other flavonoid or phenylpropanoid compounds (Table 22). Although the 5-hydroxyl group of flavonoid compounds is believed to be the least reactive position due to chelation with the carbonyl group, however, it seems possible that the activity of the isoflavone 5-OMT may be due to specific binding of the flavonoid substrate and co-substrate in such a geometrical arrangement, relative to each other, such that methylation at the 5-position is preferentially catalyzed. A similar situation has been reported for the prenylation of the 4-position of tryptophan as the first step in the biosynthesis of lysergic acid (157).

The fact that the 5-O-methylation of different substituted isoflavones in yellow lupin takes place with almost equal efficiency, seems to indicate that O-methylation of isoflavones is not necessarily a later reaction (102), but may take place at different stages of isoflavonoid biosynthesis in yellow lupin (Fig. 37). Based on the substrate specificity of the 5-OMT (Table 22) and on the nature of the isoflavones which accumulate in this tissue (135), it seems likely that genistein can either be methylated at position 5 or hydroxylated at the 2′-position. Furthermore, 2′-hydroxygenistein can either be prenylated at position 8 followed by 5-O-methylation, or methylated at position 5 followed by an 8-prenylation (Fig. 37). Therefore, the physiological role of the 5-OMT seems to catalyze the 5-O-methylation of a number of isoflavones of yellow lupin roots.

Since isoflavonoids are considered to be antimicrobial compounds (158), their predominance in root tissue may play a role in the defence mechanism of yellow lupin against soil microroganisms. However, despite the fact that some flavones and flavanones have recently been reported to induce Rhizobium nod genes (159), the isoflavones, genistein and daidzein (7,4′-dihydroxyisoflavone) are believed to be among the antagonists of nod gene transcription (160). Therefore, it would of interest to find out whether 5-O-methylisoflavones play a role in the regulation of root nodulation in the yellow lupin.

The Chrysosplenium OMTs reported here mediated substrate- and position-specific methylations, thus establishing the high specificity for their flavonoid methyl acceptors. In contrast with the other OMTs of C. americanum, which accepted only hydroxylated or partially methylated aglycones, the 2′- and 5′- methylation steps seem to take place at the

Figure 37: Proposed pathway for the biosynthesis of isoflavones in *Lupinus luteus*


HO
OMeO
OH
8-Pr
cycl.
5-OMT
5-OMe Genistein
5-OMe Lupiwighteone
5-OMe Derrone
Genistein
2'-OH
2,3-dehydrokievitone
Barpisoflavone B
Barpisoflavone C
2'-OH Genistein
Barpisoflavone A

glucoside level. The partially purified 2′-/5′-OMT preparation was found to catalyze the 2′-O-methylation of A to C and the 5′-O-methylation of B to D (Fig. 19), with almost equal efficiency. These methylating activities could not be separated on a number of chromatographic supports and showed similar properties as to their pH optima, pI values, molecular weights, requirement for SH group reagents and kinetic constants as well as kinetic mechanism. This seems to indicate that both the 2′-and the 5′-methylations are catalyzed by one enzyme. Recently, a similar situation was reported for the 2′- and 5′-O-glucosyltransferases of C. americanum (Fig 19) which exhibited similar chromatographic (123) and kinetic properties (124). However, the two enzyme activities were later separated using fast protein affinity chromatography on brown-dye ligand (161). In analogy with the 2′- and 5′-O-glucosyltransferases, and in view of the strict position specificty of the 3-, 6-, 7- and 4′-OMTs of C. americanum, it is reasonable to assume that the 2′- and 5′-OMTs are distinct enzymes with identical pI values, which remain to be resolved.

The effect of the nature and pH of starting buffer, the dilution and flow-rate of Polybuffer and the presence of zwitterions was studied on the resolution and recovery of activity of the Chrysosplenium OMTs. These factors were investigated in order to separate the five position-specific OMTs of C. americanum in a one-step chromatofocusing process (Fig. 28). The results obtained show that the pI values of proteins obtained by chromatofocusing vary according to the experimental conditions used (Table 18). Therefore, it is recommended that the latter conditions be stated when reporting pI values. Furthermore, it should be noted that those values are apparent pIs, and unless certain measures

are taken to overcome these discrepancies, then isoelectric focusing remains the technique of choice for determining the true pI values of proteins.

Both the 7-OMT of Chrysosplenium and the 5-OMT of Lupinus exhibited similar steady state kinetics. Substrate interaction kinetics for both OMTs gave converging lines (Figs. 23 and 34) suggesting a sequential mechanism. The order of substrate binding and product release was determined from product inhibition patterns. Reciprocal velocities were plotted against reciprocal concentration of the variable substrate at several fixed concentrations of product. Cleland states that the slope of a reciprocal plot is affected by a substance (product or inhibitor) which associates with an enzyme form that is the same as, or is connected by a series of reversible steps to, the enzyme form with which the variable substrate combines (136-139). The intercept of a reciprocal plot, however, is affected by a substance which associates reversibly with an enzyme form other than the one with which the variable substrate combines. Cleland further adds that a change in slope indicates competitive inhibition between a substance and the variable substrate, whereas a change in intercept indicates uncompetitive inhibition. A change in both slope and intercept, however, indicates noncompetitive inhibition between a substance and the variable substrate (136-139).

A sequential mechanism can either be random or ordered binding. Based on Cleland's rules, in a random mechanism four competitive product inhibition patterns would be expected (140), except when dead-end inhibition occurs. Whereas in an ordered binding mechanism, one competitive and three noncompetitive product inhibition patterns would

be expected. The product inhibition patterns obtained for both OMTs in this study suggest an ordered binding mechansim. The fact that SAH was a competitive inhibitor of SAM (Figs. 24 and 35) suggests that SAM is the first substrate to bind to the enzyme and that SAH is the final product released (Figs. 25 and 36). This implies that SAM and SAH are the leading reaction partners and bind to the same form of the enzyme (E). The flavonoid substrate, therefore, binds second whereas the flavonoid product is released prior to the release of SAH (Figs. 25 and 36). Such a mechanism predicts one competitive pattern (SAM v/s SAH) and three noncompetitive patterns (SAM v/s flavonoid product and flavonoid substrate v/s either SAH or flavonoid product). The results obtained for both the 7- and the 5-OMT were in agreement with the above predictions. It is interesting to note that the OMTs of this study followed the same kinetic mechanism as that of the other OMTs of C. americanum (117) despite their distinct substrate and position specificity.

The K_m values for the flavonol substrate and SAM, the co-substrate for the 7-OMT were similar to those of the 3-, 6- and 4'-OMTs of Chrysosplenium (Table 25). Furthermore, the inhibition constant for SAH was the same order of magnitude as those for the other three OMTs. The inhibition constant for the flavonoid product, however, more closely resembled that for the 4'-OMT than the 3- or 6-OMT (Table 25). The fact that the inhibition constant of the flavonoid product for the 3- and the 6-OMTs was one order of magnitude higher than that for the 7- or 4'-OMT indicates that the 7- and 4'-OMTs are more sensitive for inhibition by the flavonoid product. This could provide further support for the view that both substrate and intermediate products of the OMT sequence play an important role in the regulation of each enzyme and thus, the whole

Table 25: Kinetic constants of Chrysosplenium OMTs.

Enzyme	Substrate	Product	Parameter Km (μM)	Parameter Ki (μM)
3-OMT[1]	SAM		114	
	Q		12	
		SAH		4.5
		3MeQ		128
7-OMT	SAM		65	
	3MeQ		7	
		SAH		10
		3,7MeQ		15
4′-OMT[1]	SAM		130	
	3,7MeQ		15	
		SAH		4.4
		3,7,4′MeQ		10
6-OMT[1]	SAM		51	
	3,7,3′MeQg		18	
		SAH		16
		3,6,7,3′MeQg		167
2′-OMT	SAM		100	
	2′-OH-substrate[2]		2	
		SAH		100
		2′-Me-product		5
5′-OMT	SAM		100	
	5′-OH-substrate[3]		2	
		SAH		100
		5′-Me-product		5

SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine; Q, quercetin, Qg, quercetagetin.

[1] From ref. 117.

[2] 5,2′-Dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-O-glucoside.

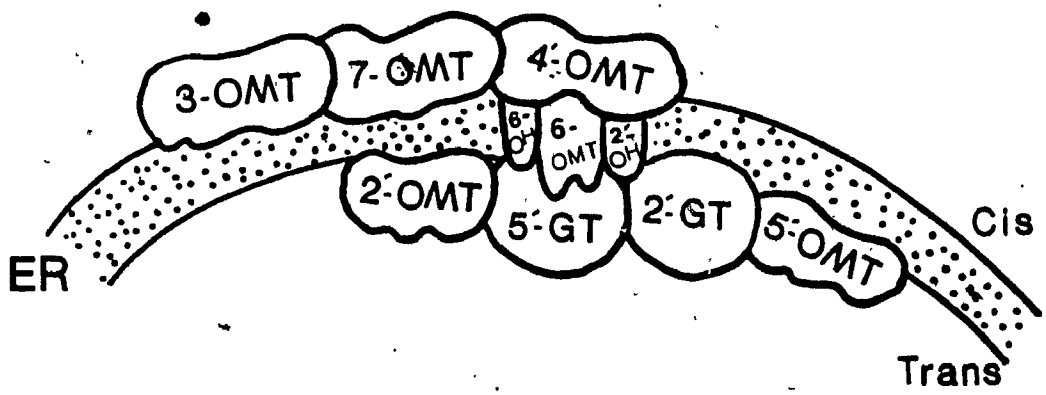
[3] 5,5′-Dihydroxy-3,7,4′-trimethoxyflavone-2′-O-glucoside.

pathway of flavonoid biosynthesis in this tissue.

Whereas the enzymes studied in this pathway were found, after tissue homogenization, in the cytosolic fraction, it is difficult to determine whether they are actually soluble, or easily solubilized enzymes. This may be attributed to the loose association of the proteins with each other or to an organelle, such that this association may be easily destroyed upon cell disintegration. However, in view of the coordinated sequence of O-methylation and O-glucosylation of this pathway (Fig. 19), it is tempting to postulate that the synthesis of polymethylated flavonol glucosides in Chrysosplenium may take place on the surface of an aggregated, membrane-associated, multienzyme system (162). It is evident that if such system exists in vivo, its component enzymes would be loosely associated, or membrane bound by non-covalent forces, unlike the membrane-bound pathway of cyanogenic glycoside biosynthesis (163), or the multifunctional protein of the arom complex (164). Such a compartmentation model facilitates the formation of metabolic chains of intermediates and allows for increased catalytic activity. Furthermore, it permits the physical differences inherent to the microenvironments of the different enzymes of the pathway, such as changes in pH, changes in solubility of substrates and products; especially if the last steps of biosynthesis involve methylation and/or glucosylation of relatively lipophilic intermediates (165), as is the case in Chrysosplenium. A multienzyme system catalyzing the methylation-glucosylation sequence in this tissue could not be isolated, since attempts to fractionate tissue homogenates resulted in the recovery of both sets of enzymes from the supernatant fraction (166). However, various lines of evidence tend to support the concept of a multienzyme

system; these are: (a) the incorporation of [^{14}C]cinnamate into the final products, after 5-10 min pulse, without labelling of the low methylated intermediates (167); (b) the absence of low methylated intermediates among the products which accumulate in this tissue (134); (c) the sequential multistep O-methylation of positions 3,7, and 4′ by cell-free extracts, and of other O-methylated intermediates at position 6,2′ and 5′ (115, 116); (d) the similarity of the kinetic mechanisms of the enzymes studied (117, 124); (e) the regulation of these enzymes by very specific range of substrate and product concentrations; (f) the varied microenvironment of the different O-methyltransferases with respect to pH optima (116) and utilization of aglycones or glucosides as substrates; and (g) inhibition of the O-methyltransferases and O-glucosyltransferases studied by their flavonoid substrates (117, 124). Whereas this indirect evidence does not unequivocally demonstrate the existance of a multienzyme system, it is tempting to propose a model for the compartmentation of this pathway in which the 3-, 7- and 4′-OMTs are loosely associated with the cis 'forming' face (168) of the endoplasmic reticulum (ER) membrane (Fig. 38). Partially O-methylated intermediates could easily be hydroxylated at position 6 and/or 2′ by specific hydroxylases, which are embedded in the membrane (162, 165), and further methylated at position 6. Since the 6-OMT is the only enzyme of this pathway which requires Mg^{2+} (115) and that its substrate scarcely accumulates in the flavonoid pool, it seems possible, therefore, that it may also be embedded in the membrane. Partially O-methylated intermediates could then be transported across the membrane for further glucosylation and methylation at positions 2′ and/or 5′. The final products, thus formed, may accumulate at the trans 'maturing'

Figure 38: Proposed model of the multienzyme, membrane-associated complex of Chysosplenium americanum.


3-OMT
7-OMT
4'-OMT
6'-OH
6-OMT
2'-OH
2'-OMT
5'-GT
2'-GT
5'-OMT
ER
Cis
Trans

face, which in turn, may form cisternal channels or vesicles (169) such that the partially methylated glucosides are sequestered before being secreted (170). Very recently, it has been shown that the epidermal cell walls appear to be the site of accumulation of flavonoids in Chrysosplenium (171-173), which supports the above view. Despite the attractive features of this proposed model, however, immunocytochemical techniques using antibodies against the individual enzymes, should provide unequivocal evidence for the compartmentation of the enzymes of this pathway.

F. CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

1. A cell suspension culture of Cinchona succirubra was initiated and the anthraquinone glucosides produced in vitro were identified. The conditions for optimal growth and production of anthraquinones were determined.

2. Five, anthraquinone-specific glucosyltransferases were resolved by chromatofocusing. These enzymes expressed preference for different anthraquinones as glucosyl acceptors, indicating their specificity towards these substrates, and was consistent with the accumulation of their glucosides in this tissue.

3. A novel enzyme, the 2′-/5′-OMT, was isolated and partially purified from Chrysosplenium americanum. The 7-OMT was further purified from the same tissue and its kinetic analysis was undertaken. A model was proposed for the compartmentation of the multi-step pathway involved in flavonoid synthesis and accumulation in this tissue.

4. Another novel OMT, which catalyzed the 5-O-methylation of isoflavones was partially purified and its kinetic mechanism studied in yellow lupin roots. The study of substrate specificty of this enzyme reaction made it possible to propose a pathway for the biosynthesis of the isoflavone derivatives which accumulate in this tissue.

G. PERSPECTIVE FOR FUTURE WORK

Since Cinchona succirubra cell suspension culture produces a variety of anthraquinone glucosides in vitro, it would serve as an ideal system for studying the elicitation of these metabolites by fungal cell wall extracts (155). Furthermore, the fact that this culture produces quinoline alkaloids in vitro (132) makes it valuable in studying the regulatory mechanisms involved in the biosynthesis of both groups of natural products.

Despite the scarcity of ample supply, C. americanum has proven to be an ideal system for the enzymatic studies of polymethylated flavonol glucosides (174). However, preliminary work by this author demonstrated the difficulty in isolating the multienzyme complex of Chrysosplenium despite the use of gel filtration on a number of supports with molecular weight exclusion limits greater than 10^6 (H. E. Khouri, unpublished results). This may be attributed to the loose association of these enzymes with each other, and the organelles concerned in vivo as was mentioned earlier (p.161). However, evidence for the association of these enzymes can be derived from isotopic studies using protoplasts prepared from leaves of Chrysosplenium. Catalytic facilitation of the enzymes may be demonstrated using [^{3}H]-quercetin and [^{14}C]-labeled intermediates such as 3,7,4′-trimethylquercetin by one of the following feeding experiments:

(a) The overall rate of synthesis of polymethylated flavonol glucosides is greater than when starting with an intermediate such as 3,7,4′-trimethylquercetin.

(b) Addition of [^{14}C]3,7,4′-trimethylquercetin to limiting

concentrations of [^{3}H]-quercetin does not increase the rate of the reaction.

(c) Comparison of $^{3}H/^{14}C$ ratios of product and intermediate after incubation with substrate limiting amounts of ^{3}H-quercetin and [^{14}C]3,7,4′-trimethylquercetin. Ratios greater than 1.0 are usually taken as evidence for catalytic facilitation (162).

This will indicate that only intermediates which are derived from quercetin can be channelled for further methylation and glucosylation to give rise to polymethylated flavonol glucosides in Chrysosplenium americanum. However, unequivocal evidence for the compartmentation of the enzymes of this complex should be derived from immunocytochemical studies. Antibodies raised against individual enzymes of this pathway can be used to localize the enzymes involved in the biosynthesis of this pathway. Since the O-glucosyltransferases and O-methyltransferases of this tissue have different molecular weights, they may be separated by gel filtration. The one-step chromatofocusing process of OMTs described in this study (Fig. 28) may serve as an ideal procedure for the separation of individual enzymes of this pathway. Polyclonal antibodies can then be raised in rabbits for their use in in vivo localization of these enzymes by immunocytochemical methods and to verify the model proposed for their compartmentation (Fig. 38).

H. REFERENCES

1. Bonner, J. & Varner, J. E. (1976) Plant Biochemistry. Academic Press, New York, p. 278.
2. Mann, J. (1980) Secondary Metabolism. Clarendon Press, Oxford, p. 1.
3. Luckner, M. (1984) Secondary Metabolism in Microorganisms, Plants, and Animals. Springer-Verlag, Berlin, Heidellberg, New York.
4. Goodwin, T. W. & Mercer, E. I. (1983) Introduction to Plant Biochemistry. Pergamon Press, New York, p. 227.
5. Bell, E. A. (1981) In The Biochemistry of Plants: A Comprehensive Treatise (Conn, E. E., ed.), Vol 7. Academic Press, New York, p. 1.
6. Bennett, J. W. & Ceigler, A. (eds.) (1983) Secondary Metabolism and Differentiation in Fungi. Marcel Dekker, Inc., New York.
7. Krumphanzl, V., Sikyta, B. & Vanek, Z. (eds.) (1982) Overproduction of Microbial Products. Academic Press, London, New York.
8. Scherer, P. J. (ed.) (1978-1983) Marine Natural Products: Chemical and Biological Perspectives. Vols. 1-5. Academic Press, New York.
9. Robinson, T. (1983) The Organic Constituents of Higher Plants. 5th ed. Cordus Press, MA.
10. Staba, E. J. (ed.) (1980) Plant Tissue Culture as a Source of Biochemicals. CRC-Press, Boca Raton, Florida.

11. Tabata, M. (1977) In Plant Tissue Culture and Its Bio-Technological Applications (Barz, W., Reinhard, E. & Zenk, M. H., eds.). Springer-Verlag, Berlin, Heidelberg, New York, p. 3.

12. Zenk, M. H. (1978) In Fronteirs of Plant Tissue Culture (Thorpe, T. A., ed.), p. 1. University of Calgary Press.

13. Harborne, J. B. & Mabry, T. J. (eds.) (1982) In The Flavonoids: Advances in research. Chapman and Hall, London, New York.

14. Thomson, R. H. (ed.) (1971) Naturally Occurring Quinones. Academic Press, London, New York.

15. Bently, R. & Campell, I. M. (1974) In The Chemistry of Quinoid Compounds (Patai, S., ed.), p. 683. Wiley, New York.

16. Leistner, E. (1981) In The Biochemistry of Plants: A Comprehensive treatise (Conn, E. E., ed.). Vol 7, p. 403. Academic Press, New York.

17. Mulchandani, N. B. & Hassarajani, S. A. (1977) Planta Med., 32: 357.

18. Agrawal, G. D., Rizve, S. A. I., Gupta, P. C. & Teware, J. D. (1972) Planta Med., 21: 150.

19. Koshioka, M. & Takino, Y. (1978) Chem. Pharm. Bull., 26: 1343.

20. Imre, S. & Oztune, A. (1976) Z. Naturforsch., 31C: 403.

21. Muller, W.-U. & Leistner, E. (1978) Phytochemistry, 15: 407.

22. Ghaleb, H., Rizk, M. M., Hammouda, F. M. & Abdel-Gawed, M. M. (1972) Qual. Plant. Mater. Veg., 21: 237.

23. Miyase, T. Ruedi, P. & Eugster, C. H. (1978) Helv. Chim. Acta, 60: 2770.

24. Rao, R. S. & Werra Reddy, G. C. (1977) Indian J. Chem., Sect. B 15: 497.

25. Burnett, A. R. & Thomson, R. H. (1968) J. Chem. Soc. (C) p. 850.

26. Burnett, A. R. & Thomson, R. H. (1968) J. Chem. Soc. (C) p. 2437.

27. Burnett, A. R. & Thomson, R. H. (1968) J. Chem. Soc. (C), p. 854.

28. Blakrishna, S., Seshadri, T. R. & Venkataramani, B. (1961) J. Sci. Ind. Res. (India), 20B: 331.

29. Muller, H. (1911) J. Chem. Soc., 99: 967.

30. Schutzenberger, P. (1965) Bull. Soc. Chim. France, 4: 12.

31. Perkin, A. G. & Hummel, J. J. (1983) J. Chem. Soc., 63: 1157.

32. Burnett, A. R. & Thomson, R. H. (1968) Phytochemistry, 7: 1421.

33. Beilstein, F. (1882) Chem. Ber., 15: 901.

34. Beal, G. D. & Akey, R. E. (1919) J. Amer. Chem. Soc., 41: 693.

35. Tschirch, A. & Pool, J. F. A. (1908) Arch. Pharm., 246: 320.

36. Fairbairn, J. W. & Shestha, A. B. (1967) Phytochemistry, 6: 1203.

37. Mahran, G. H., Sayed, M. D. & El-Keiy, M. A. (1958) Egyptian Pharm. Bull., 40: 149.

38. Aschan, J. (1903) Arch. Pharm., 241: 340.

39. Tutin, F. & Clewer, H. W. B. (1911) J. Chem. Soc., 99: 946.

40. Liddell, R. W., King, C. G. & Beal , G. D. (1942) J. Amer. Pharm. Assoc., 31: 161.

41. Tschirch, A. & Heipe, E. (1900) Arch. Pharm., 238: 427.

42. Schratz, E. (1960) Planta Med., 8: 301.

43. Tsukida, K., Suzuki, N. & Yakota, M. (1954) J. Pharm. Soc. Japan, 74: 224.

44. Anchel, M. (1949) J. Biol. Chem., 177: 169.

45. Perkin, A. G. & Hummel, J. J. (1983) J. Chem. Soc., 63: 1160.

46. Simonsen, J. L. (918) J. Chem. Soc., 113: 766.

47. Murti, V. V. S., Neelakantan, S., Seshardi, T. R. & Verkataramani, B. (1959) J. Sci. Indian Res., 18B: 367.

48. Perkin, A. G. & Hummel, J. J. (1894) J. Chem. Soc., 65: 851.

49. Shibata, S. & Takido, M. (1852) J. Pharm. Soc. Japan, 72: 1311.

50. Tschirch, A. & Weil, F. (1912) Arch. Pharm., 250: 20.

51. Tsukida, K. & Yoneshige, M. (1954) J. Pharm. Soc. Japan, 74: 223.

52. Briggs, L. H. Jacombs, F. E. & Nicholls, G. A. (1953) J. Chem. Soc., p. 3069.

53. Kariyone, T. T. & Tsukida, K. (1954) J. Pharm. Soc. Japan, 74: 223.

54. Perkin, A. G. (1895) J. Chem. Soc., 67: 1084.

55. Hanschild, G., Steiner, M. & Glonbitza, K. W. (1968) Naturwissenschaften, 55: 346.

56. King, N. M. (1957) J. Amer. Pharm. Assoc., 46: 271.

57. Schulte, U., El-Shagi, H. & Zenk, M. H. 91984) Plant Cell Rep., 3: 51.

58. Inoue, K., Nayeshiro, H., Inoue, H. & Zenk, M. H. (1981) Phytochemistry, 20: 1693.

59. Leistner, E. (1975) Planta Med. Suppl., 33: 214.

60. Zenk, M. H., El-Shagi, H., Schulte, U. (1975) Planta Med. Suppl., 33: 79.

61. Zenk, M. H., Schulte, U. & El-Shagi, H. (1984) Naturwissenschaften, 71: 266.

62. Bauch, H-J. & Leistner, E. (1978) Planta Med., 33: 105.

63. Inoue, K., Shiobara, Y., Nayeshiro, H., Inoue, H., Wilson, G. [redacted], M. H. (1984) Phytochemistry, 23: 307.

64. Wilson, G. & Marron, P. (1978) J. Exp. Botany, 29: 837.

65. Suzuki, H., Matsumato, T. & Obi, Y. (1982) In Proc. 5th Intl. Cong. Plant Tissue and Cell Culture (Fujiwara, A., ed.), p. 285.

66. Suzuki, H., Matsumoto, T. Mikami, Y. (1984) Agric. Biol. Chem., 48: 603.

67. Suzuki, H., Matsumoto, T. & Mikami, Y. (1985) Agric. Biol. Chem., 49: 519.

68. Robins, R. J., Payne, J. & Rhodes, M. J. C. (1986) Phytochemistry, 25: 2327.

69. Verpoorte, R., Wijnsma, R., Mulder-Krieger, Th., Harkes, P. A. A., & Baerheim Svendsen, A. (1985) In Primary and Secondary Metabolism of Plant Cell Cultures, (Neumann, K., ed.), p. 196. Springer-Verlag, Berlin, Heidelberg.

70. Mulder-Krieger, Th., Verpoorte, R. & Baerhaim Svendsen, A. (1982) Planta Med., 44: 237.

71. Wijnsma, R., Go, J. T. K. A., Harkes, P. A. A., Verpoorte, R. & Baerheim Svendsen, A. (1986) Phytochemistry, 25: 1123.

72. Mulder-Krieger Th., Verpoorte, R., de Water, A., van Gesel, M. & van Oeveren, B. C. J. A. (1982) Planta Med., 46: 19.

73. Wijnsma, R., Verpoorte, R., Mulder-Krieger, Th. & Baerheim Svendsen, A. (1984) Phytochemistry, 23: 2307.

74. Harkes, P. A. A., Krijbolder, L., Libbenga, K. R., Wijnsma, R., Aremge, T. N. & Verpoorte, R. (1985) Plant Cell Tissue Organ Culture, 4: 199.

75. Zenk, M. H. & Leistner, E. (1968) Lloydia, 31: 275.

76. Bently, R. (1975) Biosynthesis, 3: 181.

77. Leistner, E. (1980) In Pigments in Plants (I.-C. Czygan, ed.), pp. 352-369, G. Fischer-Verlag, Stuttgart, New York.

78. Walker, J. E. & Abraham, E. P. (1970) Biochem. J., 118: 563.

79. Heide, L. & Leistner, E. (1983) Phytochemistry, 22: 659.

80. Heide, L., Kolkman, R., Arendt, S. & Leistner, E. (1982) Plant Cell Rep., 1: 180.

81. Bentley, R. & Maganathan, R. (1982) Microbiol. Rev., 46: 241.

82. Weische, A., Garrert, W. & Leistner, E. (1987) Arch. Biochem. Biophys., 256: 223.

83. Rai, P. P. (1978) Lloydia, 41: 114.

84. Anton, R. (1980) Pharmacology, 20 Suppl. 1: 104.

85. Longo, R. (1980) Bull. Chim. Farm., 119: 669.

86. Chang, P. & Lee, K. H. (1984) Phytochemistry, 23: 1733.

87. Wijnsma, R., Go, J. T. K. A., van Weerden, I. N., Harkes, P. A. A., Verpoorte, R. & Baerheim Svendsen, A. (1985) Plant Cell Rep., 4: 241.

88. Hahlbrock, K. (1981). In The Biochemistry of Plants: A Comprehensive Treatise. (Conn, E. E., ed.), Vol 7, pp. 425-456. Academic Press, New York.

89. Weirmann, R. (1981) In The Biochemistry of Plants: A Comprehensive Treatice. (Conn. E. E., ed.), Vol 7, p. 85. Academic Press, New York.

90. Hahlbrock, K. & Grisebach, H. (1975) In The Flavonoids. (Harborne, J. B., Mabry, T. J. & Mabry, H., eds.), p. 866. Chapman & Hall, London.

91. Ebel, J. & Hahlbrock, K. (1977) Eur. J. Biochem., 75: 201.

92. Kreuzaler, F. & Hahlbrock, K. (1975) Eur. J. Biochem., 56: 204.

93. Kreuzaler, F. & Hahlbrock. K. (1975) Arch. Biochem. Biophys., 169: 84.

94. Hrazdina, F. Kreuzaler, F. & Hahlbrock, K. (1976) Arch. Biochem. Biophys., 175: 392.

95. Saleh, N. A. M., Fritsch, H., Kreuzaler, F. & Hahlbrock, K. (1978) Phytochemistry, 17: 183.

96. Hahlbrock, K., Wong, E., Schill, L. & Grisebach, H. (1970) Phytochemistry, 9: 949.

97. Forkmann, G., Heller, W. & Grisebach, H. (1980) Z. Naturforsch., 35C: 691.

98. Stotz, G. & Forkmann, G. (1981) Z. Naturforsch., 36C: 737.

99. Britsch, K., Heller, W. & Grisebach, H. (1981) Z. Naturforsch., 36C: 742.

100. Stotz, G. & Forkmann, G. (1982) Z. Naturforsch., 37C: 19.

101. Hinderer, W., Flentje, U. & Barz, W. (1987) FEBS, 214: 101.

102. Poulton, J. E. (1981) In Biochemistry of Plants: A Comprehensive Treatise. (Conn, E. E., ed.), Vol 7, p. 667. Academic Press, New York.

103. Poulton, J. E., Hahlbrock, K. & Grisebach, H. (1977) Arch. Biochem. Biophys., 180: 543.

104. Sutfeld, R. & Wiermann, R. (1978) Biochem. Physiol. Pflanzen, 172: 111.

105. Ebel, J., Hahlbrock, K. & Grisebach, H. (1972) Biochem. Biophys. Acta, 268: 313.

106. Jonsson, L. M., Aarsman, M. E., Schram, A. W. & Bennink, G. J. (1982) Phytochemistry, 21: 2457.

107. Wengenmayer, H. Ebel, J. & Grisebach, H. (1974) Eur. J. Biochem., 50: 135.

108. Kuroki, G. & Poulton, J. E. (1981) Z. Naturforsch., 36C: 916.

109. Tsang, Y. F. & Ibrahim, R. K. (1979) Phytochemistry, 18: 1113.

110. Tsang, Y. F. & Ibrahim, R. K. (1979) Z. Naturforsch., 34C: 46.

111. Knogge, W. & Weissenboch, G. (1984) Eur. J. Biochem., 140: 113.

112. Jay, M. De Luca, V. & Ibrahim, R. K. (1985) Eur. J. Biochem., 153: 321.

113. Brunet, G., Saleh, N. A. M. & Ibrahim, R. K. (1978) Z. Naturforsch., 33C: 786.

114. Brunet, G. & Ibrahim, R. K. (1980) Phytochemistry, 19: 741.

115. De Luca, V. & Ibrahim, R. K. (1982) Phytochemistry, 21: 1537.

116. De Luca, V. & Ibrahim, R. K. (1985) Arch. Biochem. Biophys., 238: 596.

117. De Luca, V. & Ibrahim, R. K. (1985) Arch. Biochem. Biophys., 238: 606.

118. Hösel, W. (1981) In The Biochemistry of Plants: A Comprehensive Treatise (Conn, E. E., ed.), Vol 7, p.725. Academic Press, New York.

119. Ibrahim, R. K., Khouri, H. E., Brisson, L., Latchinian, L., Barron, D. & Varin, L. (1986) Bull. Liaison Groupe Polyphenols, 13: 3.

120. Sutter, A. & Grisebach, H. (1973) Biochim. Biophys. Acta, 309: 289.

121. Van Brederode, J., Van Nigtevecht, G. & Kamsteeg, J. (1977) Heredity, 35: 429.

122. Jourdan, P. S. & Mansell, R. L. (1982) Arch. Biochem. Biophys., 213: 434.

123. Bajaj, K. L., De Luca, V., Khouri, H. & Ibrahim, R. K. (1983) Plant Physiol., 72: 891.

124. Khouri, H. & Ibrahim, R. K. (1984) Eur. J. Biochem., 142: 559.

125. Robberecht, R. & Caldwell, M. M. (1978) Oecologia, 32: 277.

126. Grisebach, H. & Ebel, J. (1978) Angew. Chem., 50: 668.

127. Pusztai, R., Beladi, I., Bakai, M., Muysci, I. & Kukan, E. (1966) Acta Microbiol. Acad. Sci. Hung., 13: 113.

128. Freedman, L. & Merrit, A. (1963) Science, 139: 344.

129. Menkin, V. (1959) Amer. J. Physiol., 196: 1205.

130. Wilson, R., Booth, A. & De Eds, F. (1951) Proc. Soc. Exp. Biol. Med., 76: 540.

131. Hara, M. & Janku, I. (1958) Arch. Intl. Pharmacodyn., 118: 23.

132. Sejourne, M. Resplandy, G., Viel, C., Chenieux, J. C. & Rideau, M. (1986) Fitoterapia, 57: 121.

133. Gamborg, O. L., Miller, R. A. & Ojima, K. (1968) Exp. Cell Res., 50: 151.

134. Collins, F. W., De Luca, V., Ibrahim, R. K., Voirin, B. & Jay, M. (1981) Z. Naturforsch., 36C: 730.

135. Tahara, S., Hashidoko, Y., Ingham, J. L. & Mizutani, J. (1986) Agric. Biol. Chem., 50: 1809.

136. Cleland, W. W. (1963) In The Enyzmes (Boyer, P. D., ed.), Vol 2, p. 1. Academic Press, New York.

137. Cleland, W. W. (1963) Biochim. Biophys. Acta, 67: 104.

138. Cleland, W. W. (1963) Biochim. Biophys. Acta, 67: 173.

139. Cleland, W. W. (1963) Biochim. Biophys. Acta, 67: 188.

140. Segal, I. H. (1975) Enzyme kinetics. Wiley, New York.

141. Bradford, M. M. (1976) Anal. Biochem., 72: 248.

142. Recommendations of the International Union of Biochemistry (1973) Compar. Biochem., 13: 26.

143. Hansen, S. A. (1975) J. Chromatogr., 107: 224.

144. van den Berg, A. J. J. & Labadie, R. P. (1978) Planta Med., 33: 105.

145. Chromatofocusing with Polybuffer and Polybuffer Exchanger (1980) Pharmacia, Uppsala.

146. Sluyterman, L. A. AE. & Elgersma, O. (1978) J. Chromatogr., 150: 17.

147. Sluyterman, L. A. AE. & Wijdenes, J. (1981) J. Chromatogr., 206: 429.

148. Sluyterman, L. A. AE. & Wijdenes, J. (1981) J. Chromatogr., 206: 441.

149. FPLC Ion Exchange and Chromatofocusing (1985) Pharmacia, Uppsala.

150. Dewick, P. M. (1982) In The Flavonoids: Advances in Research (Harborne, J. B. & Mabry, T. J., eds.) p. 535. Chapman and Hall, London.

151. Ingham, J. L. (1983) Forsch. Chem. Org. Nat.-Stoffe, 43: 266.

152. Constabel, F., Stryluk, J. & Gamborg, O. (1971) Planta, 96: 306.

153. Tabata, M., Mizukami, H., Hiraoka, N. & Konoshima, M. (1976) 12th Phytochem. Symp. Japan, Kyoto, p.1.

154. Ibrahim, R. K. (1987) In Cell Culture and Somatic Cell Genetics of Plants (Constabel, F. & Vasil, I., eds.). Vol 4, p. 77. Academic Press, New York.

155. Di Cosmo, F. & Towers, G. H. N. (1984) In Recent Advances in Phytochemistry (Timmermann, B., Steelink, C. & Loewus, F., eds.). Vol 18, p. 95. Plenum Press, New York and London.

156. Ayabe, S., Yoshikawa, T., Kobayashi, M. & Furuya, T. (1980) Phytochemistry, 19: 2331.

157. Floss, H. G. (1976) Tetrahedron, 32: 873.

158. Smith, D. A. & Banks, S. W. (1986) Phytochemistry, 25: 979.

159. Firman, J. L., Wilson, K. E., Rossen, L. & Jonston, A. W. B. (1986) Nature, 324: 90.

160. Redmond, J. W., Batley, M., Djordjevic, M. A., Innes, R. W., Kuempel, P. L. & Rolfe, B. G. (1986) Nature, 323: 623.

161. Latchinian, L., Khouri, H. E. & Ibrahim, R. K. (1987) J. Chromatogr., 388: 235.

162. Stafford, H. A. (1981) In The Biochemistry of Plants (Conn, E. E., eds.). Vol 7, p. 188. Academic Press, New York.

163. Cutler, A. J. & Conn, E. E. (1982) Rec. Adv. Phytochem., 16: 249.

164. Gaertner, F. H. & Cole, K. W. (1977) Biochem. Biophys. Res. Commun., 75: 259.

165. Luckner, M., Dietrich, B. & Lerbs, W. (1977) In Progress in Phytochemistry (Reihold, L., Harborne, J. B. & Swain, T., eds.). Vol 6, p. 103. Pergamon Press, London.

166. De Luca, V. (1984) Enzymology of Flavonoid Methylation, Ph.D. Thesis, Concordia University, Montreal.

167. Khouri, H. E. (1983) Purification, Characterization and Kinetic Analysis of a Flavonol-Ring B O-Glucosyltransferase from Chrysosplenium americanum, M.Sc. Thesis, Concordia University, Montreal.

168. Harris, N. (1986) Annu. Rev. Plant Physiol., 37: 73.

169. Chrispeels, M. J. (1980) In The Biochemistry of Plants (Talbert, N. E., eds.). Vol. 11, p. 39. Academic Press, New York.

170. Tsukuda, M. & Tabata, M. (1984) Planta Med., 51: 338.

171. Brisson, L., Vacha, W. E. K. & Ibrahim, R. K. (1986) Plant Sci., 44: 169.

172. Marchand, L. Charest, P. M. & Ibrahim, R. K. (1987) J. Plant Physiol., 131: 339.

173. Charest, P. M., Brisson, L. & Ibrahim, R. K. (1987) Protoplasma, 134: 95.

174. Ibrahim, R. K., De Luca, V., Khouri, H. E., Latchinian, L., Brisson, L. & Charest, P. M. (1987) Phytochemistry, 26: 1237.

I. APPENDICES

Appendix I: Inorganic macronutrient components of selected culture media (mg/l) [1].

Constituents	MS	B5	White's	Heller's
KCl	-	-	65	750
$NaNO_3$	-	-	-	600
$MgSO_4.7H_2O$	370	250	720	250
$NaH_2PO_4.H_2O$	-	150	16.5	125
$CaCl_2.2H_2O$	440	150	-	75
KNO_3	1900	2500	80	-
Na_2SO_4	-	-	200	-
$(NH_4)_2SO_4$	-	134	-	-
NH_4NO_3	1650	-	-	-
KH_2PO_4	170	-	-	-
$Ca(NO_3).4H_2O$	-	-	300	-

[1] Adapted from Yeoman, M. M. & Macleod, A. J. (1972) In Plant Tissue and Cell Culture (Street, H. E., ed.); Vol 11, p. 31. University of California Press, Berkeley and Los Angeles.

Appendix II: Inorganic micronutrient components of selected culture media (mg/l) [1].

Constituents	MS	B5	White's	Heller's
$FeSO_4.7H_2O$	27.8	27.3	-	-
$MnSO_4.H_2O$	-	10.0	-	-
$MnSO_4.4H_2O$	22.3	-	7	0.01
KI	0.83	0.75	0.75	0.01
$NiCl_2.6H_2O$	-	-	-	0.03
$CoCl_2.6H_2O$	0.025	0.025	-	-
$ZnSO_4.7H_2O$	8.6	2.0	3	1.0
$CuSO_4.5H_2O$	0.025	0.025	-	0.03
H_3BO_3	6.2	3.0	1.5	1.0
$FeCl_3.6H_2O$	-	-	-	1.0
$Na_2MoO_4.2H_2O$	0.025	0.025	-	-
$AlCl_3$	-	-	-	0.03
$Fe(SO_4)_3$	-	-	2.5	-

[1] See foot note of Appendix I.

Appendix III: Organic constituents of selected culture media (mg/l) [1].

Constituents	MS	B5	White's	Heller's
Glycine	2	-	3	-
Myo-inositol	100	100	-	-
Cysteine	-	-	1.0	-
Vitamine B_1	0.1	10	0.1	-
Vitamine B_6	0.5	1.0	0.1	1.0
Nicotinic acid	0.5	-	0.5	-
EDTA (disodium salt)	37.3	37.3	-	-
Ca D-pantothenic acid	-	-	1.0	-

[1] See foot note of Appendix I.