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# BIOCHEMICAL AND IMMUNOLOGICAL ASPECTS OF O-GLUCOSYLTRANSFERASES INVOLVED IN FLAVONOID GLUCOSIDE BIOSYNTHESIS

Lilian Latchinian

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## ABSTRACT

BIOCHEMICAL AND IMMUNOLOGICAL ASPECTS
OF O-GLUCOSYLTRANSFERASES
INVOLVED IN FLAVONOID GLUCOSIDE BIOSYNTHESIS

Lilian Latchinian, Ph.D.
Concordia University, 1990

Chrysosplenium americanum (Saxifragaceae) is known to contain a flavonol O-glucosyltransferase (UDP-glucose:flavonol 2'-/5'-O-glucosyltransferase, E.C. 2.4.1.-) which attacks the 2'- or 5'-positions of partially methylated flavonols. Both enzyme activities could not be resolved by conventional and fast protein liquid chromatography on gel filtration, ion-exchange or chromatofocusing columns, suggesting similar chromatographic properties. However, the combined use of UDP-glucuronic acid-agarose and a dye-ligand affinity support (reactive brown 10-agarose), with a fast protein liquid chromatographic system, allowed the separation of the two flavonol-ring B-specific O-glucosyltransferases. Both enzyme activities were desorbed from the dye-ligand column by a pH-salt gradient, and exhibited position-specificity towards their respective substrates.

A monoclonal antibody to the 2'-O-glucosyltransferase was produced by in vitro immunization of spleen cells from Balb/c mice, followed by fusion with mouse myeloma cells. The majority of the immunoglobulin-producing hybridomas were IgM-secretors. Two of the IgM-producing clones, highly

immunoreactive with partially purified enzyme preparation, were expanded *in vitro* and further characterized by inhibition studies and immunoblotting. The culture supernatant of one of these clones inhibited the 2'- but not the 5'-*O*-glucosyltransferase. The native form of the 2'-enzyme was essential for recognition, thus suggesting that the epitope recognized by the antibody is a conformational discontinuous one. Attempts to use this antibody for localization studies were not successful.

The flavonol 2'-/5'-*O*-glucosyltransferases were localized in the leaves of *C. americanum* by applying a post-embedding immunogold labeling technique. The 2'-/5'-enzymes were co-purified to apparent homogeneity and used to produce polyclonal antibodies. *In situ* localization studies of the enzymes revealed the deposition of gold particles in the periplasmic region of epidermal and mesophyll cells. The gold label was mainly associated with vesicle-like structures, as well as with the poorly preserved plasmalemma and accompanying cytoplasmic membranes. These vesicles and membranes most probably originated from the endoplasmic reticulum, since the Golgi apparatus was free from labeling. These results seem to indicate that the site of *O*-glucosylation is associated with the endoplasmic reticulum membrane, and support a previously proposed model (Ibrahim *et al*. *Phytochemistry* 26:1237, 1987) for flavonoid biosynthesis in this plant.

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

To my husband Hossam for his truthful love, patience and assistance.

To my baby boy Jack Sam Jr. who is my inspiration.

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To my sister Nairy and her family for their consistent help and support.

To the memory of my father Hagop who would have been real happy this day.

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# ABBREVIATIONS

| | |
|---|---|
| Ab | Antibody |
| Ag | Antigen |
| Bis-acrylamide | N,N'-Methylene*bis*acrylamide |
| Bis-Tris | *Bis*-(2-hydroxyethyl)imino-*tris*-(hydroxymethyl)methane |
| BSA | Bovine serum albumin |
| CHS | Chalcone synthase |
| CoA | Coenzyme A |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| EM | Electron microscopy |
| FBS | Fetal bovine serum |
| FPLC | Fast protein liquid chromatography |
| GT | *O*-Glucosyltransferase |
| HAT | Hypoxanthine-aminopterin-thymidine |
| HR | High resolution |
| I.D. | Internal diameter |
| Ig | Immunoglobulin |
| IMDM | Iscove's modified Dulbecco's medium |
| kD | Kilodalton |
| mAb | Monoclonal antibody |
| OH | Hydroxylase |
| OMT | *O*-Methyltransferase |
| PAGE | Polyacrylamide gel electrophoresis |

| | |
|---|---|
| PAL | Phenylalanine ammonia lyase |
| PEG | Polyethylene glycol |
| pI | Isoelectric point |
| SDS | Sodium dodecyl sulfate |
| TLC | Thin layer chromatography |
| Tris | Tris(hydroxymethyl)aminomethane |
| UDP | Uridine 5'-diphosphate |
| UDP-Glc | Uridine 5'-diphospho-glucose |

## A. INTRODUCTION

Compounds common to all organisms such as carbohydrates, lipids, proteins, and nucleic acids are known as primary metabolites since they are central to the chemistry of life processes (growth, development, reproduction and survival) (1). In addition to these primary metabolites, most plants and microorganisms synthesize such compounds as alkaloids, phenylpropanoids, quinones, flavonoids, steroids, tannins, lignins, and volatile oils. These metabolites are usually referred to as "secondary plant products" or "natural products" since they are not implicated in the vital metabolic processes of the organism that produces them (1,2). However, there are no sharp lines dividing primary and secondary metabolism. Primary metabolism provides a number of building blocks, such as shikimic acid, amino acids and acetate, which are utilized as precursors for secondary metabolic pathways (1,3,4).

Most plants, unlike animals, lack the ability to move and must have evolved alternative means for defense and survival. The synthesis of secondary compounds may, therefore, serve to deter predators, compete with other plant species, or attract pollinators (5,6). Secondary metabolites are, therefore, very essential to plant life because they provide a defense mechanism comparable to the immune system of animals (7). Furthermore, phytohormones such as auxin, cytokinins, abscisic

acid and gibberellins may be considered secondary metabolites that are mainly concerned with the regulation of plant growth processes (7).

Apart from their ecological role in plant-vertebrate, plant-insect, plant-plant, and plant-microbe interactions (5), secondary products are useful to man and other animals. Hence, plants have been used as good sources of essential vitamins and pharmacologically active drugs (7).

The increase in the number of identified secondary plant products has been the result of the recent advances in chromatographic techniques. Tracer experiments, on the other hand, allowed the elucidation of the biosynthetic pathways of most secondary products which, in turn, was followed by the study of their enzymology. Compartmentation of these metabolites and the enzymes involved in their biosynthesis has provided another field of research (8).

Secondary plant metabolites may be toxic to the organism synthesizing them, therefore, they are usually sequestered into compartments surrounded by membranes which isolate them from the protoplasm. Alternatively, they may be detoxified by conjugation with other groups. Hence, the accumulation of these products depends on the co-evolution of structural or biochemical systems for their safe storage.

In most instances, synthesis of secondary plant products requires multistep reactions. The complexity of the whole process of secondary metabolism becomes apparent if we

consider the numerous enzymes or multienzyme complexes involved (9). Consequently, control mechanisms are essential for the regulation of secondary metabolite synthesis (8). These control mechanisms include the synthesis and degradation of enzymes, regulation of their activity, and compartmentation in relation to their precursors, intermediates and end products within cells and tissues. Furthermore, the understanding of these mechanisms requires a good knowledge of the biosynthetic pathways as well as the enzymology of secondary metabolism.

Flavonoids, a group of plant secondary metabolites, are widely distributed in higher plants. Their biosynthesis, enzymology and localization have been the subject of a number of reviews (10-14). Furthermore, the biological and medicinal properties of these metabolites have received special attention (15). However, the complete picture of all the enzymes involved in the biosynthesis of flavonoids as well as the compartmentation of these enzymes/enzyme systems is still far from clear.

In this laboratory, Chrysosplenium americanum, a semi-aquatic plant which accumulates a variety of polymethylated flavonol O-glucosides, provided an ideal system to study the enzymes involved in later steps in the biosynthesis of these compounds (for review see refs. 12,13). The site of accumulation of these flavonol metabolites within the plant tissues was also examined (for review see ref. 13).

Nevertheless questions concerning the specificity of some enzymes in the biosynthetic pathway as well as their localization required further investigation.

## B. REVIEW OF LITERATURE

### B.1. Flavonoid structure

The chemical structure of flavonoids is based on a $C_{15}$ skeleton consisting of a chroman ring bearing a second aromatic ring (ring B) at position 2 (most flavonoids), 3 (isoflavonoids), or 4 (neoflavonoids or 4-phenylcoumarins) (Fig. 1). Flavonoids are classified according to the oxidation state of the pyran ring C, as well as its substitution pattern. Examples of the major classes of flavonoids are shown in Fig. 2.

Flavonoids occur either as free aglycones or as conjugates, ranging from structures which are lipophilic, to those which are water soluble. Hydroxylation patterns of rings A and B (Fig. 1), and their O-methylation lead to various aglycone structures within each flavonoid subgroup. An almost complete list of flavone and flavonol aglycones with hydroxyl and/or methoxyl substitution has recently been published (16). Acylated flavone- and flavonol aglycones are rather rare in nature (16). Other frequent modifications of the flavonoid skeleton are due to glycosylation of hydroxyl groups (flavonoid O-glycosides) and to acylation of these sugar moieties (acylated flavonoid O-glycosides) (17). Less frequent modifications include, C-glycosylation (flavonoid C-glycosides) (18), C-methylation (C-methylflavonoids) (16), and O-sulfation (flavonoid O-sulfates) (19).

Figure 1. Basic structure of most flavonoids.

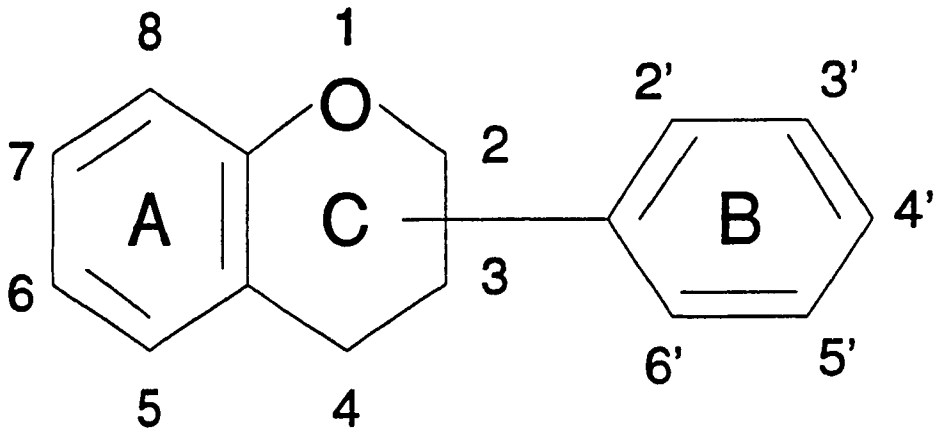
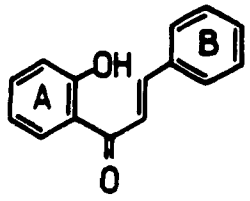
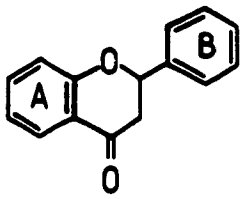

8
1
2'
3'
O
7
2
A
C
B
4'
6
3
6'
5'
5
4

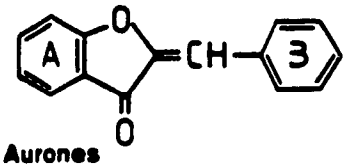
Figure 2. The major classes of flavonoids.



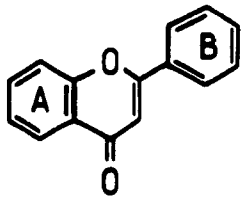

Chalcones



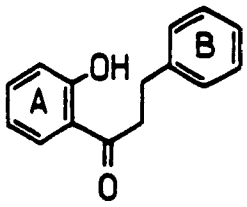

Flavanones



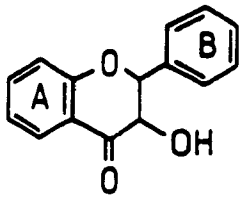

Aurones



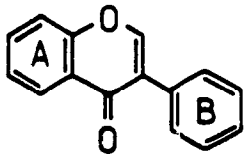

Flavones



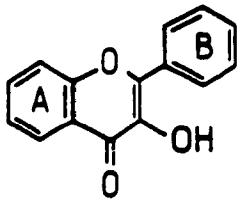

Dihydrochalcones



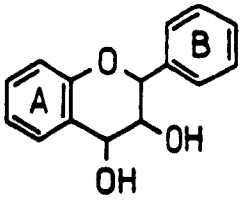

Dihydroflavonols



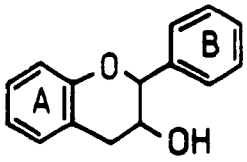

Isoflavones



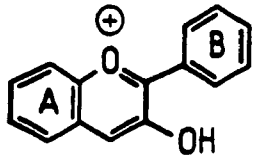

Flavonols




Flavan-3,4-diols
(Leucoanthocyanidins)




Flavan-3-ols
(Catechins)




Anthocyanidins

## B.2. Occurrence, distribution and significance of flavonoids

Flavonoid compounds constitute one of the most ubiquitous classes of natural plant products. At the latest count, over 4000 flavonoids have been identified in vascular plants (17,20). They occur in all plant parts including seeds, stems, roots, leaves, flowers, fruits, wood and bark. Many plant species contain complex flavonoid mixtures of up to 50 structures and the flavonoid pattern may vary in different tissues and within tissues (20). The major flavonoids usually occur in higher plants and to a lesser extent in lower plant groups (21).

Different classes of flavonoids vary in their biological functions (22). For example, anthocyanins serve as flower and fruit pigments; flavonols act as feeding deterrents in leaves, and as co-pigments in flowers. Flavones (23,24) and chalcones (25) have recently been reported as extracellular signal molecules for the activation of nodulation genes in nitrogen fixing bacteria. Several other flavonoids have been shown to block the induction of nodulation genes by flavones (26). Isoflavonoids are known to act as phytoalexins in response to microbial attack in members of the Leguminoseae (27,28). In addition, a group of flavonoids have recently been reported to act as regulators of polar auxin transport in a variety of plant tissues (29).

The intracellular levels of flavonoids may vary greatly during different stages of plant development (30). The

accumulation of these metabolites in the epidermal layers of young green leaves (31) was suggested to function as a protective screen against uv radiation. This is supported by the fact that flavonoids absorb strongly in the 265 and 340 nm regions, and their synthesis can be induced by uv light (10).

Apart from their roles in plant growth, development and defense, flavonoids are natural constituents of dietary substances which may affect humans and other animals. Different flavonoids have been reported to have antiallergic, antiviral, antiinflammatory and anticarcinogenic properties which may lead to applications in the treatment of various diseases. In fact, flavonoids constitute the active ingredient of many "folk" medicines, including rutin (vitamin P) still being used in various parts of the world. The biological as well as the medicinal properties of flavonoids have recently been reviewed (15).

## B.3. Biosynthesis of flavonoids

Our present knowledge of the field of flavonoid biosynthesis is based on earlier results from isotopic *in vivo* studies (for review see ref. 32) and from *in vitro* enzymic studies (for review see refs. 10,33). In the past few years, considerable progress has been made in the enzymology of flavonoid biosynthesis (for review see ref. 14). With few exceptions, the essential steps of the biosynthetic pathway of major flavonoid classes are now clear. It is well established

that all classes of flavonoids derive their carbon skeleton from intermediates of cell metabolism through two consecutive pathways namely, the general phenylpropanoid (group I enzymes) and flavonoid (group II enzymes) pathways (Fig. 3).

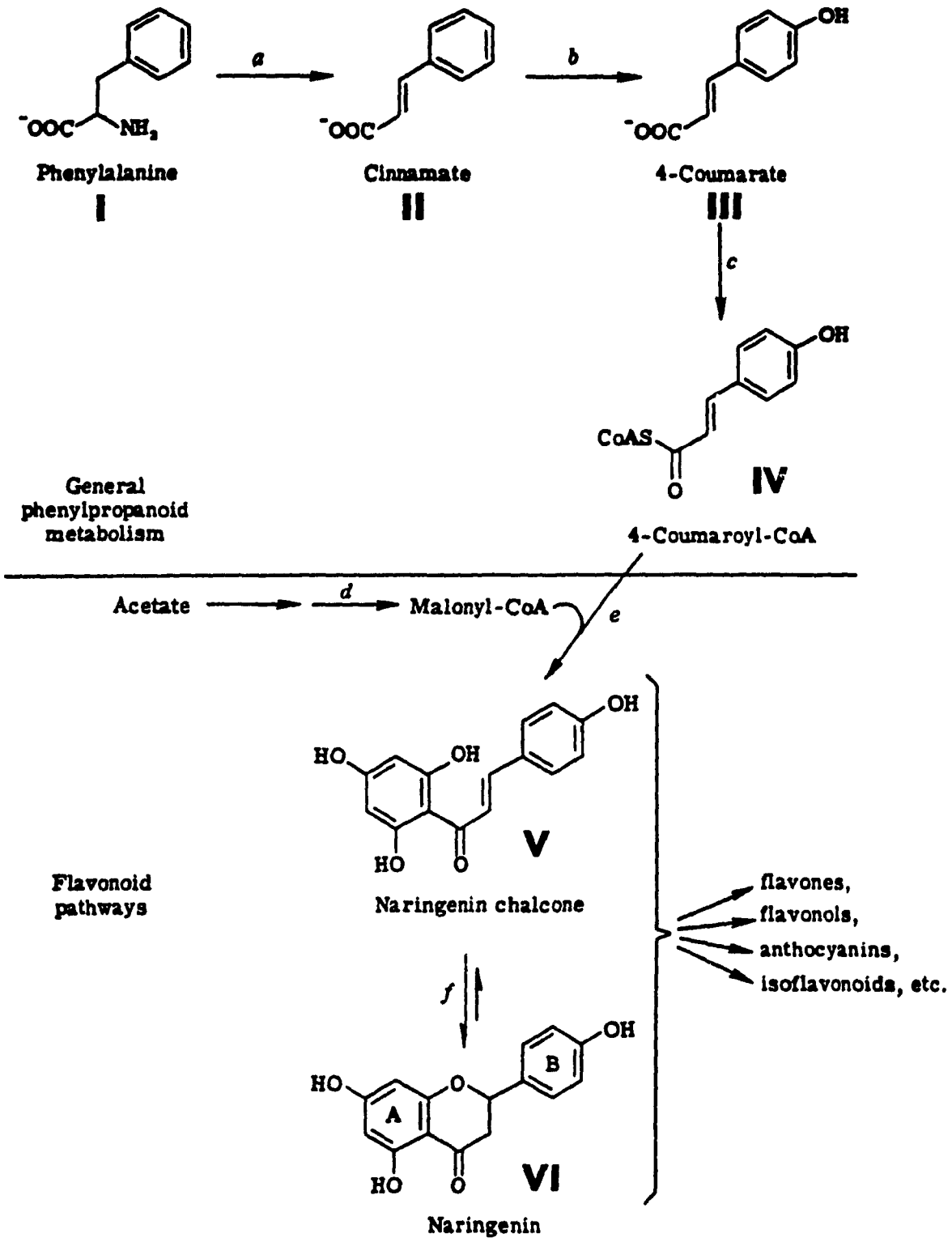
The general phenylpropanoid pathway is shared with some other biosynthetic routes (for review see ref. 34), and it will be briefly mentioned in the first part of this section. Chalcone synthase (CHS), the first committed enzyme in flavonoid biosynthesis, and O-glycosyltransferase, a later enzyme in the pathway will be discussed in some detail. Other individual, enzyme-catalyzed steps of flavonoids and their modifications have been summarized in a number of reviews (10,14,35).

### B.3.1. Origins and pathways of flavonoid precursors

Earlier experiments using radioactively labeled precursors have established that the carbon skeleton of all flavonoids is derived from acetate and phenylalanine (Fig. 3). Ring A is formed by head-to-tail condensation of three acetate units derived from malonyl-coenzyme A (CoA), whereas phenylalanine gives rise to ring B and the three carbons of the heterocyclic ring C (Fig. 3). The results of these tracer studies have been summarized in several reviews (e.g. 32,36).

Both flavonoid precursors are derived from carbohydrate metabolism. Malonyl-CoA is formed from acetyl-CoA by the action of acetyl-CoA carboxylase (reaction d, Fig. 3), whereas

**Figure 3.** Common steps in the biosynthesis of flavonoids. (a) Phenylalanine ammonia lyase (PAL); (b) cinnamate 4-hydroxylase; (c) 4-coumarate:CoA ligase; (d) acetyl-CoA carboxylase; (e) chalcone synthase (CHS); and (f) chalcone isomerase (10).


Phenylalanine
I
Cinnamate
II
4-Coumarate
III
4-Coumaroyl-CoA
IV
General phenylpropanoid metabolism
Acetate
Malonyl-CoA
Flavonoid pathways
Naringenin chalcone
V
Naringenin
VI
flavones,
flavonols,
anthocyanins,
isoflavonoids, etc.

the second precursor, 4-coumaroyl-CoA (IV) originates from the shikimate/arogenate pathway. The latter constitutes the main route for the formation of the aromatic amino acids, phenylalanine and tyrosine (37). Phenylalanine (I) enters the general phenylpropanoid metabolism by the action of phenylalanine ammonia lyase (PAL, reaction a) and gives rise to trans-cinnamate (II) (Fig. 3). Aromatic hydroxylation of cinnamate by cinnamate 4-hydroxylase (reaction b) leads to the formation of 4-coumarate (III) which is further transformed to 4-coumaroyl-CoA (IV) by the action of 4-coumarate:CoA ligase (reaction c) (Fig. 3). Enzymes of the general phenylpropanoid metabolism as well as acetyl-CoA carboxylase have been reviewed (10,14). Moreover, the physiology and molecular biology of the general phenylpropanoid metabolism and its functional significance in relation to other branch pathways have recently been discussed (34).

### B.3.2. Chalcone synthase (E.C. 2.3.1.74)

The formation of the flavonoid $C_{15}$ skeleton by stepwise condensation of three units of malonyl-CoA with 4-coumarate-CoA (or related cinnamic acid esters) is catalyzed by the key enzyme, chalcone synthase (reaction e) (Fig. 3). The chalcones (e.g. naringenin chalcone, V), thus formed, are central intermediates in flavonoid biosynthesis.

For all CHSs tested from various plant sources, 4-coumaroyl-CoA (IV) was found to be the best substrate with

naringenin chalcone (V) as the immediate product (Fig. 3) (14). This indicated that further substitution of ring B in flavonoids takes place at the $C_{15}$ stage. The occurrence of specific flavonoid ring B-hydroxylases and -methyltransferases supports the latter reasoning.

CHS activity is developmentally controlled and is induced by different stimuli in various species thus leading to the accumulation of a vast array of distinct flavonoid end products. The regulation and structure of CHS genes in various plant species have recently been reviewed by Dangl et al. (38). The latter review includes examples of CHS induction in response to uv irradiation, fungal infection, elicitation, and wounding, as well as the developmental signals involved in early leaf development and floral morphogenesis.

Using immunological methods, the localization of CHS has been investigated at the tissue/subcellular level in several tissues (for review see ref. 39). In buckwheat hypocotyls, both the biochemical and immunological evidence seem to suggest that CHS is associated with the endoplasmic reticulum (ER) membranes (40). On the other hand, CHS in spinach leaves has been reported to be a cytosolic enzyme (41). It is necessary, therefore, to further investigate the localization of this key enzyme in other plant species in order to better understand its compartmentation within plant cells. Furthermore, it is possible that CHS is associated with different subcellular compartments in different plant species.

### B.3.3. O-Glycosylation of flavonoids

#### B.3.3.1. Physiological role of O-glycosylation

O- Glycosylation of flavonoid aglycones is believed to be a detoxification mechanism of the chemically reactive hydroxyl groups. This explains why glycosides, rather than free aglycones, accumulate in plant tissues (42). Furthermore, it is generally agreed that compounds which are hydrophilic in nature, due to the presence of glycosyl residues, are found soluble in the cellular vacuole. In fact, localization studies of flavonoid- as well as other phenolic glycosides indicated that these sugar conjugates are mainly sequestered in the vacuoles of epidermal cells (for review see ref. 11). On the other hand, flavonol glucosides which are partially hydrophilic (due to the presence of one glucosyl moiety), but mostly lipophilic (due to their high level of O-methylation) have recently been reported to accumulate within the walls of epidermal cells and, to a lesser extent, in mesophyll cell walls of Chrysosplenium americanum (13).

#### B.3.3.2. Nucleotide-activated sugars as glycosyl residue donors

Almost all enzymatic glycosylation reactions of flavonoids, as well as those of other secondary plant metabolites, are catalyzed by transferases utilizing nucleotide-activated sugars. The use of "low energy" sugar

donors, such as α-D-glucose-1-phosphate, failed to show any enzymatic activity (43). With few exceptions, UDP derivatives have proven to be the best glycosyl donors (42). Their adenine, cytosine or guanine analogs were shown to have little or no activity. The specificity of O-glycosyltransferases with respect to the sugar residue seemed to be very high, since no activity could be detected whenever the right UDP-sugar was replaced by other UDP-sugars (42).

### B.3.3.3. O-Glycosyltransferases

The transfer of the glycosyl moiety from nucleotide-activated sugars to the hydroxyl group of flavonoid acceptors is catalyzed by O-glycosyltransferases. O-Glycosylation has been considered to be a terminal step in flavonoid biosynthesis (32,44). However, glycosylation may be followed by acylation of preexisting sugar moieties with malonic acid (45-47), and to a lesser extent with aromatic acyl groups (48). Malonylation in parsley cell cultures has been reported to be catalyzed by position-specific malonyltransferases (49,50). O-Methylation of the 2'- and 5'-hydroxyl groups of partially methylated flavonol O-glucosides in C. americanum shoot tips has recently been shown to be the final step in flavonoid biosynthesis (51).

#### B.3.3.3.1. Purification, properties and localization

Except for a recent report (57), most flavonoid O-glycosyltransferases have only been partially purified using

one or more steps of conventional chromatography. However, the combined use of the recently introduced high resolution columns and high performance liquid chromatographic systems has allowed the purification (to apparent homogeneity) of a number of enzymes of secondary metabolism which occur in very low abundance.

Properties of O-glycosyltransferases from different sources have been summarized in a number of reviews (11,14,33,42). The molecular weight of most characterized flavonoid O-glycosyltransferases varies between 40,000 and 55,000, and their pH optima ranges between 7.0 and 9.0, with few exceptions. Various transferases respond differently to divalent cations; some do not show any requirement for such ions, whereas others are inhibited by them.

O-Glycosyltransferases were thought to possess broad substrate specificity (57a). However, recent reports tend to indicate their strict specificity towards flavonoid acceptors. In fact, there are several well-characterized O-glycosyltransferases with varying substrate-specificities (for review see refs. 11,14,33,42). All well-characterized flavonoid O-glycosyltransferases exhibit pronounced specificity not only with regard to the substrate, but also for a particular position on the flavonoid ring system, as well as for the sugar moiety.

Until recently, our knowledge of O-glycosylation of flavonoids has been restricted to positions 5 and 7 of ring A

and to position 3 of ring C of the flavonoid structure (Fig. 1). A number of flavonoid-specific O-glycosyltransferases have been reported to catalyze single or multiple (sequential) O-glycosylation steps of positions 3 and 7 of flavonols and flavones, 3 and 5 of anthocyanins, and 7 of isoflavones (for review see refs. 11,14,33,42). More recently, a novel flavonol ring B-specific O-glucosyltransferase from C. americanum has been reported to attack the 2'- or 5'-positions of partially O-methylated flavonols (52,53). However, it was not known, at the time, whether O-glucosylation of these two positions was catalyzed by one or two distinct enzymes. Moreover, a flavone 4'-O-glucuronyltransferase was partially purified and separated from two other 7-O-glucuronylating enzymes in the primary leaves of rye (54).

Novel flavanone 7-O-glucosyltransferases have recently been demonstrated in cell-free extracts derived from young leaves and fruits of Citrus mitis and C. maxima (55) as well as in the seedlings of grapefruit (C. paradisi) (56). In the former study, the flavanone 7-O-glucoside was reported to be further rhamnosylated at position 2" of the glucose upon addition of exogenous UDP-glucose and NADH to the crude extracts. In grapefruit seedlings, the flavanone-specific 7-O-glucosyltransferase was resolved from other O-glucosylating activities. This new enzyme was highly purified and exhibited strict specificity for the 7-position of the flavanones naringenin (VI, Fig. 3) and hesperetin (5,7,3'-trihydroxy-4'-

O-methoxyflavanone). It did not accept other flavone- or flavonol aglycones.

Very few studies have been carried out on the localization of flavonoid O-glycosyltransferases either at the tissue or subcellular level (58-60). These investigations seem to indicate that flavonoid O-glycosyltransferases are generally associated with the mesophyll and/or epidermal cells. At the subcellular level, the enzyme activity was found to be either cytosolic (58) or associated with ER (60). However, these studies were confined to subcellular fractionation of intact petal or protoplast preparations (58,60), and to manual dissection of intact leaves to epidermal and mesophyll layers (59). In addition, such techniques usually suffer from the lack of proper identification of tissues or cellular compartments associated with the enzyme activity due to the high risk of contamination. A more promising technique is the use of highly specific antibodies raised against the homogeneous enzyme protein. To date, almost all reports dealing with immunolocalization of the enzymes of secondary plant metabolism involved those of phenolic and flavonoid metabolism (39), however, none of them as yet has dealt with flavonoid O-glycosyltransferases.

## B.4. The *Chrysosplenium*-flavonoid system

O-Methylated flavonoids are widely distributed in the

plant kingdom. The genus Chrysosplenium (Saxifragaceae), however, is characterized by the presence of highly O-methylated flavonoids which are of restricted distribution in this family (61,62). Furthermore, several species of this genus have additional hydroxyl and/or methoxyl substituents at positions 6 and/or 2' (61-63). One member of this genus, C. americanum, accumulates a variety of tri- to penta-O-methylflavonol O-glucosides which are derived from 6- and/or 2'-hydroxyquercetin (see Fig. 4 for the structure of quercetin) (63). The enzymatic synthesis as well as the localization of these highly O-methylated flavonol compounds have been thoroughly investigated in this laboratory and will be reviewed in this section.

### B.4.1. Structure and proposed pathway for the biosynthesis of the major polymethylated flavonoids of C. americanum

The major flavonoid constituents which accumulate in C. americanum (A-F, Fig. 4) have been identified by UV, NMR and mass spectroscopy, as well as the analysis of their hydrolysis products (64). These compounds consist of two 2'-O-glucosides of partially methylated 2'-hydroxyquercetin (A, B, Fig. 4), and four 5'-O-glucosides of either partially methylated 6-hydroxyquercetin (quercetagetin) (C, F, Fig. 4), or its 2'-hydroxy derivative (E, F, Fig. 4). Tracer experiments using [2-$^{14}$C]cinnamate (II, Fig. 3) administered to young shoots labelled the six major methylated flavonoid O-glucosides (A-F,

Figure 4. Postulated pathway for the enzymatic synthesis of polymethylated flavonol O-glucosides in C. americanum. GT, O-glucosyltransferase; OH, hydroxylase; and OMT, O-methyltransferase (12).

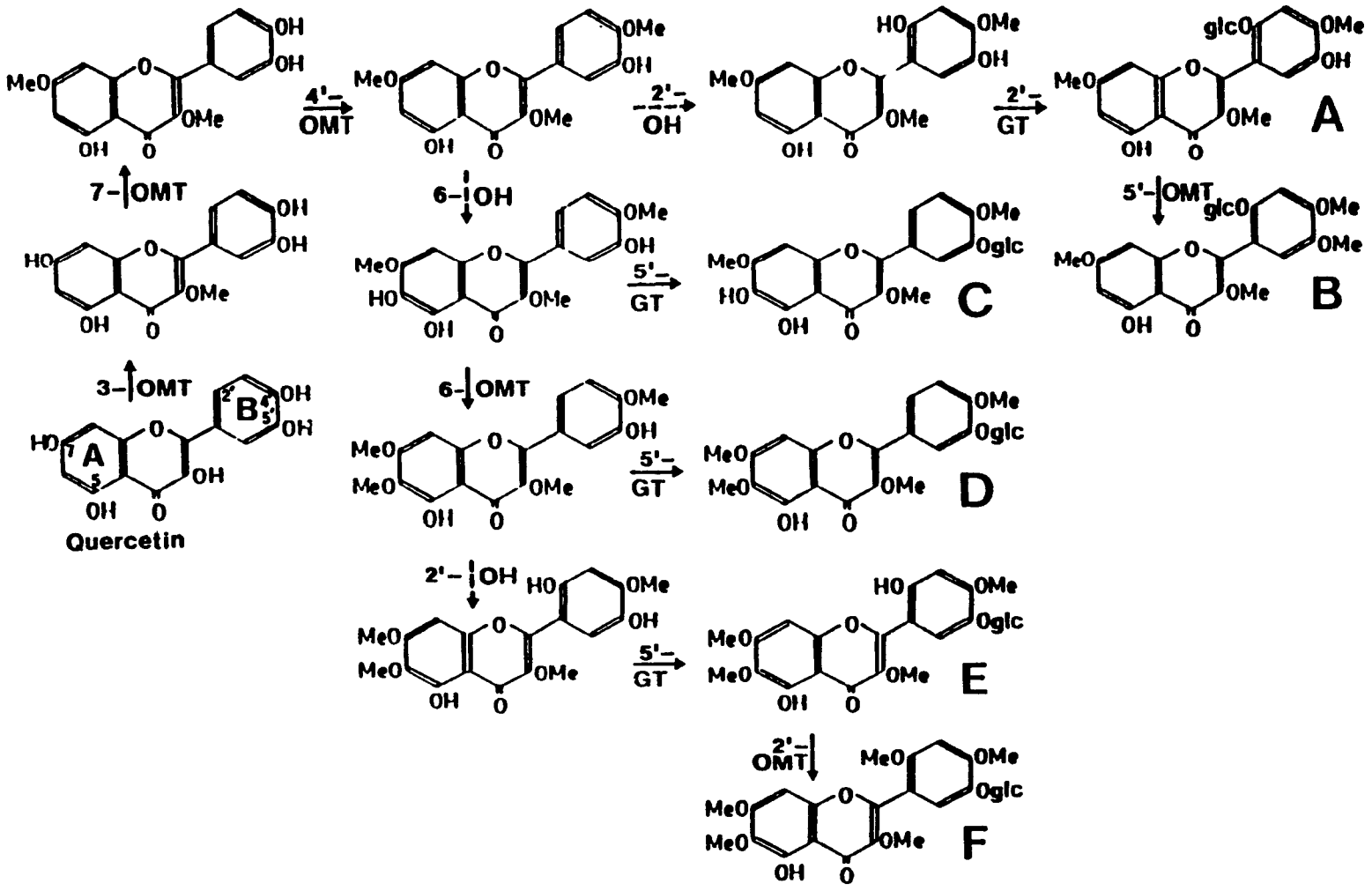

Quercetin
A
B
C
D
E
F
4'–OMT
2'–OH
2'–GT
7–OMT
6–OH
5'–GT
5'–OMT
3–OMT
6–OMT
2'–OH
2'–OMT

Fig. 4), but none of the lower methylated intermediates of the pathway (for review see refs. 12,13). The enzymatic synthesis of these lower intermediates could only be demonstrated in vitro using cell-free extracts and quercetin as substrate (64). These results suggested the existence in C. americanum of the enzyme complement necessary for the biosynthesis of these metabolites, and prompted the isolation and characterization of the individual enzymes of the proposed biosynthetic pathway (Fig. 4).

### B.4.2. Enzymatic O-methylation and O-glucosylation

Five distinct position-specific O-methyltransferases, namely 3-, 6-, 7-, 2-'/5'- and 4'-O-methyltransferases were highly purified from C. americanum shoot tips by applying conventional column chromatography alternatively with fast protein liquid chromatography (FPLC) (51,65-67). The different O-methyltransferases were resolved on the basis of the inherent differences in their apparent isoelectric points (pI) (66). The partially purified enzymes have been characterized (for review see ref. 12), and their kinetic constants as well as kinetic mechanisms, elucidated (67,68).

A novel O-glucosyltransferase which catalyzes the transfer of glucose from UDP-glucose to positions 2' or 5' of partially methylated flavonols has also been isolated from the shoot tips of C. americanum. The enzyme was partially purified (52) and its kinetic mechanism was investigated (53). The data

obtained has indicated the close similarity in both chromatographic (52) and kinetic (53) properties of the 2'- and 5'-O-glucosylating activities. However, in view of the high substrate- and position specificity of the O-methyltransferases in this tissue and of O-glycosyltransferases to their flavonoid acceptors in general, it was considered of interest to determine whether O-glucosylation of the 2'- and 5'-positions is catalyzed by one or two distinct enzymes.

### B.4.3. Localization of C. americanum flavonoids

Whereas the flavonoids of C. americanum possess some hydrophilic properties (due to the 2'-/5'-sugar moiety), they are quite lipophilic due to the presence of three to five methoxyl groups (A-F, Fig. 4). This dual solubility property prompted the study of their intracellular localization. The latter was achieved using three different techniques which involved electron microscopy, immunofluorescence and immunogold labeling (for review see refs. 12,13). The results obtained from these localization studies indicated that Chrysosplenium flavonoids were deposited within the walls of the epidermal and mesophyll cells of leaves. The physiological significance of the site of localization of these metabolites was consistent with their possible role in the defense mechanism against predators and/or protection against uv radiation.

In this laboratory, based on biosynthetic, enzymic and kinetic evidence, it has recently been postulated that the biosynthesis of flavonoids in C. americanum may take place on the surface of an aggregated, membrane-associated multienzyme system (12). Although this hypothetical model seems to corroborate with the ultrastructural and localization studies of the final metabolites, it must await unequivocal evidence for the compartmentation of the key enzymes involved in their biosynthesis.

## B.5. Immunochemistry

The binding reaction between an antibody (Ab) and its antigen (Ag) is sufficiently specific to allow their use as "molecular probes" for the detection of the corresponding Ags (macromolecules or haptens) either in situ or in vitro (69). Consequently, immunochemical methods have become widely used in plant as well as animal research for the isolation, identification and localization of proteins, polysaccharides and other metabolites. The sensitivity of immunochemical methods is such that the detection of individual Ags within plant/animal cells and tissues is now possible, for example, by immunocytochemistry (39,70,71).

The antigenic determinant of a protein (epitope) is the particular site on the surface of the Ag responsible for binding the Ab. Experiments with synthetic peptides, as well as with protein antigens, have revealed that the antigenic

site usually consists of only three to eight amino acid residues. These residues may form part of a continuous stretch of a peptide sequence (continuous antigenic site), or may be separated from each other in the primary protein sequences, although they are brought together in the native folded protein by protein conformation (discontinuous topographic antigenic site) (72,73). More recent studies have suggested that most, if not all, determinants are discontinuous to some extent (74).

A conventional antiserum (polyclonal) not only has Abs to several determinants, but also a family of Abs of different structure and avidity which compete for each individual determinant on the Ag molecule. Monoclonal Abs (mAb) produced by applying the hybridoma technology (75,76), on the other hand, are homogeneous because they are selected for their ability to bind to a single, unique determinant. The production and characterization of polyclonal and monoclonal Abs, their advantages and disadvantages, as well as their use in immunocytochemical labeling techniques will be briefly reviewed in this section.

### B.5.1. Production and characterization of antibodies

The principles and practice of eliciting polyclonal and monoclonal Abs have been described in many books and reviews (77-80). The process of producing polyclonal Abs is quite simple and standard. It requires nothing more than a pure Ag,

an adjuvant (for example, Freund's adjuvant), a suitable animal species, and an appropriate immunization protocol. The hybridoma technology, however, involves a considerable investment of effort, time and expense. The process is more complex and requires careful judgement before its adoption in preference to conventional antisera. A general procedure used by many laboratories for producing mAbs is shown in Figure 5a. Figure 5b compares *in vivo* immunization with the novel *in vitro* technique (79).

A critical step in the production of mAbs is the screening of hybridoma cultures for hybrids secreting useful Abs. This requires a rapid and sensitive assay to screen hundreds of hybrids. Various assays have been developed for screening (81) of which, the enzyme linked immunosorbent assay (ELISA) (82) is the most suitable, but it requires the use of pure Ag. Alternatively, biological assays are usually sensitive and accurate, although they can be very time consuming. For example, Abs to an enzyme may be identified by inhibition of its activity (83,84). Further characterization of mAbs may be accomplished by immunoblotting (Western blotting) (85) following nondenaturing- or SDS-polyacrylamide gel electrophor sis (PAGE). The above assay techniques are also applied to assess the specificity of conventional polyclonal antisera.

The fully characterized antibodies, whether monoclonal or polyclonal, can be purified by applying different

Figure 5a. The general procedure of monoclonal antibody production in mice (88).

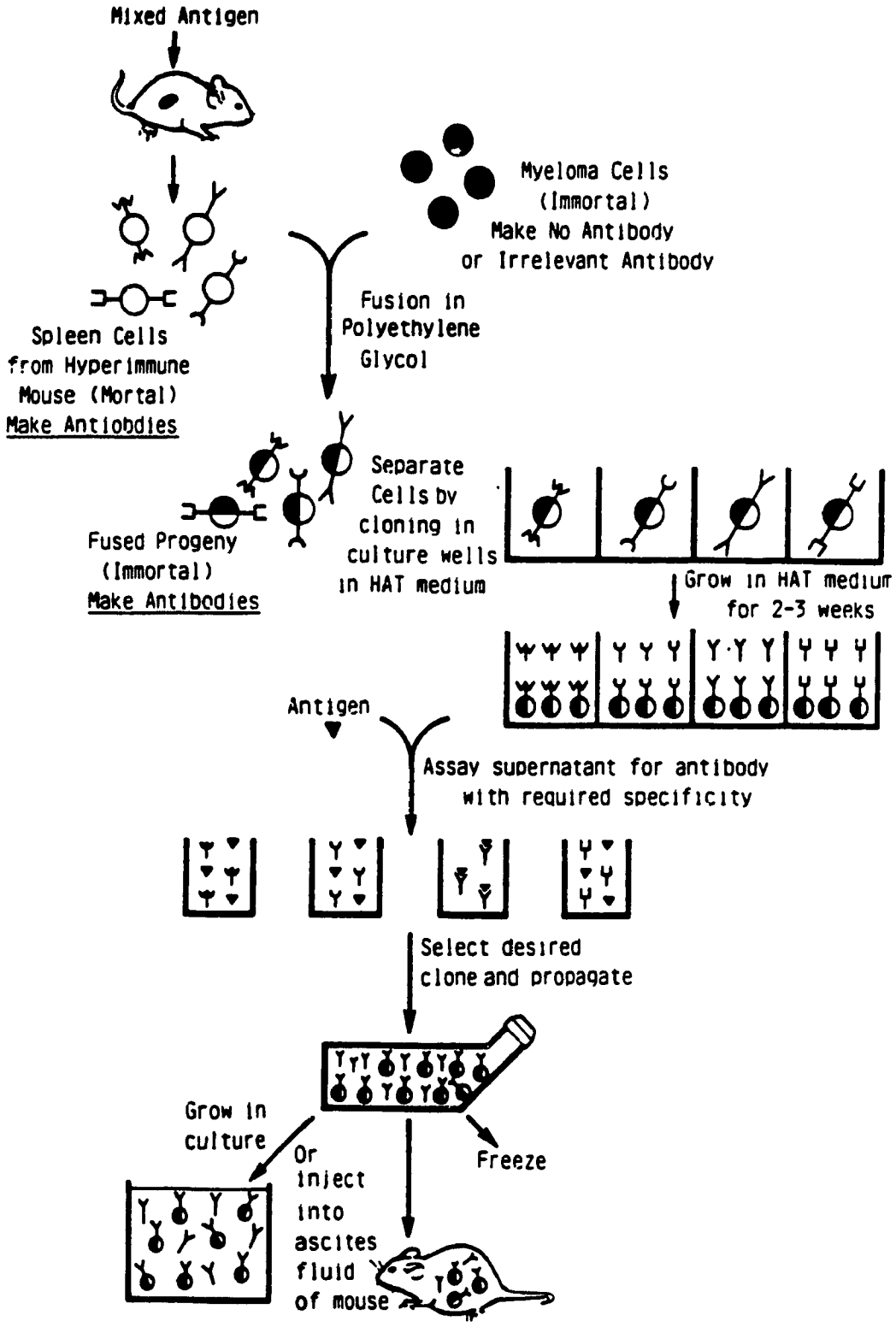
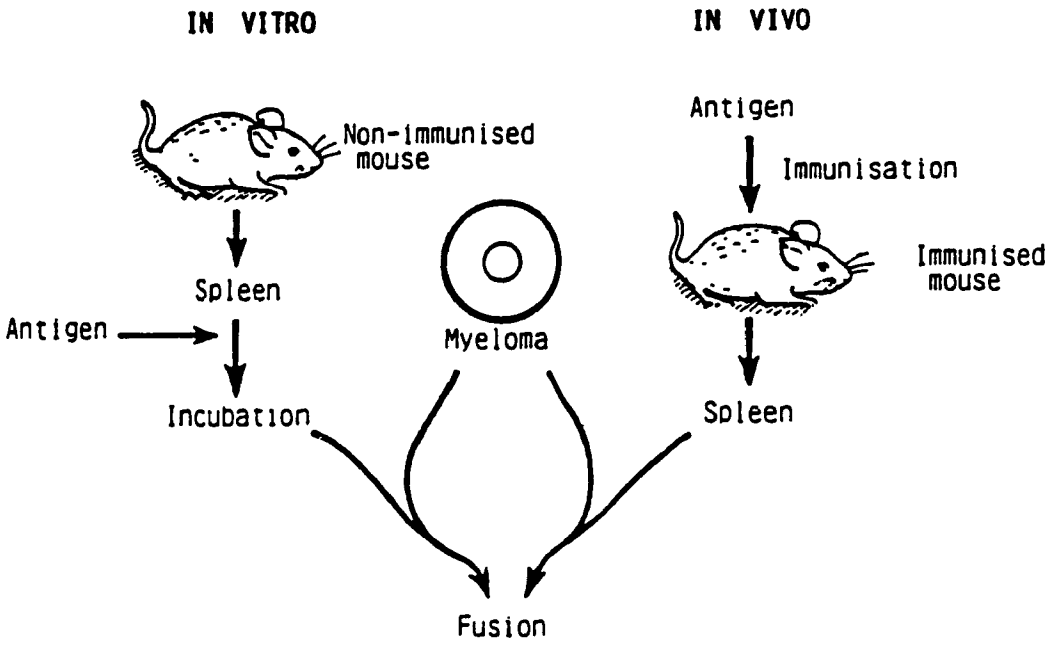

Mixed Antigen
Spleen Cells
from Hyperimmune
Mouse (Mortal)
Make Antibodies
Myeloma Cells
(Immortal)
Make No Antibody
or Irrelevant Antibody
Fusion in
Polyethylene
Glycol
Fused Progeny
(Immortal)
Make Antibodies
Separate
Cells by
cloning in
culture wells
in HAT medium
Grow in HAT medium
for 2-3 weeks
Antigen
Assay supernatant for antibody
with required specificity
Select desired
clone and propagate
Grow in
culture
Or
inject
into
ascites
fluid
of mouse
Freeze

**Figure 5b.** A comparison of *in vivo* and *in vitro* immunization (79).


IN VITRO
IN VIVO
Non-immunised mouse
Antigen
Immunisation
Immunised mouse
Spleen
Antigen
Myeloma
Incubation
Spleen
Fusion

chromatographic techniques (86,87) in order to isolate the IgG (IgM) pool from culture supernatants, ascites fluids or crude sera.

### B.5.2. Polyclonal versus monoclonal antibodies: advantages and disadvantages

The choice between polyclonal and monoclonal Abs depends on the desired application and requires an understanding of the differences between conventional and monoclonal serology (Table 1) (88,89).

The specificity of polyclonal antisera is the result of a consensus of hundreds of different clones, which bind to antigenic determinants covering most of the external accessible surface of the Ag (73,90). As a consequence, small changes in the structure of the Ag usually have little, or no effect on polyclonal Ab binding. This is favourable whenever the antigenic structure is slightly changed due to denaturation, for example, during tissue processing for immunocytochemistry (70,91). In contrast, mAbs usually bind to a single and unique site on the Ag molecule, alteration of such site would affect the Ab binding ability of the Ag. Variation of the antigenic structure of proteins may result from a difference in one epitope and not the others, giving rise to highly similar but distinct molecules. A mAb specific to such an epitope is useful to distinguish between the two protein species; the same variation may be unrecognized when

**Table 1.** Comparison between conventional sera and monoclonal antibodies (88).

| | Conventional antiserum | Monoclonal antibody |
|---|---|---|
| Determinant | - Several | - Single |
| Specificity | - Variable with animal and bleed<br>- Partial cross-reactions with common determinants<br>- Seldom too specific | - Standard<br>- Unexpected cross-reactions may occur<br>- May be too specific for requirements |
| Affinity | - Variable with bleed | - May be selected during cloning |
| Yield of useful antibody | - Up to 1 mg/mL | - Up to 100 µg/mL in tissue culture<br>- Up to 20 mg/mL in ascitic fluid |
| Contaminating immunoglobulin | - Up to 100% | - None in culture<br>- 10% in ascitic fluid |
| Purity of antigen | - Either pure antigen or serum absorption | - Some degree of antigen purification desirable but not essential |
| Approximate minimum cost | - Usually below £100 | - Capital cost £10,000<br>- Running cost £10,000 p.a. |

dealing with polyclonal Abs.

One of the most important differences between conventional and monoclonal Abs lies in the extent of purity of the Ag used in the immunization (88). In the production of monospecific polyclonal antisera, it is a prerequisite to purify the antigen to electrophoretic homogeneity before immunizing the animal. On the other hand, mAb production does not require a pure Ag, since the desired Ab is selected from the different cell lines by an appropriate and specific method of screening. Thus, it is possible to produce a mAb to a minor component of a complex mixture of protein Ags which would be impossible or difficult to separate by protein purification techniques.

### B.5.3. Immunocytochemistry

The specificity of individual Abs can be used as an analytical tool to detect given epitopes in tissues and cells. The use of Abs as reagents/probes to specifically detect determinants within sections prepared for microscopy is the domain of immunocytochemistry (39,70,71). In this technique, the <u>in situ</u> visualization of the immune complex is accomplished by either a "direct" or an "indirect" method. The direct method involves the reaction between a labeled Ab and its cell-/tissue-bound Ag. In the indirect immunocytochemical technique, the primary Abs are used unlabeled and, after they have reacted with the corresponding Ag, the complex is

revealed by a secondary reagent. Protein A, protein G or anti-immunoglobulin (Ig) raised in another animal species are labeled with a marker and utilized as secondary reagents. Using tagged secondary Abs in the indirect labeling, enhances the sensitivity of the detection by several folds, due to the fact that each primary Ab contains several epitopes that can be bound by the labeled anti-Abs (71).

The most common markers employed in immunocytochemistry are either fluorescent labels (immunofluorescence labeling), enzymes (e.g. immunoperoxidase labeling) or colloidal gold particles (immunogold labeling). These labeling techniques as well as the initial preparative steps (fixation, tissue pretreatment, embedding, etc.) have been critically discussed in previous books and reviews (39,70,71,92).

In order to localize Ags at the subcellular level by electron microscopy (EM), the second Ab (or protein A or G) should be conjugated to an electron-dense material such as colloidal gold particles. The latter has been adopted for use in post-embedding assays (for review see refs. 91,93), but has also been used in pre-embedding assays (e.g. 94). Post-embedding labeling has the particular advantage of being a simple and reproducible routine assay method. In this procedure, tissues are fixed, dehydrated and embedded in plastic using protocols similar to those of conventional electron microscopy. The choice of embedding resin, however, represents a major compromise between structural preservation

and retention of antigenicity (39,70). Since specifically sized colloidal gold particles may be isolated by density gradient centrifugation (95), therefore, double- (96) or triple- (97) labeling experiments may be performed using two or three distinctly different sizes of gold probes, respectively. The use of colloidal gold as a probe has the following advantages: high electron density, accuracy of detection by EM, little tendency for non-specific binding, and amenability to quantitation. Consequently, immunogold labeling has been successfully applied to the localization of a large number of plant proteins and metabolites (for review see ref. 70) including those of secondary plant metabolism (for review see ref. 39).

## C. AIM OF WORK

This dissertation deals with some biochemical and immunological aspects of flavonol O-glucosyltransferase in Chrysosplenium americanum. The specific aim of this work is two-fold:

**a. Is O-glucosylation of the 2'- and 5'-positions of polymethylated flavonols catalyzed by one or two distinct enzymes?**

In our laboratory, a novel flavonol 2'-/5'-O-glucosyltransferase was partially purified from Chrysosplenium americanum tissues. Previous work on this enzyme indicated the close similarity in both chromatographic (52) and kinetic (53) properties of the 2'- and 5'-O-glucosylating activities. The novelty of O-glucosylation at the 2'- and 5'-positions deserved futher investigation. In view of the high substrate and position specificities of O-glucosyltransferases for their flavonoid acceptors (11,42), it was considered important to determine whether O-glucosylation of the para-oriented, 2'- and 5'-positions was catalyzed by one or two distinct enzymes. In this work, more recent and advanced chromatographic techniques such as: FPLC, affinity and dye-ligand chromatography were applied to answer the above question. The unique specificity of monoclonal Abs to their Ags provided another ideal tool to distinguish between these closely related proteins.

**b. What is the site of localization of O-glucosyltransferase in the leaves of C. americanum?**

Since the partially methylated flavonol O-glucosides of C. americanum were shown to accumulate mainly within the cell walls (13), it was considered important, therefore, to study the compartmentation of a later enzyme in the biosynthetic pathway, for example the O-glucosyltransferase, in relation to the final metabolites. Very few studies have been carried out on the localization of flavonoid O-glucosyltransferases (11), none of these have been at the subcellular level. Data obtained from previous studies were subject to debate, mainly because they utilized conventional fractionation techniques which suffered from a number of limitations (see Section B.3.3.3.1). Immunocytochemistry provides a reproducible and accurate means to localize enzymes. The technique makes use of the highly specific interaction between the Ag (enzyme) to be localized and its specific Ab. This approach was applied in this work to localize Chrysosplenium flavonol O-glucosyltransferase.

The results of the in situ localization of the O-glucosyltransferases would be discussed in view of a previously proposed model for the site of flavonoid biosynthesis in this plant tissues (12).

## D. MATERIALS AND METHODS

### D.1. Plant material

Chrysosplenium americanum Schwein ex Hooker (Saxifragaceae) (Fig. 6) was collected from St. Anicet, Province of Québec. This semi-aquatic plant was maintained in a separate compartment in the green house under controlled conditions simulating its natural habitat with respect to light, temperature, and humidity. The compartment was equipped with a netting in order to decrease the light intensity. The temperature was regulated by an automatic cooling unit which maintained the temperature between 15 and 20°C at all times. The plants were grown in flat plastic trays (6 x 25 x 36 cm) filled with potting soil-peat moss mixture and watered with a controlled running stream to near flooding.

### D.2. Chemicals and equipment

UDP-[U-$^{14}$C]Glc (specific activity ca. 292 mCi/mmole) and reference mouse serum were purchased from ICN Biomedicals (Costa Mesa, CA). UDP-agarose, UDP-glucuronic acid-agarose, reactive brown 10-agarose, o-phenylenediamine, 4-chloro-1-naphthol, Polyclar AT, adjuvant peptide, Iscove's modified Dulbecco's medium (IMDM), Freund's complete and incomplete adjuvants, goat anti-rabbit IgG-gold, and protein A-gold were from Sigma (St. Louis, MO). Sephadex G-25, Sephacryl S-200 and Superose 12 (prep grade) chromatographic media, prepacked

**Figure 6.** Photograph of Chrysosplenium americanum. The plant was maintained in the green house under conditions that have been controlled for low light, low temperature, and high humidity.

Superose 12 (analytical), Mono P and Mono Q columns, C 10/10 and HR 16/50 columns, Polybuffer 74, and the FPLC system (see below) were obtained from Pharmacia (Uppsala, Sweden). Stirred cell ultrafiltration systems, PM30 membrane, and dye-ligand chromatography Matrex Gel media were purchased from Amicon (Danvers, MA). Polyethylene glycol (PEG 4000) was from Merck (Darmstadt, Germany). Dowex 1-X2, protein reagent, Mini-Protean II dual slab cell, Mini Trans-Blot cell, nitrocellulose membrane, and all electrophoresis reagents were from Bio-Rad (Richmond, CA). Lowicryl K4M was from Chemische Werke Lowi (Waldkraiburg, Germany) and LR White was from Analychem Corp. Ltd. (Markham, Ontario). HAT, glutamine, sodium pyruvate, antibiotic-antimycotic solution, and tissue culture flasks were obtained from Gibco/BRL (Burlington, Ontario). Affinity purified goat anti-mouse/rabbit IgG/IgM, unlabeled or labeled with peroxidase, were purchased from Kirkegaard & Perry Labs. (Gaithersburg, MD). EL308 microplate reader was from Bio-Tek (Burlington, VT). Fetal bovine serum (FBS) and tissue culture plates were obtained from Flow Labs. (Mississauga, Ontario) and Immulon-2 plates were from Dynatech Labs. (Alexandria, VA). The flavonoid compounds were from our laboratory collection. All other chemicals and solvents were of analytical grade reagents.

### D.3. Animals

Balb/c and CD1 mice, 6 to 8 weeks old, were obtained from

Charles River Canada Inc. (St. Constant, Québec). New Zealand white rabbits, 2-2.5 kg, were from Laka Biological Science Supplies (St. Basile-le-Grand, Québec).

**D.4. Myeloma cell line**

Mouse non-Ig producing myeloma cell line P3X63 Ag8.653 (98) was kindly provided by Dr. J.M. Leclerc, St. Justine Hospital, Montréal, Québec.

**D.5. Buffers**

The following buffers were used: (A) 0.2 M Tris-HCl (pH 7.5) containing 14 mM 2-mercaptoethanol, 5 mM EDTA, and 10 mM diethylammonium diethyldithiocarbamate; (B) 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol; (C) 25 mM bis-Tris-iminodiacetic acid (pH 7.0) containing 14 mM 2-mercaptoethanol; (D) Polybuffer 74-iminodiacetic acid (diluted with water 1:10, v/v) (pH 4.0) containing 14 mM 2-mercaptoethanol; (E) 0.2 M Tris-HCl (pH 8.0) containing 14 mM 2-mercaptoethanol; (F) 25 mM imidazole (pH 6.4) containing 14 mM 2-mercaptoethanol; (G) 25 mM imidazole (pH 8.0) containing 0.2 M potassium chloride and 14 mM 2-mercaptoethanol; and (H) 20 mM Tris-HCl (pH 7.4).

**D.6. Preparation of crude plant extract**

All extraction steps were carried out at 4°C. C. americanum shoot tips (10 to 100 g), comprising terminal buds

and leaves from the first two nodes, were homogenized in presence of Polyclar AT (10%, w/w) with buffer A (1:3, w/v). The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 10,000 g for 15 min. The supernatant was collected and gently stirred for 20 min with Dowex 1-X2 (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 fraction which precipitated between 35 and 70% salt saturation was collected by centrifugation at 15,000 g for 20 min.

### D.7. Enzyme purification

All purification steps were performed at 4°C.

#### D.7.1. Fast protein liquid chromatography (FPLC) system

The 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 REC-482 chart recorder, a pH monitor with a flow-through electrode, and a Frac 100 fraction collector. All buffers used with the system were filtered through 0.22 μm Millipore filter and degassed under vacuum.

#### D.7.2. Gel filtration

The 35-70% ammonium sulfate pellet was suspended in a minimal volume of buffer B and subjected to gel filtration either on Sephadex G-25 (160 x 25 mm I.D.), Sephacryl S-200

(940 x 24 mm I.D.), Superose 12 (prep grade) HR 16/50 (500 x 16 mm I.D.), or Superose 12 (analytical) HR 10/30 (300 x 10 mm I.D.) columns depending on the purification protocol that will follow. The columns were preequilibrated and developed with buffer B. In the case of Sephadex G-25 column, the unfractionated desalted protein peak was collected by monitoring protein absorbance at 280 nm. Superose 12 columns, analytical and prep grade, were run at a flow rate of 0.5 mL/min using the FPLC system, and 1- and 3-mL fractions were collected, respectively. The Sephacryl S-200 column was developed overnight at a flow rate of 0.45 mL/min and 5.5-mL fractions were collected. The fractions collected were assayed for O-glucosyltransferase activity.

**D.7.3. Ion-exchange chromatography**

Desalted protein was subjected to ion-exchange chromatography on Mono Q HR 5/5 (50 x 5 mm I.D.) column which had previously been equilibrated with buffer B. The column was washed with five bed volumes of the same buffer to eliminate all unbound proteins. The bound proteins were eluted using a linear salt gradient of 0-0.5 M potassium chloride in buffer B. The flow rate was adjusted to 0.5 or 1 mL/min and 1-mL fractions were collected and assayed for O-glucosyltransferase activity.

**D.7.4. Chromatofocusing**

Chromatofocusing of the enzyme protein was performed by chromatography on a Mono P HR 5/20 (200 x 5 mm I.D.) column

which had been equilibrated with buffer C. Desalted protein was applied to this column and the bound proteins were eluted with 50 mL of buffer D, which generated a linear gradient between pH 7.0 and 4.0. The flow rate was 0.3 mL/min and 1-mL fractions were collected in tubes containing 0.5 mL of buffer E, and assayed for O-glucosyltransferase activity.

### D.7.5. Affinity chromatography

Binding of the 2'-/5'-O-glucosyltransferase to different dye-ligand supports, UDP-agarose, and UDP-glucuronic acid-agarose, was tested using a crude protein extract which has been desalted by gel filtration on Sephadex G-25 column (see Section D.7.2). Two buffers, 50 mM Tris-HCl and 50 mM imidazole (at different pH values) were used to desalt the crude protein extract and to preequilibrate the affinity columns. After applying the desalted extract, unbound proteins were washed off and the bound proteins were eluted by stepwise potassium chloride gradient of 0.5, 1, and 2 M in the equilibration buffer.

#### D.7.5.1. Chromatography on UDP-glucuronic acid-agarose

The desalted enzyme protein was chromatographed on a UDP-glucuronic acid-agarose column (70 x 10 mm I.D.) which had been equilibrated with buffer B. The column was washed with five bed volumes of the same buffer, and the bound proteins were eluted with a linear salt gradient of 0-0.4 M potassium chloride in buffer B. Fractions of 1-mL were collected and assayed for O-glucosyltransferase activity.

### D.7.5.2. Chromatography on reactive brown 10-agarose

The desalted O-glucosyltransferase active protein was subjected to dye-ligand affinity chromatography on reactive brown 10-agarose. The latter was packed in a C 10/10 column (100 x 10 mm I.D.) and run using the FPLC system. The protein was loaded onto the column which had previously been equilibrated with buffer F. The column was washed with five bed volumes of the same buffer and the bound proteins were eluted using a linear gradient of 0-100% buffer G in buffer F. The flow rate was adjusted to 0.2 mL/min and 0.5-mL fractions were collected and assayed for O-glucosyltransferase activity.

### D.7.6. Ultrafiltration

Concentration and/or desalting of O-glucosyltransferase active protein pools were achieved by ultrafiltration under nitrogen in an ultrafiltration unit (10 or 50 mL) using PM30 membrane. The enzyme activity of the concentrate as well as the filtrate was tested using the standard O-glucosyltransferase assay.

### D.8. Flavonol O-glucosyltransferase assay

The standard assay mixture consisted of 10 μM of the flavonoid substrate (10 μL, in 50% dimethylsulfoxide, v/v), 10 μL of UDP-[U-$^{14}$C]Glc (25 nCi), and the enzyme protein (in buffer B), which was added last, to give a total volume of 100 μL. The assay mixtures were incubated for up to 30 min at

30°C. The enzymic reaction was stopped by adding 10 μL of 6 N HCl and the reaction products were extracted with 250 μL of ethylacetate. An aliquot (100 μL) of the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid. The flavonoid substrates used were 5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone (I, Fig. 7) and 5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone (II, Fig. 7) for the 2'- and 5'-O-glucosylating activities, respectively (Fig. 7.).

### D.9. Product identification

The organic phase from several enzyme assays was pooled, evaporated to dryness, and subsequently dissolved in a small volume of methanol (ca. 20 μL). The latter was chromatographed on Polyamid-6 MN TLC plates using benzene - methyl ethyl ketone - methanol (8:1:1, v/v/v) as the solvent system. The identity of O-glucosylated products was confirmed by co-chromatography with reference compounds, visualization in UV light followed by autoradiography on X-ray film.

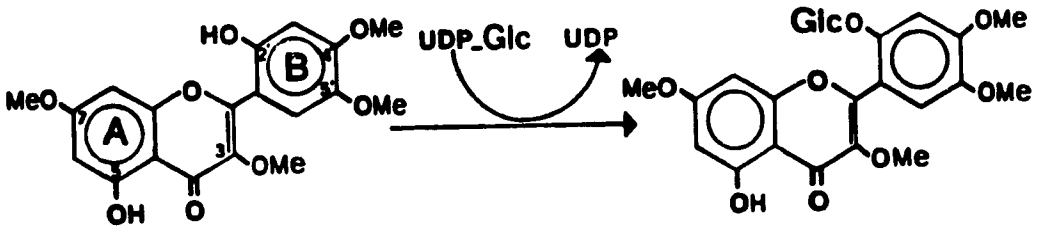
### D.10. Gel electrophoresis

The Bio-Rad Mini-Protean II dual slab cell electrophoretic system was used for nondenaturing- and denaturing- (SDS-) polyacrylamide gel electrophoresis (PAGE).

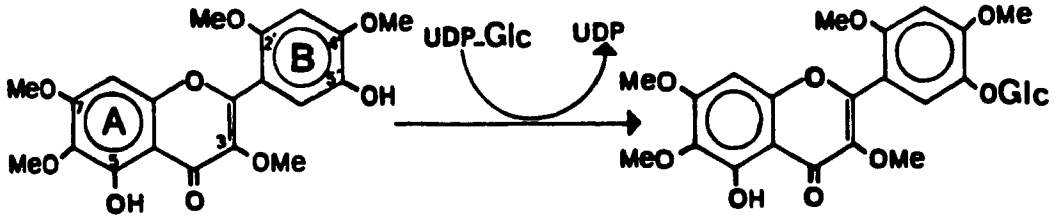
#### D.10.1. Nondenaturing-gel electrophoresis

Standard 7.5% polyacrylamide slab gels (99) with 4%

**Figure 7.** O-Glucosylation of the 2'- and 5'-positions of partially methylated (Me = methyl group) flavonols of *Chrysosplenium americanum*. I, 5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone; II, 5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone.


HO
OMe
UDP-Glc
UDP
GlcO
OMe
MeO
A
B
O
OMe
OMe
OMe
OH
O
MeO
OMe
OH
O

I


MeO
OMe
UDP-Glc
UDP
MeO
OMe
MeO
A
B
O
OH
OMe
OGlc
MeO
OH
O
MeO
OMe
OH
O

II

stacking gel were loaded with partially purified enzyme protein and electrophoresed at constant voltage (150 v) at 4°C. In order to localize O-glucosyltransferase activity, gels were sliced horizontally into 2-mm strips. Each strip was transferred into an Eppendorf tube, macerated in buffer B using a glass rod, incubated overnight at 4°C, and assayed for enzyme activity.

D.10.2. Denaturing-gel electrophoresis

SDS-polyacrylamide gels with 4% stacking and 12% resolving gels (100) were used to fractionate proteins from different stages of purification under denaturing conditions. The SDS-PAGE molecular weight standards used were: bovine serum albumin (BSA, 66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), and trypsinogen (24 kD). The gels were stained with Coomassie brilliant blue R, alternatively with silver nitrate. The molecular weight of the apparently homogeneous O-glucosyltransferase was estimated according to the method of Weber and Osborn (101).

11. Molecular weight determination by gel filtration

The molecular weight of Chrysosplenium O-glucosyltransferase was determined by gel filtration on Superose 12 HR 16/50 column using the FPLC system. The column was preequilibrated and developed with buffer B, and was calibrated with the following proteins: BSA (66 kD), ovalbumin

(45 kD), chymotrypsinogen A (25 kD), and ribonuclease A (13.7 kD). The void volume of the column was determined by the elution of a sample of blue dextran (2,000 kD). The different protein peaks were monitored by measuring their absorbance at 280 nm, whereas the O-glucosyltransferase was monitored by the standard assay. The apparent molecular weight of this enzyme was estimated from its elution volume from the column according to Andrews (102).

### D.12. Determination of protein concentration

Protein concentration was estimated according to the method of Bradford (103) using the Bio-Rad protein reagent and BSA as a standard protein.

### D.13. Definition of enzyme unit

Enzyme unit is expressed in katals (kat) as recommended by the International Union of Biochemistry (104). One katal is defined as the catalytic activity that will raise the rate of reaction by one mole per second in a specified assay system.

### D.14. Production of anti-O-glucosyltransferase monoclonal antibodies

#### D.14.1. Source of antigen

Crude protein extracts of C. americanum shoot tips were prepared as described in Section D.6. Partial purification of the 35-70% ammonium sulfate pellet was performed by gel

filtration on Superose 12 column followed by ion-exchange chromatography on Mono Q column (see Section D.7). The protein fractions which contained 2'-/5'-O-glucosyltransferase activities were pooled and used as the source of Ag.

D.14.2. Cell count and viability

Cell number was determined by counting on a haemocytometer, and cell viability was estimated by trypan blue (0.4%, w/v) dye exclusion.

D.14.3. Spleen cell harvest

Spleens from three unimmunized Balb/c mice were removed under sterile conditions and washed with IMDM. Intact spleens were teased by a scalpel and filtered through sterile nylon mesh in order to harvest lymphocytes. The latter were pooled and washed with IMDM.

D.14.4. *In vitro* immunization of spleen cells

*In vitro* immunization was performed according to a recently reported procedure (105). Briefly, the lymphocyte pellet was suspended at $7.5 \times 10^6$ cells/mL in IMDM containing 10% FBS, 50 $\mu$M 2-mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate, and 1% antibiotic-antimycotic solution. Adjuvant peptide, namely N-acetylmuramyl-L-alanyl-D-isoglutamine (10 $\mu$g/mL) (106) and partially purified O-glucosyltransferase preparation (2.5 or 12.5 $\mu$g/mL) were added to a 20-mL suspension of lymphocytes. The suspension was divided into two aliquots, each of which was transferred to a tissue culture flask (25 $cm^2$) and incubated at 37°C in a humidified incubator

containing 8% carbon dioxide for 5 or 7 days prior to fusion.

**D.14.5. Macrophage preparation (feeder cells)**

CD1 mice were injected intraperitoneally with 5 mL IMDM, and after massaging for 5 min, the abdominal fluid was withdrawn by a syringe. The macrophage pellet obtained by centrifugation was washed with IMDM, suspended in selective HAT (hypoxanthine, aminopterin and thymidine) medium containing 15% FBS, and distributed into 96-well tissue culture plates at $6.4 \times 10^3$ cells/well.

**D.14.6. Production of hybridomas**

Fusion partners, namely mouse myeloma cells and in vitro sensitized spleen cells, were hybridized following a recently described method (107) at a ratio of 1:2, lymphocytes to myeloma cells. The fusion solution (pH 7.0) contained 5 mL PEG 4000, 0.5 mL dimethylsulfoxide, and 4.5 mL phosphate-buffered saline. One mL of the fusion solution was added to the mixed cell pellet over a 45-sec period while gently shaking the tube. The fusion process was stopped by slowly adding 50 mL of IMDM. Fused cells were centrifuged gently, the cell pellet was suspended in selective HAT medium containing 15% FBS, distributed at $2.1 \times 10^5$ total cells per well on feeder cells in the 96-well plates, and incubated at 37°C in a humidified atmosphere containing 8% carbon dioxide. Control wells with unfused sensitized spleen cells or myeloma cells were also included. Two fusions were performed, one on day 5 and the other on day 7 after in vitro immunization.

**D.14.7. Screening of hybridomas and cloning**

Screening for Ig-producing hybridomas was performed 10 days after each fusion by ELISA (see below). ELISA was also applied to screen for hybridomas which react with the partially purified O-glucosyltransferase preparation. Highly immunoreactive hybridomas were cloned twice by limiting dilution at one cell per well, retested by ELISA for reactivity, and if found positive were expanded *in vitro*. Further characterization of immunoreactive mAb-producing clones was performed by Western blot analysis and enzyme inhibition studies (see below).

**D.15. Production of anti-O-glucosyltransferase polyclonal antibodies**

**D.15.1. Source of antigen**

*C. americanum* O-glucosyltransferase was extracted and purified by successive chromatography on Sephacryl S-200, UDP-glucuronic acid-agarose, Mono P, Superose 12, and Mono Q columns as described in Sections D.6 and D.7. This purification protocol resulted in an apparently homogenous enzyme protein which was used as the source of Ag to immunize rabbits.

**D.15.2. Immunization**

Antibodies to purified 2'-/5'-O-glucosyltransferase were raised in New Zealand white rabbits which were initially bled to obtain preimmune sera. For immunization, 75 μg of purified

Q-glucosyltransferase in one mL Tris-buffered saline was emulsified with one mL Freund's complete adjuvant and injected subcutaneously on the back of each animal at multiple sites. Primary immunization was followed by three booster injections (50 μg enzyme/injection/animal) at 3-week intervals using Freund's incomplete adjuvant. Eight days after the final booster injection, blood was collected by cardiac puncture, and the crude sera were stored at -20°C. Sera of immunized animals were tested for immunoreactivity by ELISA (see below) throughout the entire immunization period.

### D.16. Purification of antibodies

#### D.16.1. Isolation of IgMs from culture supernatants

Aliquots of different culture supernatants were concentrated and desalted on Sephadex G-25 which had been equilibrated and developed with buffer H. The desalted protein peak was subjected to gel filtration on Superose 12 column equilibrated and developed with the same buffer. The collected fractions were tested by ELISA and those containing IgM were pooled for further work.

#### D.16.2. Isolation of IgGs from rabbit sera

The globulin fraction from immune and preimmune sera was precipitated with ammonium sulfate to 40% saturation. The pellets were washed and desalted on a Sephadex G-25 column which was preequilibrated and developed with buffer H. Desalted proteins were then chromatographed on a Mono Q column

as previously described (see Section D.7.3) (87) using a linear salt gradient of 0 to 0.5 M sodium chloride in buffer H. The collected fractions were tested by ELISA and those containing IgGs were pooled, and further characterized by Western blotting (see below).

## D.17. Characterization of monoclonal and polyclonal antibodies

### D.17.1. Enzyme-linked immunosorbent assay (ELISA)

Immulon-2 microtitre plates were coated with 1 μg/mL enzyme protein, or affinity purified goat anti-mouse/rabbit IgG/IgM in carbonate buffer (pH 9.6), and the plates were incubated overnight at 4°C. All other incubations were done at room temperature in a humidified atmosphere. Tween 20 (0.05%, v/v) in phosphate-buffered saline was used to wash the plates between incubations, and BSA (2.5%, w/v) in the same buffer was added to the plates in order to block uncoated sites amenable to nonspecific interactions with the primary antibody. Culture supernatant, crude rabbit antiserum or its IgG pool were used as the primary Ab source. The reaction was revealed by peroxidase-labeled secondary antibodies using a freshly prepared solution of o-phenylenediamine in phosphate-citrate buffer (pH 5.0) containing hydrogen peroxide as the substrate. After stopping the reaction with 2.5 M sulfuric acid, the optical density was measured at 492 nm using a EL308 microplate reader.

Mouse preimmune serum, as well as myeloma cell and feeder

cell culture media were used as controls for mAbs. For polyclonal Abs, preimmune serum and its purified IgG pool were used as controls.

**D.17.2. Immunoblotting (Western blot analysis)**

Nondenaturing- and SDS-PAGE were performed as described in Section D.10. Electrophoretic transfer of protein bands from gels to nitrocellulose membrane was carried out according to Towbin et al. (85) using the Bio-Rad Mini Trans-Blot cell. The transfer buffer used was 25 mM Tris and 192 mM glycine (pH 8.3) in presence or absence of 20% methanol for SDS- or nondenaturing gels, respectively. Following transfer, nitrocellulose strips were blocked with BSA (2.5%, w/v) in Tris-buffered saline, and the strips were washed in between incubations with Tween 20 (0.05%, v/v) in the same buffer. After incubating the strips with the primary Ab (see Section D.17.1), immunoreactive bands were revealed with peroxidase-conjugated secondary Abs using 4-chloro-1-naphthol in Tris-buffered saline containing hydrogen peroxide as the substrate. The strips were then washed in water, left to dry, and stored in the dark. Controls similar to the ones used for ELISA were included.

**D.17.3. Inhibition of O-glucosyltransferase activity by monoclonal antibodies**

Initially, a partially purified enzyme preparation was stabilized with an optimum concentration of BSA (1.25 mg/mL). The preincubation mixtures which consisted of 40 μL of

stabilized enzyme protein and 160 μL of different dilutions of control and test supernatants were incubated for 30 min at room temperature. Aliquots (80 μL) from the various preincubation mixtures were then assayed for 2'- and 5'-O-glucosyltransferase activity using the standard enzyme assay (see Section D.8) and substrates I and II (Fig. 7), respectively. The controls used were mouse preimmune serum, feeder cell supernatant, and myeloma culture medium.

### D.17.4. Monospecificity of polyclonal antibodies

Specificity of the polyclonal Abs to the enzyme protein was assured by preadsorbing the IgGs with protein contaminants eluted from SDS-polyacrylamide gels of crude plant extracts. This was achieved by incubating one part of purified rabbit anti-enzyme IgG with one part of protein contaminants in Tris-buffered saline for 1 h at room temperature and for 24 h at 4°C. The preadsorption process removed all cross-reactions of the IgGs when tested by immunoblotting.

## D.18. Immunocytochemistry

### D.18.1. Processing of plant tissues for electron microscopy

Samples from leaves of C. americanum were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, v/v (pH 7.4) for 3 h at 24°C, and dehydrated before embedding in Lowicryl K4M (108) or LR White. Polymerization of Lowicryl K4M was performed under UV at -20°C for 24 h, whereas LR White was polymerized by heat-curing the resin at 60-65°C for 3 days.

Specimens to be embedded in LR White were post-fixed for 1 h in 1% osmium tetroxide at room temperature. After polymerization, ultrathin sections (gold interference colour) were mounted on Formvar-coated nickel grids (200 mesh).

**D.18.2. Immunogold labeling**

Immunostaining was carried out at room temperature in a humid atmosphere. Prior to labeling, osmified sections embedded in LR White were treated with aqueous sodium m-periodate (12%, w/v) for 30 min in order to overcome the masking of antigenic sites by osmium tetroxide (109,110). Sections mounted on the grids were blocked by floating on a drop of Tris-buffered saline containing nonfat dry milk (Blotto, 5%, w/v) for 5 min (111) before being transferred onto drops of anti-enzyme Abs. After one-hour incubation, the grids were rinsed with Tris-buffered saline and incubated for 30 min on a drop of secondary Ab conjugated with gold. Sections were then rinsed, air-dried, and post-stained with aqueous uranyl acetate (2%, w/v) for 10 min, followed by lead citrate (112) for 5 min. Sections were examined in a JEOL 1200 EX electron microscope at 80 kV.

To assess the specificity of the immunocytochemical labeling, Abs were replaced with either preimmune serum IgGs, Abs preadsorbed with enzyme protein, or Tris-buffered saline containing BSA (0.5%, w/v).

**D.18.3. Quantification of labeling**

The density of labeling of different cellular

compartments was expressed as the mean number of gold particles per $\mu m^2$ of sectioned compartments. The area of the latter was estimated directly on the electron micrographs by standard stereological techniques (113). The number of gold particles was counted manually. Test (ca. 15) and control (ca. 10) micrographs were analyzed at a final magnification of x40,000.

## E. RESULTS

### E.1. Separation of flavonol 2'- and 5'-O-glucosylating activities

#### E.1.1. Chromatography on high resolution columns

Desalted ammonium sulfate pellets of C. americanum shoot tips were subjected to chromatography on Superose 12 (Fig. 8), Mono Q (Fig. 9) and Mono P (Fig. 10) high resolution columns using the FPLC system. Although chromatography on these columns did not separate the 2'- and 5'-O-glucosyltransferase activities, it resulted in an increase of their specific activities (Fig. 8-10). Similar results have previously been obtained by conventional chromatography on gel filtration, ion-exchange and chromatofocusing columns (52).

#### E.1.2. Chromatography on specific affinity matrices

Chrysosplenium 2'- and 5'-O-glucosylating activities did not bind to UDP-agarose column under various conditions. However, they did bind to UDP-glucuronic acid-agarose both at pH 6.4 (using 50 mM imidazole) and at pH 7.8 (using 50 mM Tris-HCl). The use of the latter buffer was preferred since both 2'- and 5'-activities were more stable at pH 7.8. Upon subjecting a desalted ammonium sulfate pellet to affinity chromatography on UDP-glucuronic acid-agarose column, bound enzyme activities were not eluted by specific eluents such as UDP or UDP-Glc. On the other hand, when a 0 to 0.4 M linear potassium chloride gradient in buffer B was applied, both

**Figure 8.** Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from Superose 12 HR 10/30 column using compounds I and II as substrates, respectively. The column was equilibrated and developed with 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol, 1-mL fractions were collected, and assayed for enzyme activity.

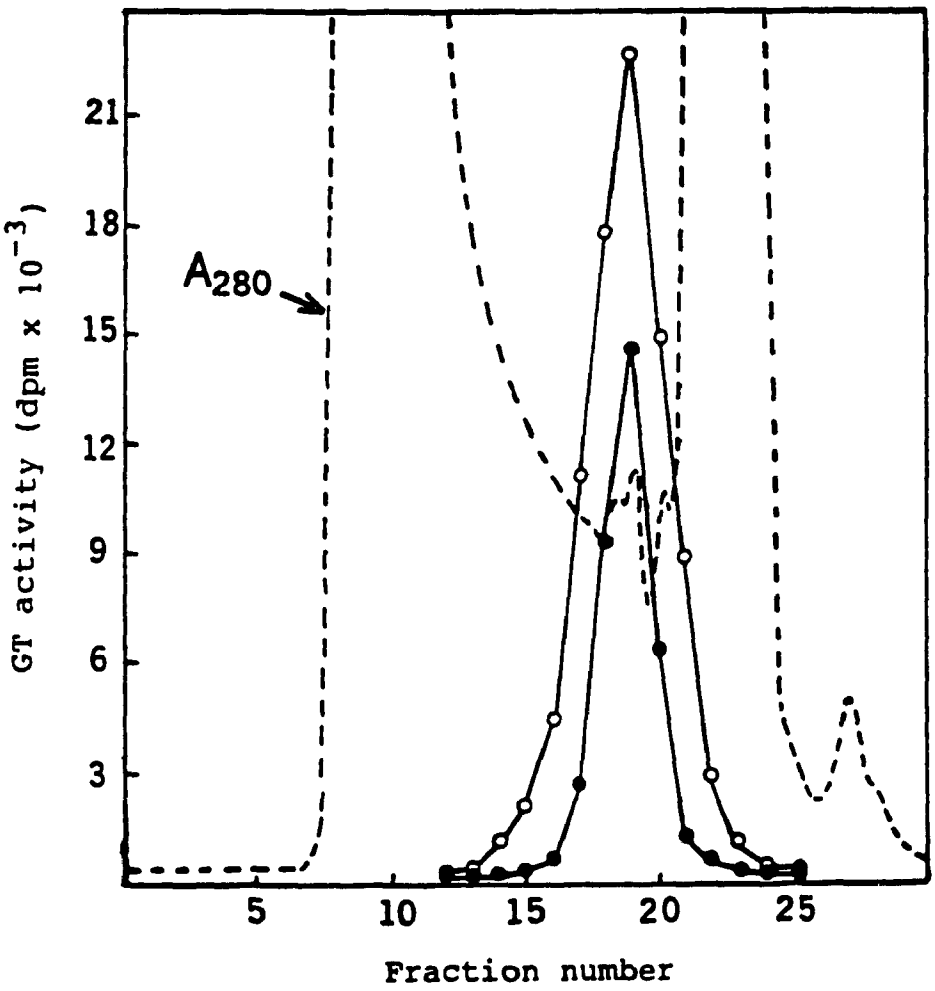

21
18
15
12
9
6
3
GT activity (dpm x $10^{-3}$)
$A_{280}$
5
10
15
20
25
Fraction number

**Figure 9.** Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from Mono Q column using compounds I and II as substrates, respectively. The desalted enzyme was applied to a column equilibrated with 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol. The bound proteins were eluted using a 20-mL linear salt gradient (0-0.5 M potassium chloride) in the same buffer. The flow rate was adjusted to 1 mL/min, 1-mL fractions were collected, and assayed for 2'- and 5'-enzyme activities.

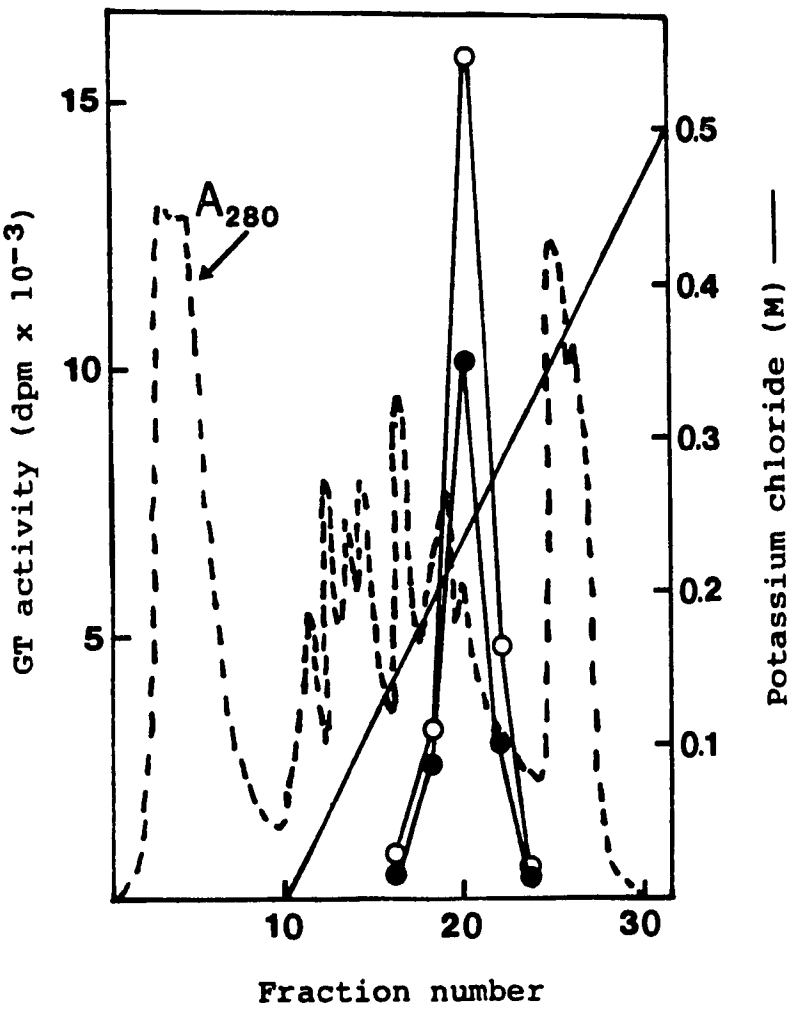
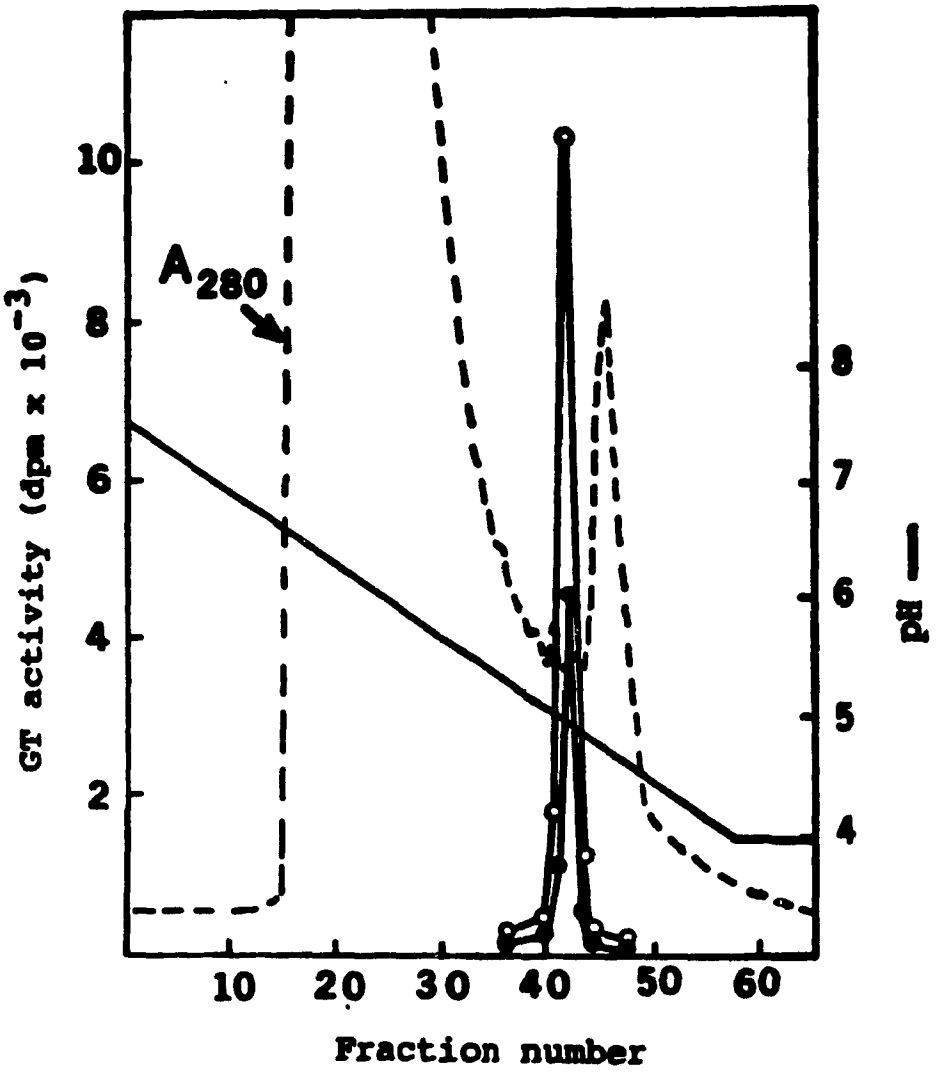

15
10
5
$A_{280}$
GT activity (dpm x $10^{-3}$)
0.5
0.4
0.3
0.2
0.1
Potassium chloride (M) ——
10
20
30
Fraction number

**Figure 10.** Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from Mono P column using compounds I and II as substrates, respectively. The desalted enzyme was loaded onto a column equilibrated with 25 mM Bis-Tris-iminodiacetic acid (pH 7.0) containing 14 mM 2-mercaptoethanol. Elution of the bound proteins was carried out using Polybuffer 74-iminodiacetic acid (diluted with water 1:10, v/v) (pH 4.0) containing 14 mM 2-mercaptoethanol. The flow rate was 0.3 mL/min, fractions of 1-mL were collected in tubes containing 0.5 mL of 0.2 M Tris-HCl (pH 8.0) containing 14 mM 2-mercaptoethanol, and were assayed for enzyme activity.


GT activity (dpm x $10^{-3}$)
$A_{280}$
pH
Fraction number
10
8
6
4
2
8
7
6
5
4
10
20
30
40
50
60

enzyme activities were eluted in a single peak at 0.26 M potassium chloride (Fig.11).

**E.1.3. Electrophoretic behaviour on nondenaturing gels**

Localization of enzyme activity on nondenaturing-PAGE of the enzyme protein eluted from UDP-glucuronic acid-agarose column is shown in Fig. 12. The 2'- and 5'-O-glucosyltransferase activities were localized in the same region of the gel suggesting that both had similar electrophoretic pattern under nondenaturing conditions.

**E.1.4. Chromatography on dye-ligand affinity supports**

Chromatography of the desalted protein pellet on a number of dye-ligand matrices was performed under conditions where the pH and ionic strength of the equilibration and elution buffers were varied. These included Matrex Gel red A, blue A, blue B, orange A, and green A, and reactive brown 10-agarose. Of the above supports, only reactive brown 10-agarose and Matrex Gel red A columns allowed binding of the two O-glucosyltransferase activities. The reactive brown 10-agarose column resulted in the resolution of the 2'- and 5'-activities. The latter was achieved under optimum conditions where the support was packed in a C 10/10 column and run using the FPLC system. A shallow linear pH-salt gradient was applied and resulted in the separation of the two O-glucosyltransferase activities. As shown in Fig. 13, the 2'- and 5'-activities eluted at pH 7.8 and 7.3, respectively. Each peak gave a single product when assayed with the respective

**Figure 11.** Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from UDP-glucuronic acid-agarose column using compounds I and II as substrates, respectively. The desalted enzyme was loaded onto a column equilibrated with 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol. The column was washed with the same buffer and the bound proteins were eluted with a linear salt gradient of 0-0.4 M potassium chloride in the same buffer. Fractions of 1-mL were collected, and assayed for O-glucosyltransferase activity.

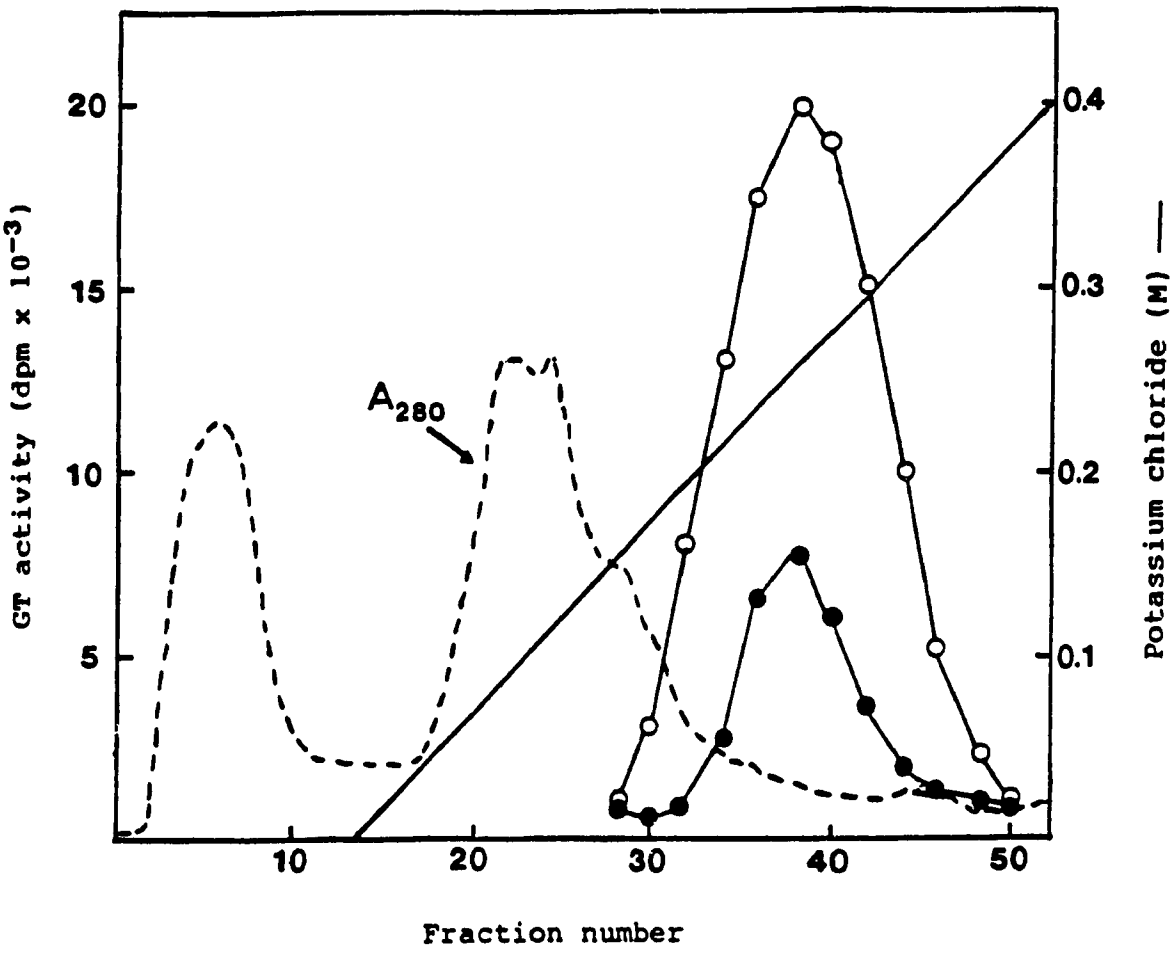

GT activity (dpm x $10^{-3}$)
20
15
10
5
$A_{280}$
10
20
30
40
50
Fraction number
0.4
0.3
0.2
0.1
Potassium chloride (M) —

Figure 12. Localization of 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities on nondenaturing-polyacrylamide gels. Partially purified enzyme was subjected to non-denaturing gel electrophoresis on 7.5% gels. The gels were sliced into 2-mm strips, macerated in 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol, and assayed against substrates I and II for the 2'- and 5'-O-glucosyltransferase activities, respectively.

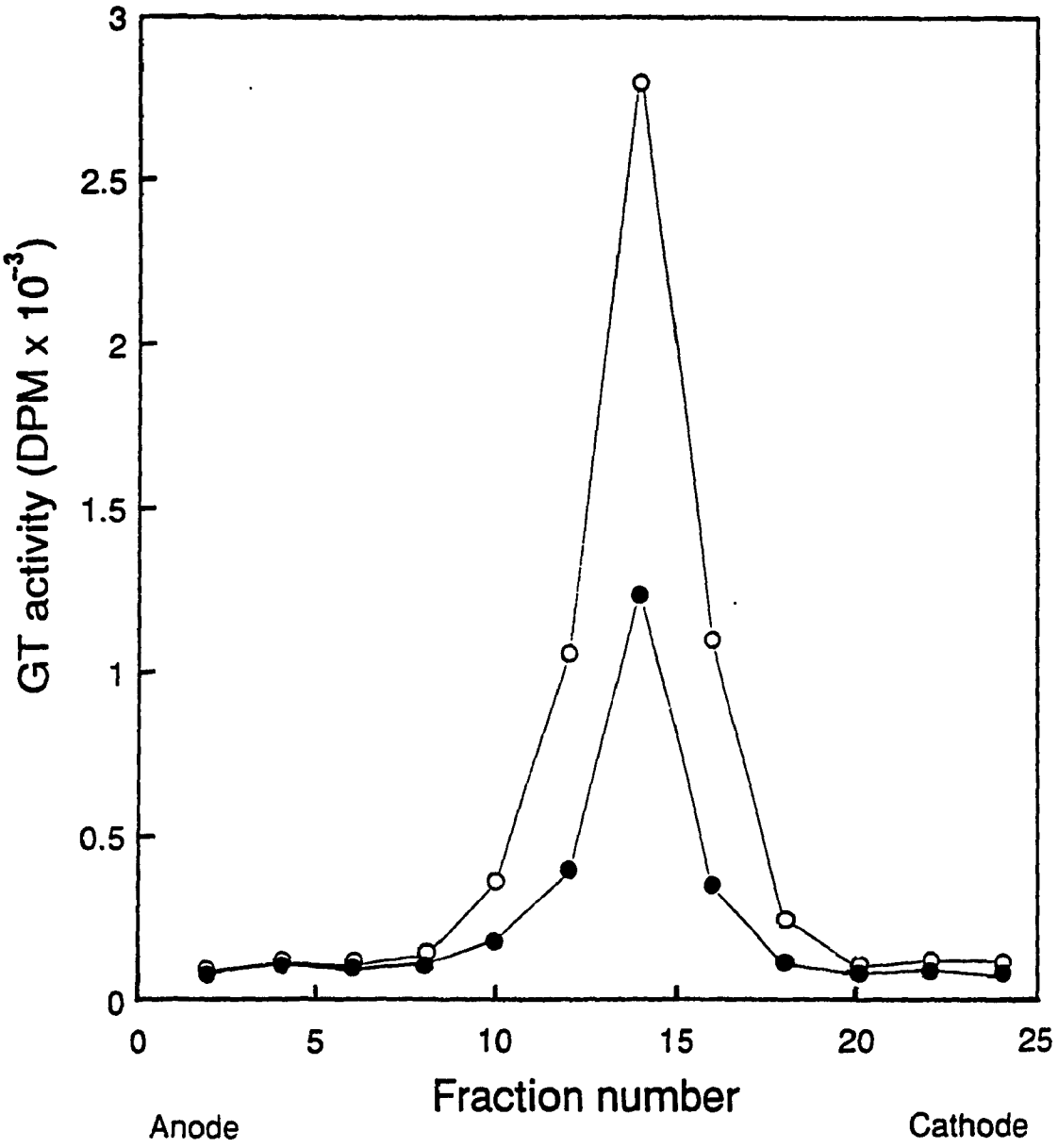
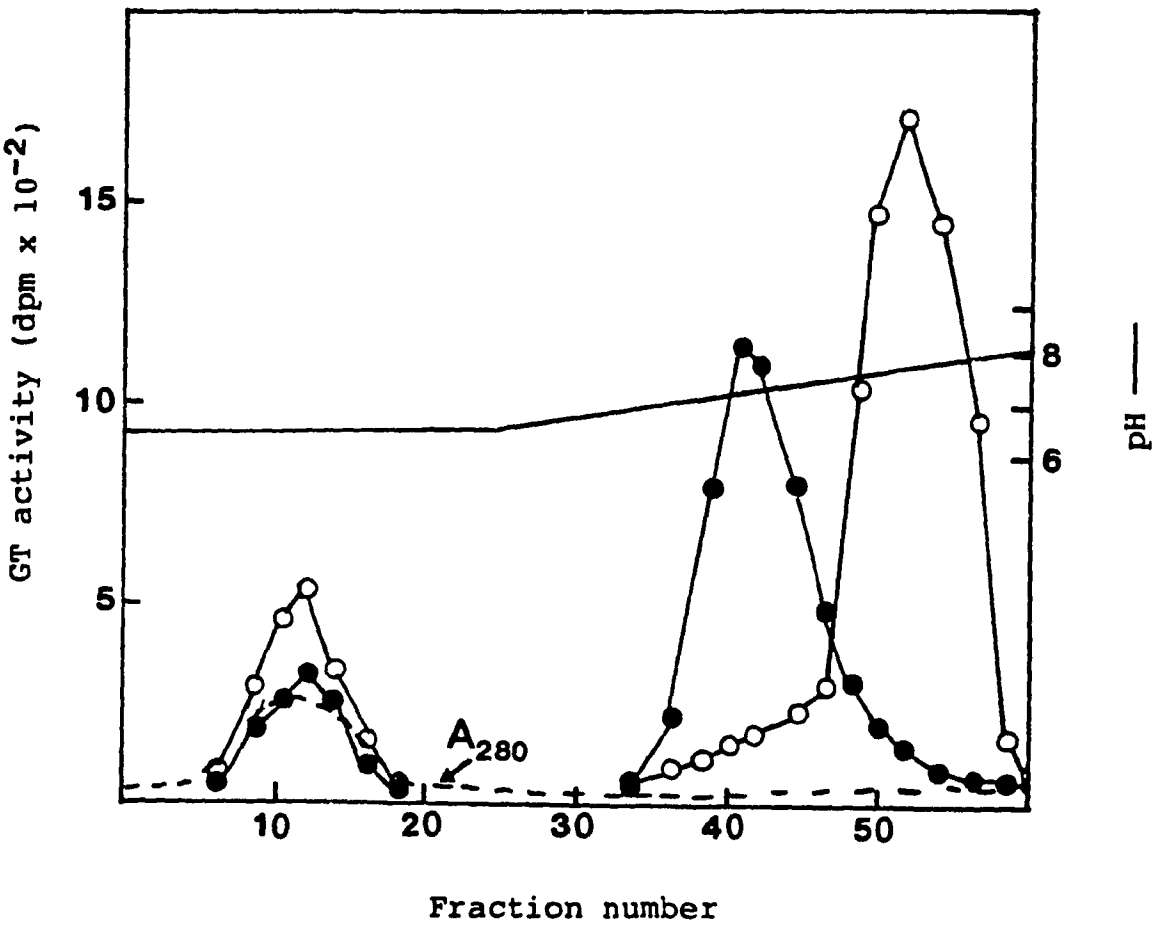

3
2.5
2
1.5
1
0.5
0
GT activity (DPM x 10$^{-3}$)
0
5
10
15
20
25
Fraction number
Anode
Cathode

**Figure 13.** Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from reactive brown 10-agarose C 10/10 column using compounds I and II as substrates, respectively. The O-glucosyltransferase active fractions following affinity chromatography and gel filtration were pooled and applied to the dye-ligand column which had been equilibrated with 25 mM imidazole (pH 6.4) containing 14 mM 2-mercaptoethanol. The bound proteins were eluted using a linear pH-salt gradient of 0-100% 25 mM imidazole (pH 8.0) containing 0.2 M potassium chloride and 14 mM 2-mercaptoethanol in equilibration buffer. The flow rate of the column was adjusted to 0.2 mL/min, 0.5-mL fractions were collected, and assayed for O-glucosyltransferase activity.


GT activity (dpm x $10^{-2}$)
15
10
5
$A_{280}$
10
20
30
40
50
Fraction number
8
6
pH

substrate as shown by autoradiography (Fig. 14).

Introducing the reactive brown 10-agarose step in a purification scheme which included affinity chromatography on UDP-glucuronic acid-agarose (Fig. 11) and gel filtration on Superose 12 (Fig. 15) resulted in an increase in specific activity of 1126- and 1011-fold for the 2'- and 5'-O-glucosyltransferases, respectively, as compared to the crude extract (Table 2). However, the low concentration of protein, the prolonged exposure of the enzymes to drastic changes of pH and ionic strength, as well as exposure to the dye-ligand resulted in poor stability of the enzymes and poor recovery of their activities.

## E.2. Production and characterization of an anti-2'-O-glucosyltransferase monoclonal antibody

Pure protein is not required as a source of Ag for the production of mAbs since the hybridomas produced give rise to cell lines secreting monospecific Abs. Furthermore, the novel technique of *in vitro* immunization (114) requires only minute quantities of soluble protein for sensitization. Therefore, in order to produce mAbs to *Chrysosplenium* flavonol O-glucosyltransferases a partially purified enzyme preparation was used as the source of Ag in an *in vitro* system.

### E.2.1. Source of antigen

The source of Ag used for immunization was a partially purified O-glucosyltransferase preparation with both 2'- and

Figure 14. Photograph of an autoradiogram of the chromatographed reaction products of the 2'- and 5'-O-glucosyltransferases eluted from the reactive brown 10-agarose column and assayed against the flavonol substrates I and II, respectively. The solvent system used was benzene - methyl ethyl ketone - methanol (8:1:1, v/v/v) on Polyamid-6 TLC plates. The products formed were co-chromatographed with the reference O-glucosides of compounds I and II.


5′–
2′–

Figure 15. Elution profile of the 2'- (o--o) and 5'- (●--●) O-glucosyltransferase (GT) activities from Superose 12 HR 16/50 column using compounds I and II as substrates, respectively. The active fractions from UDP-glucuronic acid-agarose column were pooled and applied to the column which had been equilibrated with 25 mM imidazole (pH 6.4) containing 14 mM 2-mercaptoethanol. The column was developed with the same buffer, and fractions collected were assayed for enzyme activity.

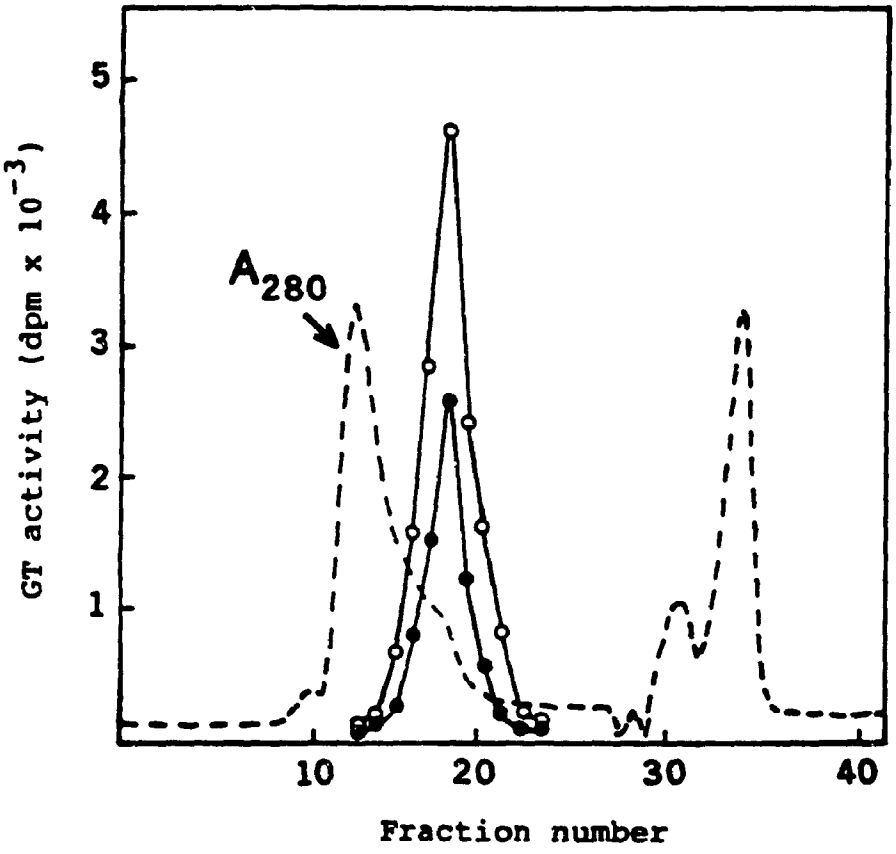

GT activity (dpm x $10^{-3}$)
5
4
3
2
1
$A_{280}$
10
20
30
40
Fraction number

Table 2. Purification of 2'- and 5'-O-glucosyltransferase activities from C. americanum.

| Purification step | Total protein (mg) | Specific activity (pkat/mg) | | Total activity (pkat) | | Purification (-fold) | | Yield (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2'- | 5'- | 2'- | 5'- | 2'- | 5'- | 2'- | 5'- |
| Crude extract [a] | 11 | 0.035 | 0.019 | 0.385 | 0.209 | - | - | 100 | 100 |
| Ammonium sulfate [b] (35-70% saturation) | 6.2 | 0.047 | 0.023 | 0.291 | 0.142 | 1.3 | 1.2 | 75 | 68 |
| Affinity chromatography [c] | 0.8 | 0.35 | 0.17 | 0.28 | 0.136 | 10 | 8.9 | 72 | 65 |
| Gel filtration [d] | 0.04 | 5.1 | 2.2 | 0.204 | 0.088 | 145 | 115 | 52 | 42 |
| Dye-ligand chromatography [e] | <0.001 | 39.4 | 19.2 | 0.039 | 0.019 | 1126 | 1011 | 10 | 9 |

[a] After treatment with Dowex 1-X2.
[b] Desalted on Sephadex G-25.
[c] On UDP-glucuronic acid-agarose column.
[d] On Superose 12 HR 16/50 column using the FPLC system.
[e] On reactive brown 10-agarose C 10/10 column using the FPLC system.

5'-activities. This was obtained by subjecting the 35-70% ammonium sulfate pellet to gel filtration and subsequent ion-exchange chromatography on Superose 12 HR 10/30 (Fig. 8) and Mono Q (Fig. 16) columns, respectively. The above purification scheme resulted in an increase in the specific activity of the flavonol O-glucosyltransferases by 187-fold as compared to the crude extract (Table 3).

**E.2.2. In vitro immunization and production of hybridomas**

In vitro sensitization of spleen cells was performed using muramyl dipeptide at 10 μg/mL as the adjuvant. The Ag was added to the culture medium at final concentrations of either 2.5 or 12.5 μg/mL. The viability and yield of sensitized spleen cells on day 0, 5, and 7 varied with different Ag concentrations (Fig. 17,18). Better viability as well as yield of cells were evident in the cultures treated with 2.5 μg/mL Ag. These results suggested that the Ag concentration may play a role in determining the fate of spleen cells.

Following immunization, two fusions were performed, one on day 5 and the other on day 7 using the in vitro culture sensitized with 2.5 μg/mL Ag. As shown in Table 4, an insignificant difference was observed in the yield of hybridomas at different time periods after in vitro immunization.

**E.2.3. Screening of hybridomas and cell lines**

Ten days after each fusion, hybridomas were screened for

**Figure 16.** Elution profile of the 2'- (●---●) O-glucosyltransferase (GT) activity from Mono Q column using compound I as substrate. The active fractions eluted from Superose 12 HR 10/30 column were pooled and applied to a column equilibrated with 50 mM Tris-HCl (pH 7.8) containing 14 mM 2-mercaptoethanol. The bound proteins were eluted using a 30-mL linear salt gradient (0-0.5 M potassium chloride) in the same buffer at a flow rate of 0.5 mL/min. One-mL fractions were collected, and assayed for 2'-O-glucosyltransferase activity.

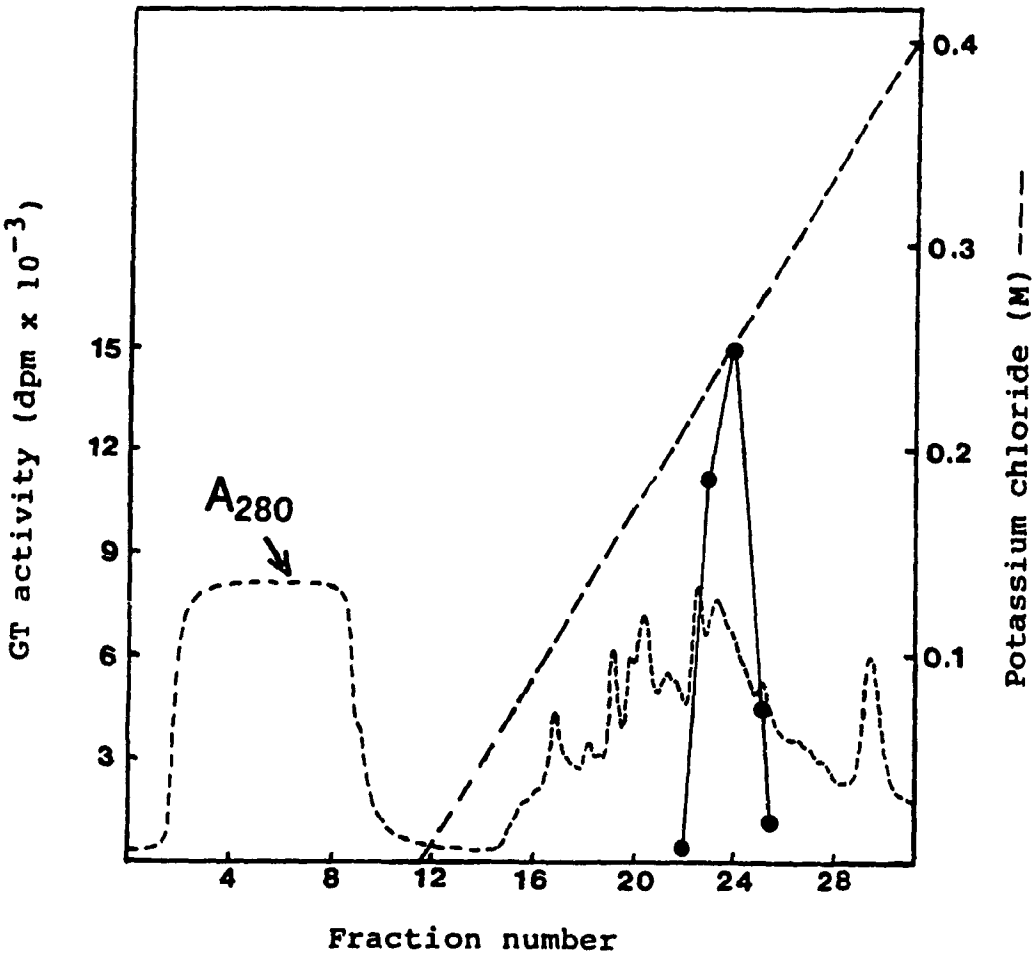

GT activity (dpm x $10^{-3}$)
15
12
9
6
3
$A_{280}$
4
8
12
16
20
24
28
Fraction number
0.4
0.3
0.2
0.1
Potassium chloride (M) ---

Table 3. Purification of Chrysosplenium flavonol O-glucosyltransferases.[a]

| Purification step | Total protein (mg) | Specific activity (pkat/mg) | Total activity (pkat) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract[b] | 59.5 | 0.08 | 4.76 | - | 100 |
| Gel filtration[c] | 3.25 | 1.1 | 3.57 | 14 | 75 |
| Ion-exchange chromatography[d] | 0.13 | 15 | 1.95 | 187 | 40 |

[a] The standard assay using substrate I was applied.
[b] After treatment with Dowex 1-X2.
[c] On Superose 12 HR 10/30 column using the FPLC system.
[d] On Mono Q column using the FPLC system.

Figure 17. Viability of *in vitro* immunized spleen cells in presence of either 2.5 μg/mL (□---□) or 12.5 μg/mL (Δ---Δ) of partially purified O-glucosyltransferases. Viability was determined by trypan blue dye exclusion.

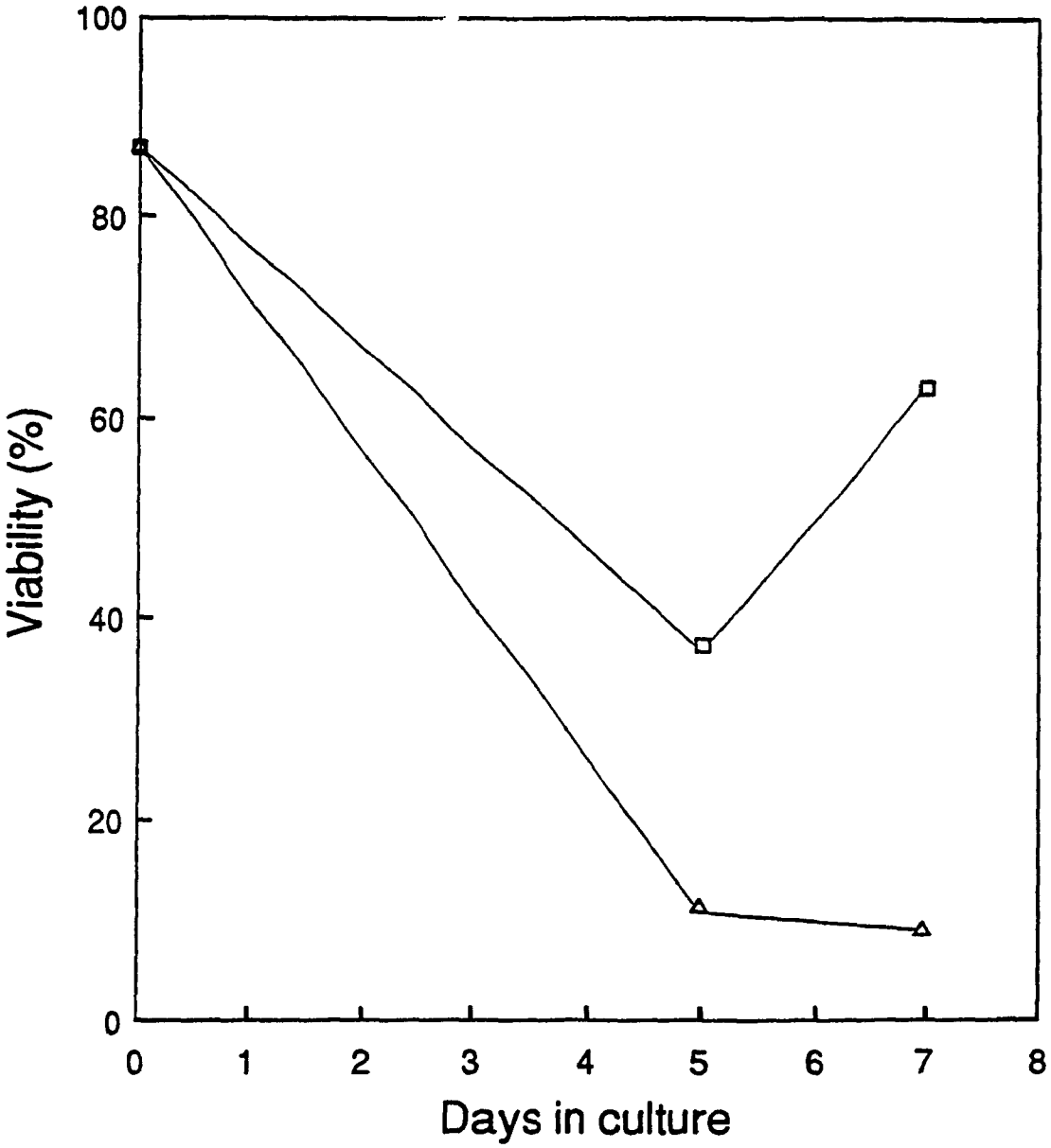

100
80
60
40
20
0
Viability (%)
0
1
2
3
4
5
6
7
8
Days in culture

Figure 18. Recovery of *in vitro* immunized spleen cells in presence of either 2.5 μg/mL (□---□) or 12.5 μg/mL (Δ---Δ) of partially purified O-glucosyltransferases. Percent recovery is based on the number of viable cells at each time point, day 0 being taken as 100%.

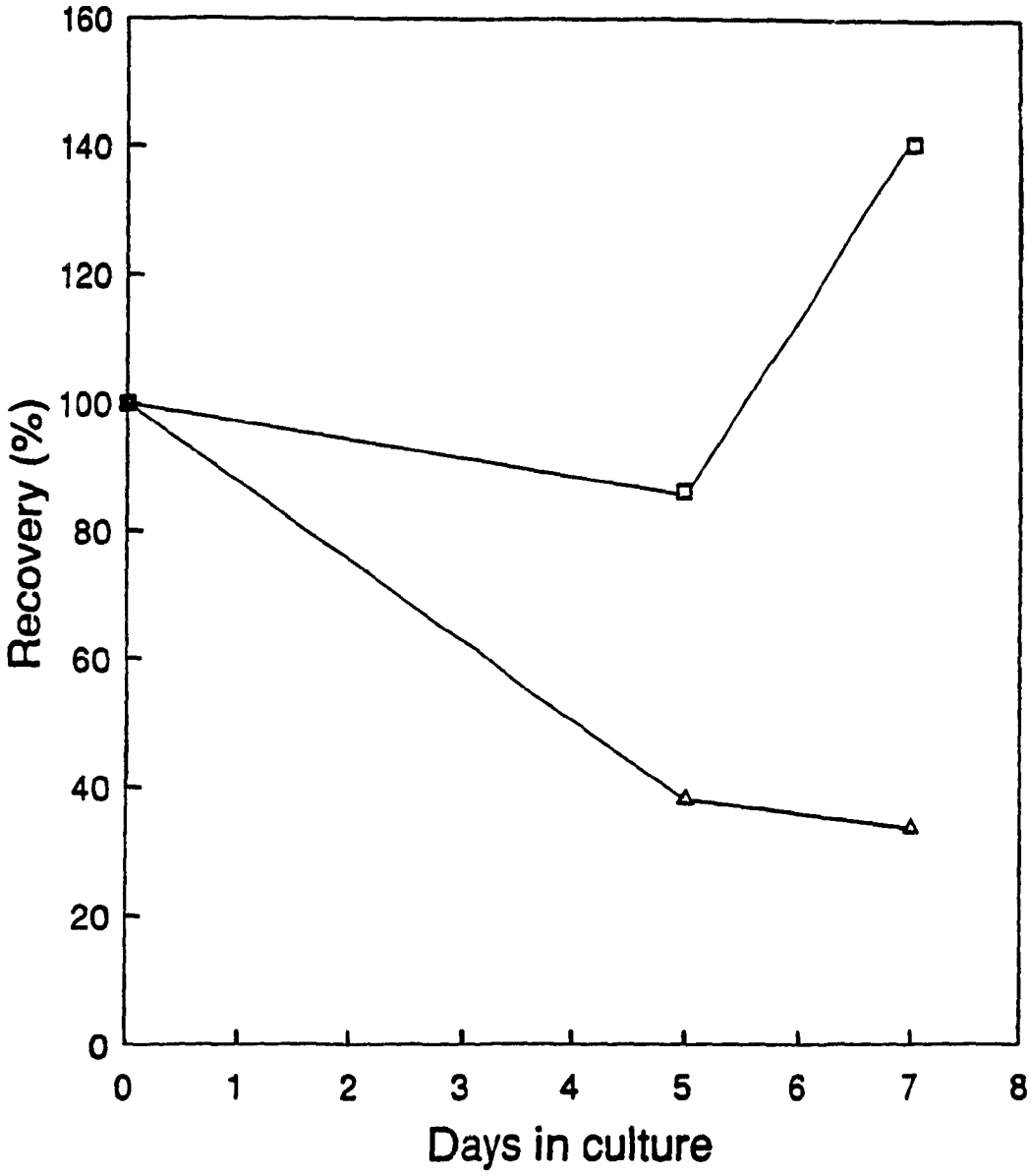

160
140
120
100
80
60
40
20
0
Recovery (%)
0
1
2
3
4
5
6
7
8
Days in culture

**Table 4. Fusion Efficiency.**[a]

| Fusion | Total number of wells | Wells with growing hybrid clones[b] (% of total) |
|---|---|---|
| DAY 5 | 192 | 69.1 |
| DAY 7 | 384 | 62.8 |

[a] Spleen cells immunized with 2.5 μg/mL antigen were fused with myeloma cells either on day 5 or on day 7 after in vitro sensitization. Fused cells were suspended in selective HAT medium and distributed at $2.1 \times 10^5$ total cells per well on feeder cells in 96-well culture plates.

[b] Growing hybrid clones were identified by microscopic examination on day 9 after each fusion.

Ig production by ELISA using affinity purified goat anti-mouse IgG or IgM, and the reaction was revealed by peroxidase-labeled secondary Abs. It has been reported that a large number of IgG-secreting hybridomas are produced by prolonging the *in vitro* immunization period (115). However, the majority of Ig-producing hybridomas in this study were IgM-secretors (Table 5), even though the sensitization period was prolonged to 7 days.

ELISA was applied for a second screening of the IgM-secreting hybridomas, but this time using a partially purified enzyme preparation with the aim of selecting hybridomas which react with the 2'- and 5'-O-glucosyltransferases (Table 5). The enzyme preparation did not show any immunoreactivity with preimmune serum as detected by ELISA.

Strongly immunoreactive IgM-producing hybridomas were cloned twice by limiting dilution, thus generating pure hybrid cell lines. Two of these cell lines, namely C3-2 and C7-1 from day 5 and day 7 fusions, respectively, were selected to be expanded *in vitro* for further characterization. In addition, a non-immunoreactive IgM-secreting hybridoma, C2-1 from day 5 fusion, was cloned twice and expanded *in vitro* to be used as a direct control. Instead of growing cell lines *in vivo* as ascitic tumours, their culture *in vitro* was preferred in this study because it offers a better chance of obtaining Abs free from irrelevant mouse Igs. Monoclonal antibodies C2-1, C3-2, and C7-1 had similar ELISA reactivities when tested with

Table 5. Specific fusion efficiency.[a]

| Fusion | Ig-producing wells[b] (% of total) | | Specific immunoreactive wells[c] (% of IgM-secretors) |
|---|---|---|---|
| | IgM | IgG | |
| DAY 5 | 50.5 | 3.6 | 22.2 |
| DAY 7 | 45.9 | 0.5 | 18.6 |

[a] Enzyme-linked immunosorbent assay (ELISA) was applied to determine the class and immunoreactivity of the antibodies produced by the hybridomas.

[b] Using goat anti-mouse IgM and IgG, and the reaction was revealed with secondary antibody labeled with peroxidase.

[c] Using partially purified O-glucosyltransferase preparation and the reaction was revealed with secondary antibody labeled with peroxidase.

goat anti-mouse IgM (Fig. 19). On the other hand, only C3-2 and C7-1 mAbs were immunoreactive when tested with O-glucosyltransferase preparation by ELISA (Fig. 20). The IgM concentration of the different clones was assessed by ELISA with goat anti-mouse IgM using reference serum containing 17.1 mg/100 mL IgMs as standard (Fig. 21). For example, C3-2 culture supernatant contained 12.5 μg/mL IgM.

**E.2.4. Characterization of IgM-secreting clones**

**E.2.4.1. Effect of BSA and FBS on O-glucosyltransferase activity**

The effect of added protein on enzyme activity was investigated using the standard assay prior to conducting the inhibition studies. BSA and FBS significantly activated (and/or stabilized) the 2'-O-glucosyltransferase activity as shown in Figure 22 and 23, respectively. Both had similar but less pronounced effect on the 5'-O-glucosyltransferase activity.

**E.2.4.2. Inhibition of enzyme activity by monoclonal antibody**

The ability of C3-2 and C7-1 culture supernatants to inhibit 2'- and/or 5'-O-glucosyltransferase activities was investigated using the standard assay. The enzyme protein was previously stabilized with an optimum concentration of BSA (1.25 mg/mL) in order to avoid protein stabilization effect by the added mAb or control supernatant. Furthermore, the protein concentration of test and control supernatants were made equal. Immunoreactive mAb C3-2 inhibited 2'-O-

Figure 19. Reactivity of monoclonal antibodies in enzyme-linked immunosorbent assay (ELISA) using 1 μg/mL of goat anti-mouse IgM. The reaction was revealed by peroxidase-labeled goat anti-mouse IgM. The antibodies (mAb) tested were C2-1 (Δ---Δ), C3-2 (●---●), and C7-1 (o---o).

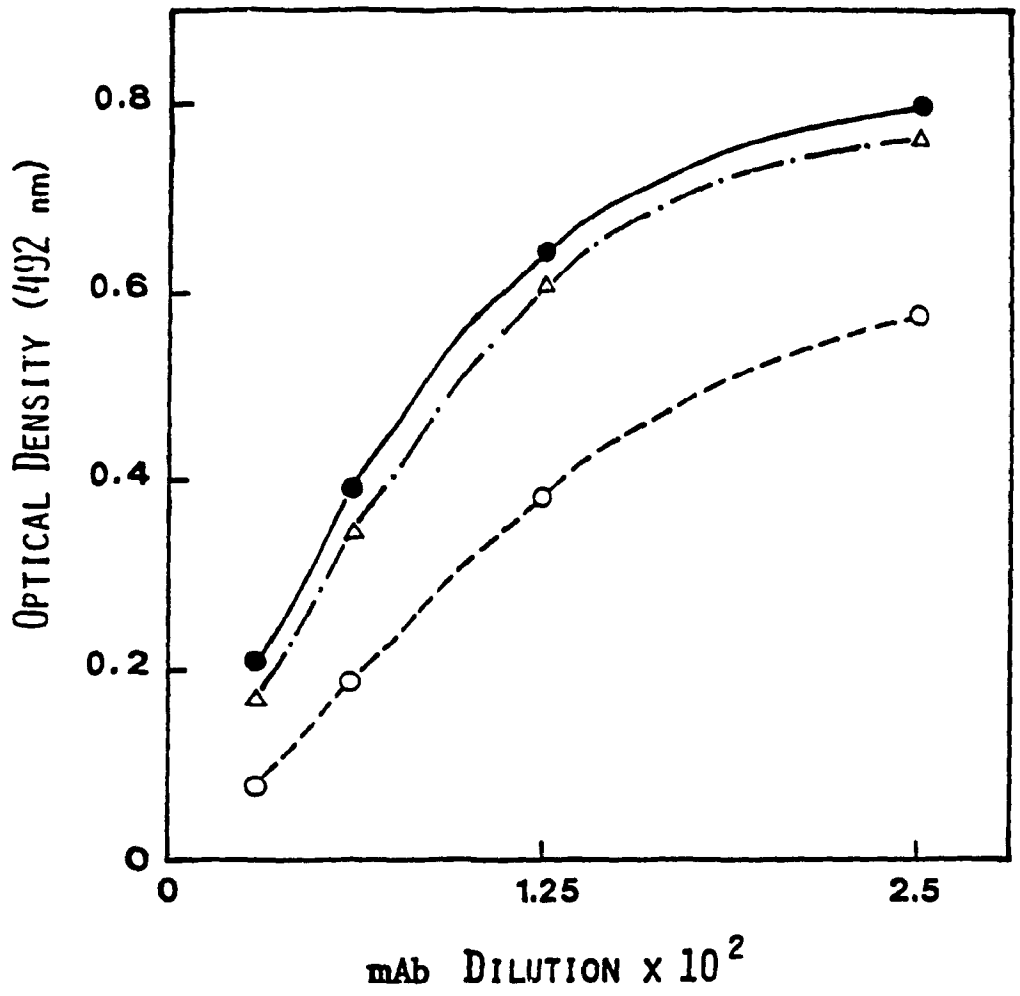

0.8
0.6
0.4
0.2
0
Optical Density (492 nm)
0
1.25
2.5
mAb Dilution x 10 2

**Figure 20.** Reactivity of monoclonal antibodies in enzyme-linked immunosorbent assay (ELISA) using 1 μg/mL of partially purified O-glucosyltransferases. The reaction was revealed by peroxidase-labeled goat anti-mouse IgM. The antibodies (mAb) tested were C2-1 (Δ---Δ), C3-2 (●---●), and C7-1 (o---o).

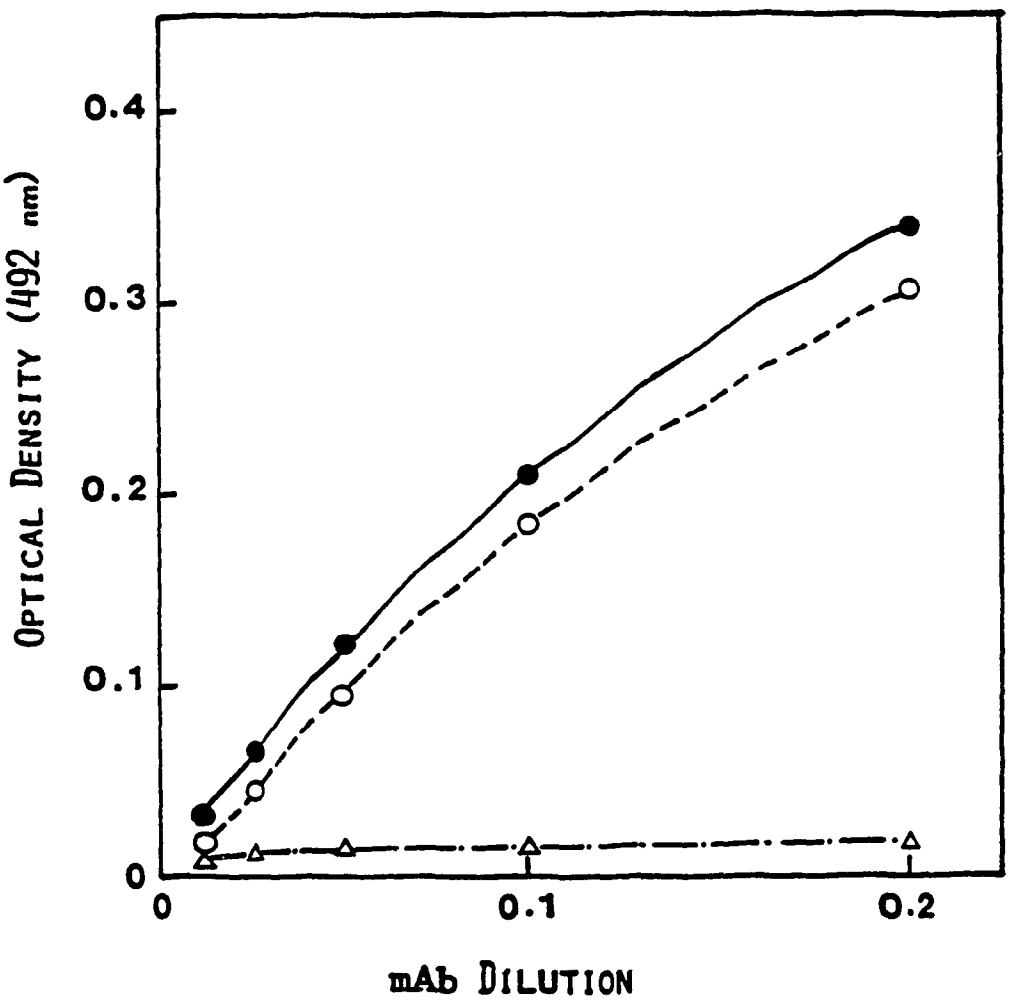

0.4
0.3
0.2
0.1
0
0
0.1
0.2
OPTICAL DENSITY (492 nm)
mAb DILUTION

**Figure 21.** Standard curve for the determination of IgM concentration. Enzyme-linked immunosorbent assay (ELISA) was applied and reference mouse serum containing 17.1 mg/100 mL IgMs was used as the standard.

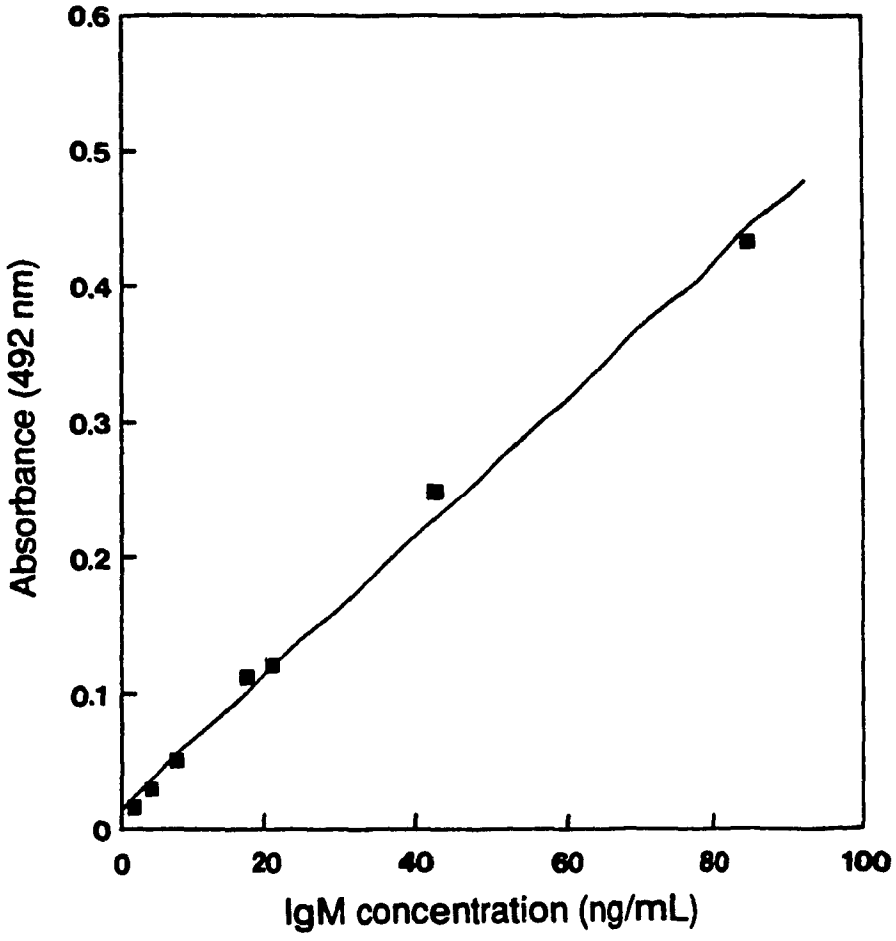

0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (492 nm)
0
20
40
60
80
100
IgM concentration (ng/mL)

**Figure 22.** Effect of bovine serum albumin on O-glucosyltransferase activity. The effect of different concentrations of bovine serum albumin (BSA) on 2'-O-glucosyltransferase (GT) activity was determined using the standard assay.

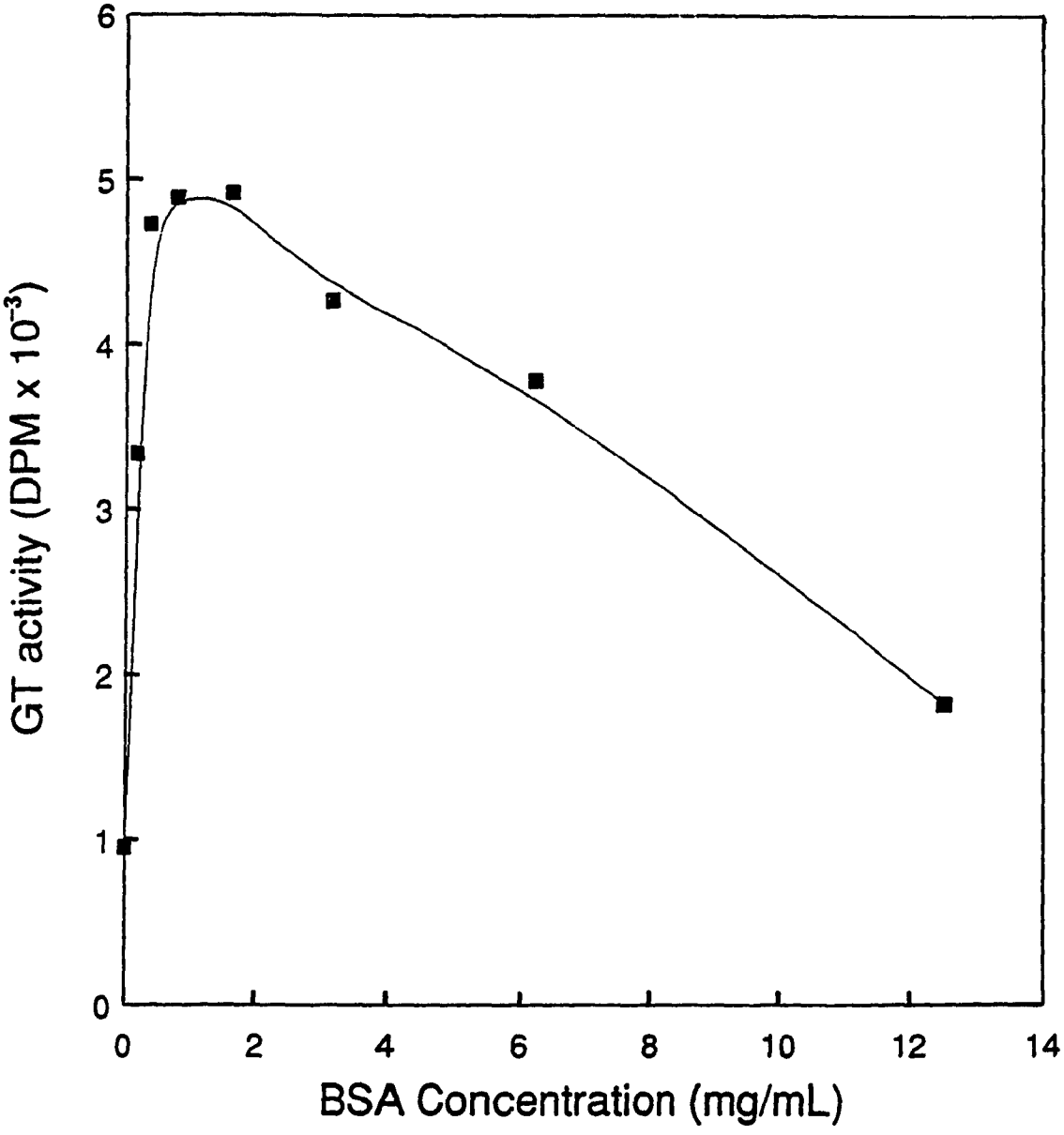
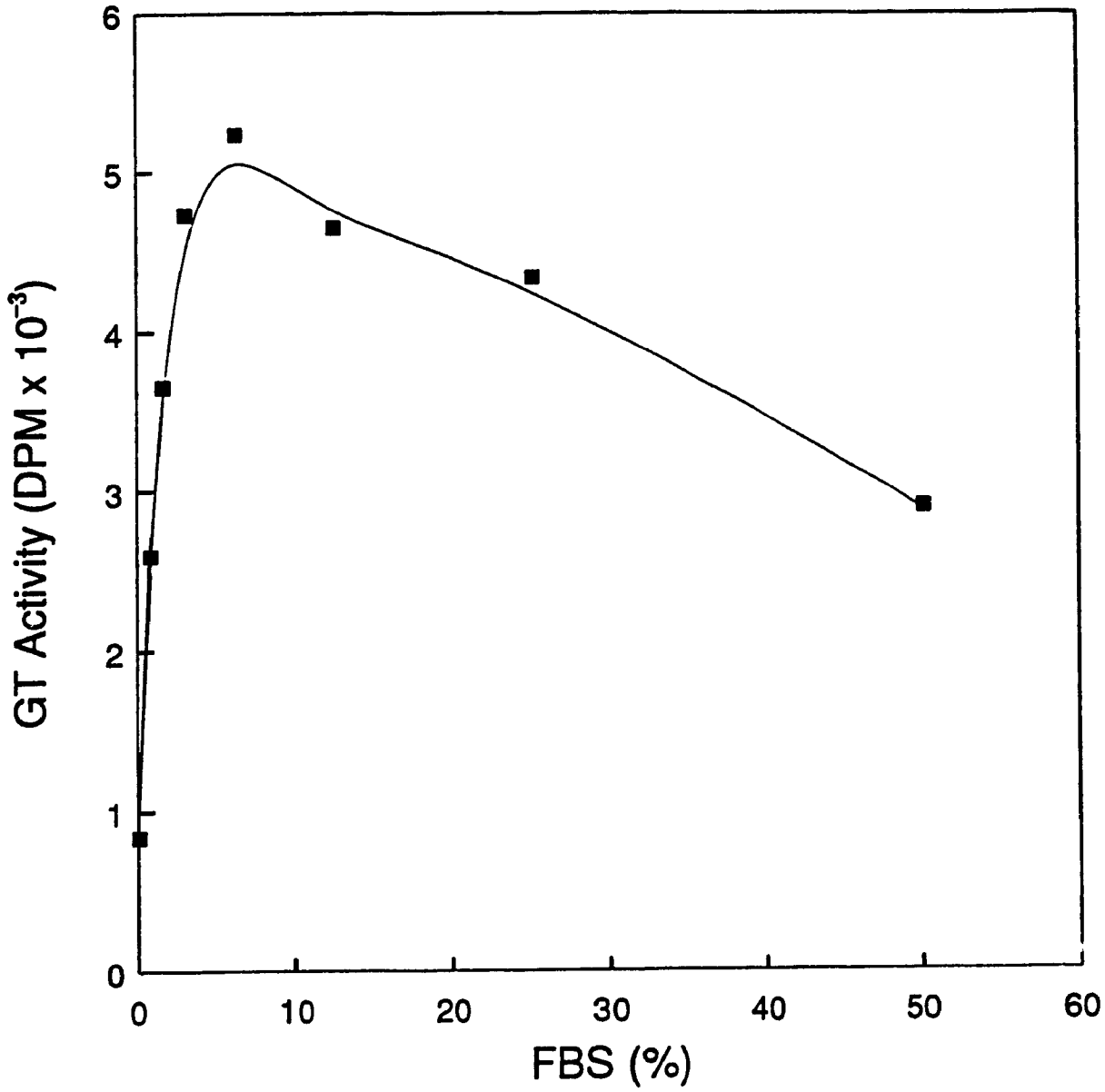

GT activity (DPM x $10^{-3}$)
BSA Concentration (mg/mL)
0
1
2
3
4
5
6
0
2
4
6
8
10
12
14

**Figure 23.** Effect of fetal bovine serum on O-glucosyltransferase activity. The effect of fetal bovine serum (FBS) on 2'-O-glucosyltransferase (GT) activity was determined using the standard assay.


GT Activity (DPM x $10^{-3}$)
6
5
4
3
2
1
0
0
10
20
30
40
50
60
FBS (%)

glucosyltransferase activity (Fig. 24), whereas immunoreactive C7-1 did not reveal any significant inhibition. In addition, undiluted C3-2 culture supernatant displayed more than 50% inhibition of the 2'-activity. The % inhibition decreased upon dilution of the supernatant (Fig. 24). Neither C3-2 nor C7-1 inhibited the 5'-O-glucosyltransferase activity. These results suggested that mAb C3-2 specifically inhibited the 2'-O-glucosyltransferase.

E.2.4.3. Immunoblotting

Further characterization of mAbs C3-2 and C7-1 was performed by Western blot analysis following nondenaturing- or SDS-gel electrophoresis. Monoclonal antibody C3-2, but not C7-1, displayed a significant immunoreactive band after electrophoretic transfer of proteins from nondenaturing-gels onto nitrocellulose (Fig. 25). This immunoreactive band corresponded with the region of 2'-O-glucosyltransferase activity on nondenaturing-gels. Immunodetection after SDS-PAGE of the enzyme preparation, using C3-2 and C7-1 culture supernatants, did not reveal immunoreactive bands. This suggested the recognition by mAb C3-2 of a determinant in the native conformation of the 2'-O-glucosyltransferase. A highly purified 5'-enzyme, obtained by applying the purification protocol described in Table 2, did not reveal any immunoreactive bands on Western blots using C3-2 or C7-1 culture supernatants. These results indicated that mAb C3-2 inhibits and binds only the 2'-form of Chrysosplenium O-

Figure 24. Inhibition of 2'-O-glucosyltransferase activity by monoclonal antibodies. The enzyme preparation was preincubated for 30 min at room temperature with different dilutions of monoclonal antibodies (mAb). ●, C3-2; o,C7-1; Δ, control supernatant. The remaining 2'-O-glucosyltransferase (2'-GT) activity was determined by the standard assay.

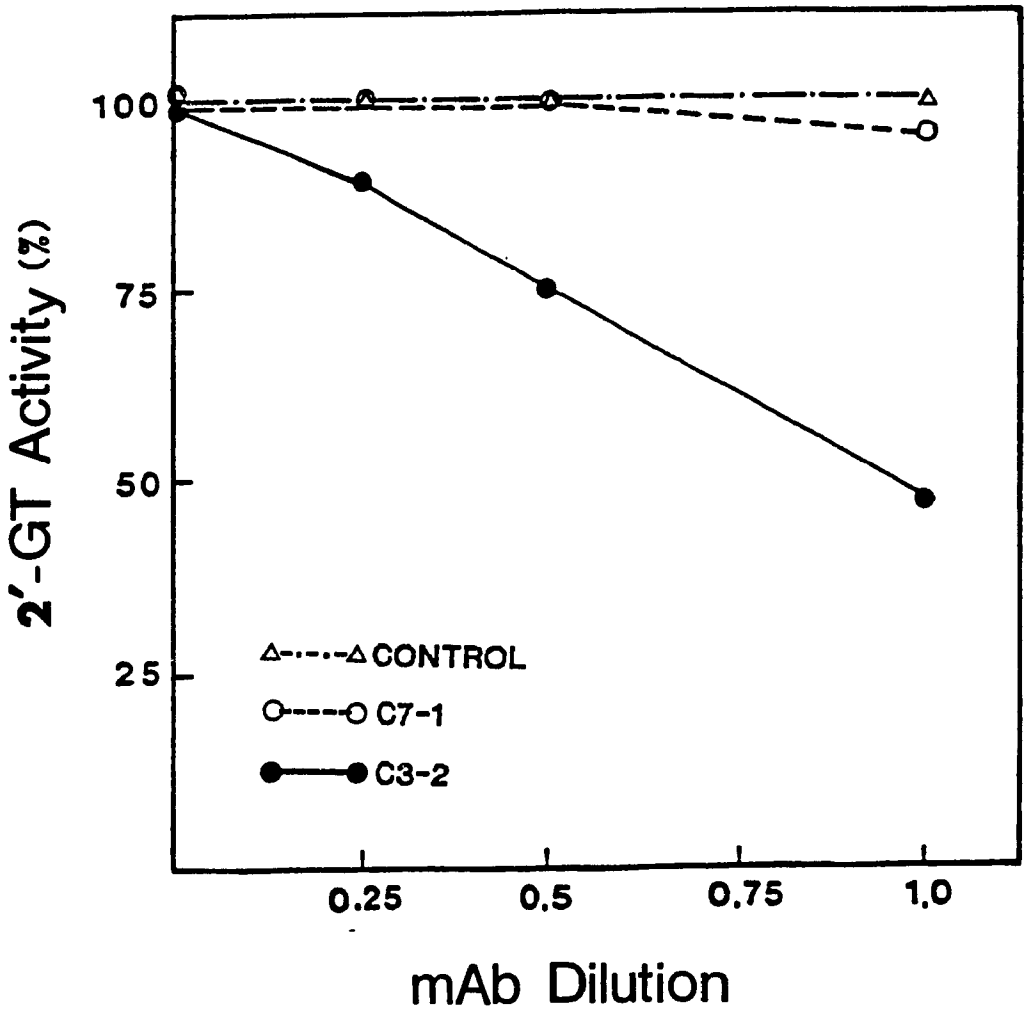

100
75
50
25
2'-GT Activity (%)
△-·-·-△ CONTROL
○----○ C7-1
●——● C3-2
0.25
0.5
0.75
1.0
mAb Dilution

Figure 25. Western blot analysis of monoclonal antibody C3-2. Partially purified O-glucosyltransferase preparation was subjected to 7.5% non-denaturing gel electrophoresis and transferred to nitrocellulose, followed by immunodetection using control supernatant (A) or monoclonal antibody C3-2 (B).

A B

glucosyltransferases.

Gel filtration is rarely used as a method for purifying IgG-type Ab. However, it is a standard technique for the separation of IgM-type Ab, since the size difference between IgM and contaminating proteins is so large that a reasonably high degree of purity can be obtained (86). Control and test supernatants were therefore subjected to purification by gel filtration on Superose 12 column (Fig. 26). Fractions containing IgM, as determined by ELISA, were pooled. Western blotting and inhibition studies using the purified IgM fractions gave results similar to the corresponding culture supernatants.

### E.3. Polyclonal anti-O-glucosyltransferase antibodies

#### E.3.1. Purification of 2'-/5'-O-glucosyltransferases to homogeneity

C. americanum 2'- and 5'-O-glucosyltransferases were co-purified to apparent homogeneity by successive chromatography on Sephacryl S-200, UDP-glucuronic acid-agarose, Mono P, Superose 12, and Mono Q columns. The above purification protocol resulted in an increase in the specific activity of flavonol O-glucosyltransferases by about 3510-fold as compared to the crude extract (Table 6). The 2'- and 5'-enzymes were not separated by applying this scheme of purification, and both appeared as a single band on SDS-gels (Fig. 27).

Figure 26. Elution profile of C3-2 IgM (●---●) from Superose 12 HR 16/50 column. The column was equilibrated and developed with 20 mM Tris-HCl (pH 7.4) and 2-mL fractions were collected and assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of IgM.

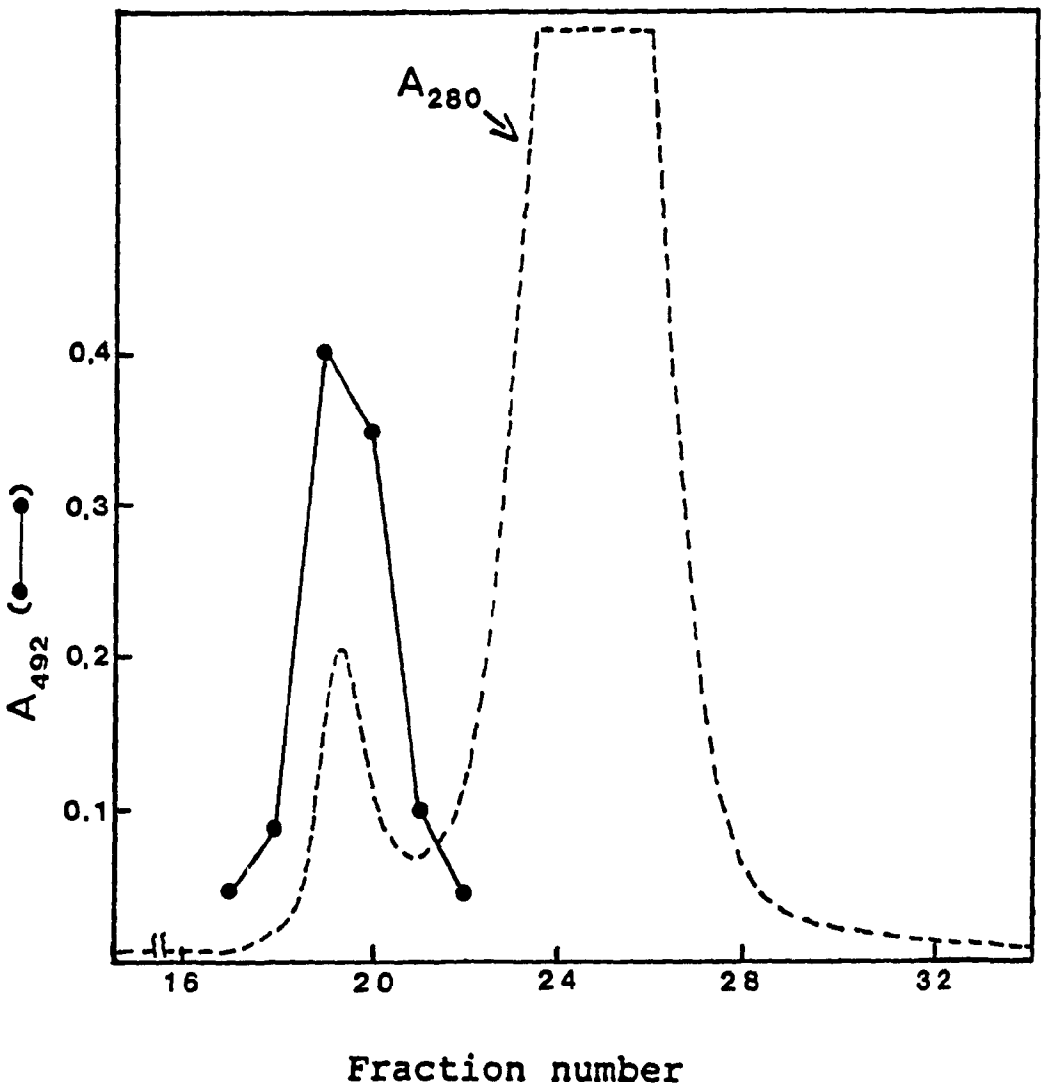
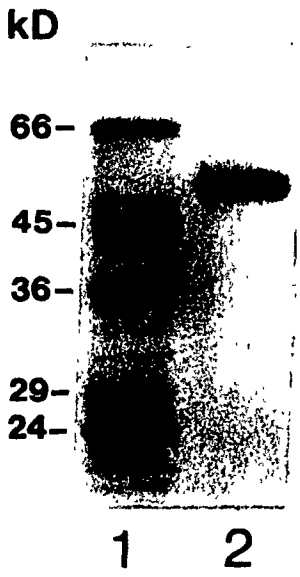

A280
0.4
0.3
0.2
0.1
A492
16
20
24
28
32
Fraction number

Table 6. Purification of Chrysosplenium flavonol O-glucosyltransferases.[a]

| Purification step | Total protein (mg) | Specific activity (pkat/mg) | Total activity (pkat) | Purification (-fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract[b] | 130 | 0.096 | 12.48 | - | 100 |
| Gel filtration[c] | 9.5 | 1.2 | 11.4 | 12.5 | 91 |
| Affinity chromatography[d] | 0.35 | 10.56 | 3.7 | 110 | 29 |
| Chromatofocusing[e] | 0.044 | 56.2 | 2.47 | 585 | 19 |
| Gel filtration[f] | 0.005 | 101.7 | 0.51 | 1059 | 4 |
| Ion-exchange chromatography[g] | <0.001 | 337 | 0.337 | 3510 | 2 |

[a] The standard assay using substrate I was applied.
[b] After treatment with Dowex 1-X2.
[c] On Sephacryl S-200 column.
[d] On UDP-glucouronic acid-agarose column.
[e] On Mono P cloumn using the FPLC system.
[f] On Superose 12 HR 10/30 column using the FPLC system.
[g] On Mono Q column using the FPLC system.

Figure 27. SDS-Polyacrylamide gel electrophoresis of purified 2'-/5'-O-glucosyltransferases. A 12% polyacrylamide gel was used. Lane 1, molecular weight standards; and lane 2, the purified O-glucosyltransferases.


kD
66-
45-
36-
29-
24-
1
2

**E.3.2. Molecular weight of 2'-/5'-O-glucosyltransferases**

The molecular weight of a highly purified O-glucosyltransferase preparation with both 2'- and 5'-activities was determined by gel filtration on a calibrated Superose 12 HR 16/50 column using the FPLC (Fig. 28). The collected fractions were assayed against substrates I and II under the normal assay conditions. Both enzyme forms had the same elution volume which corresponded to a molecular weight of 52±2.5 kD (Fig. 29). Furthermore, upon subjecting the homogeneous 2'-/5'-O-glucosyltransferases to SDS-PAGE, only one protein band was revealed by staining for protein (Fig. 27). The $R_f$ value of this band corresponded to a molecular weight of 52±2kD (Fig. 30).

**E.3.3. Production, characterization and purification of polyclonal antibodies**

The purified O-glucosyltransferase preparation was used as the source of Ag to raise polyclonal Abs in rabbits. The resulting immune serum exhibited positive reaction when tested by ELISA, and was purified by ammonium sulfate precipitation, desalted on Sephadex G-25, followed by chromatography on Mono Q column (Fig. 31). Fractions containing IgGs, as determined by ELISA, were pooled and further characterized by Western blot analysis (Fig. 32) and inhibition studies. Both 2'- and 5'-O-glucosyltransferase activities were not inhibited by the Abs. Western blotting following denaturing-PAGE of a partially purified preparation revealed a band corresponding to the O-

**Figure 28.** Elution profile of 2'-/5'-O-glucosyltransferases from Superose 12 HR 16/50 column. Purified O-glucosyltransferase preparation was applied to a calibrated Superose 12 column. The 2'-(o--o) and 5'-(●--●) activities were assayed using substrates I and II, respectively.

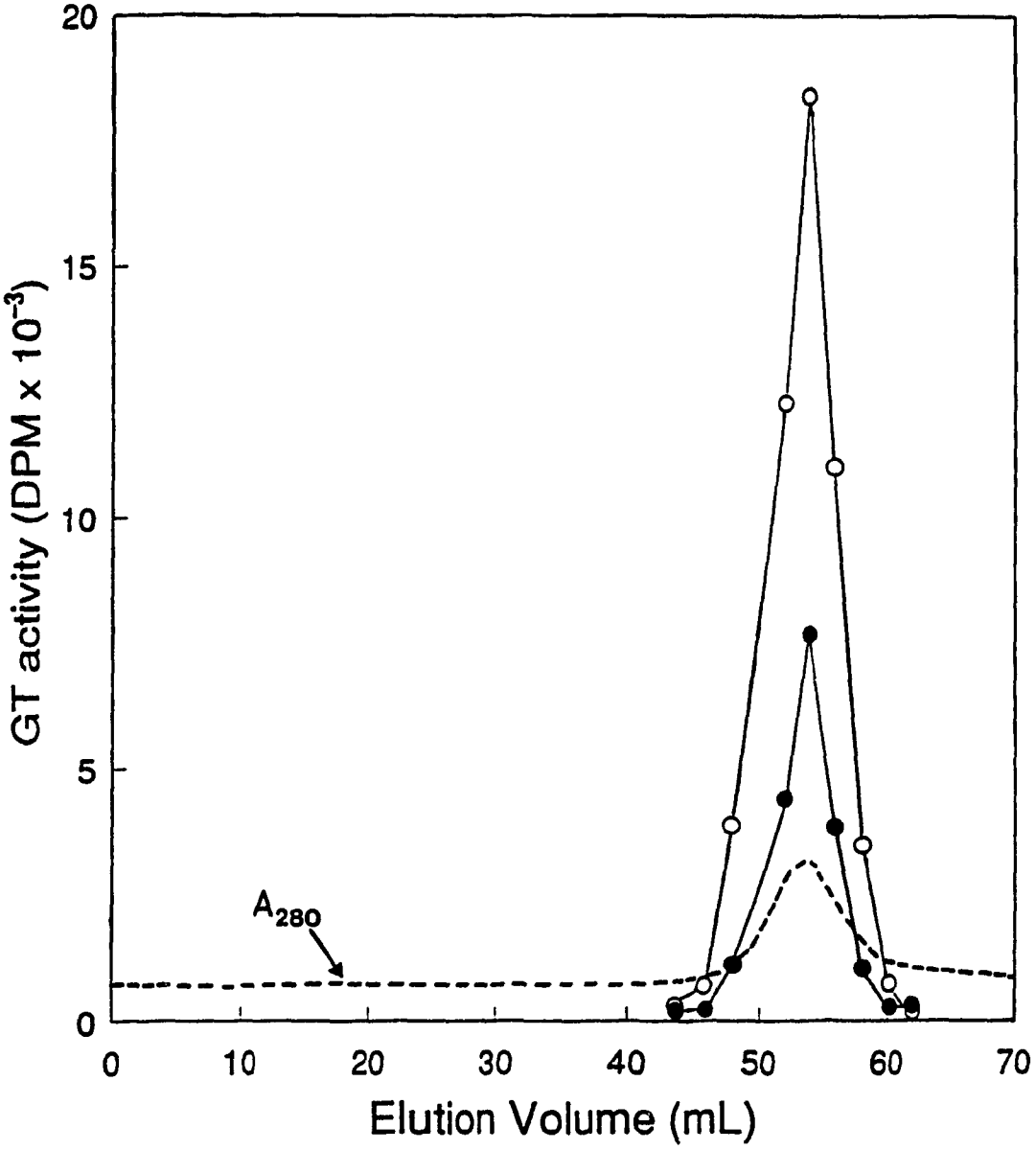

20
15
10
5
0
GT activity (DPM x 10$^{-3}$)
$A_{280}$
0
10
20
30
40
50
60
70
Elution Volume (mL)

**Figure 29.** Determination of molecular weight of native 2'-/5'-O-glucosyltransferases. A calibrated Superose 12 HR 16/50 column was used.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

where Vt = total volume; Vo = void volume; and Ve = elution volume.

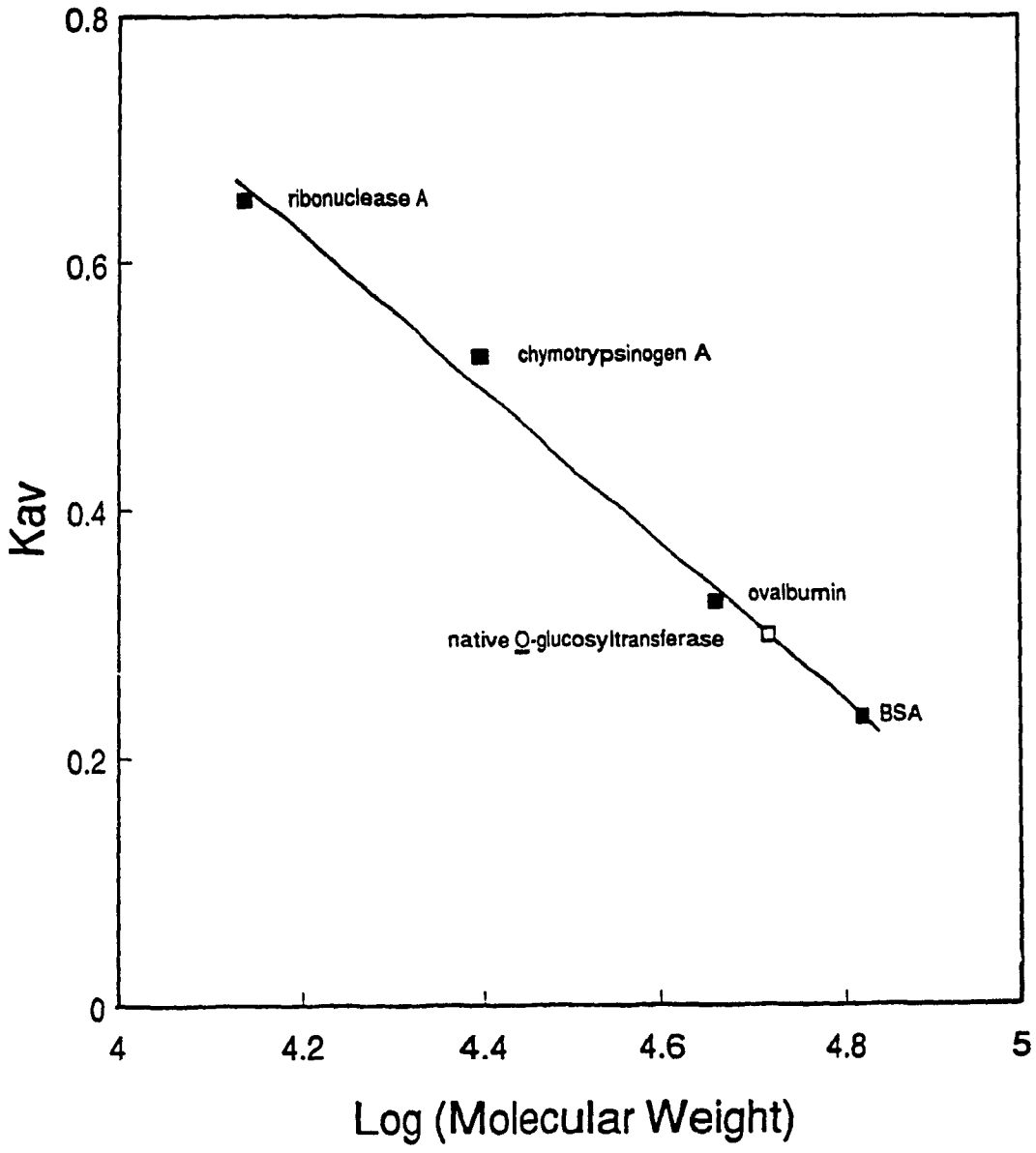

0.8
0.6
0.4
0.2
0
Kav
ribonuclease A
chymotrypsinogen A
ovalbumin
native O-glucosyltransferase
BSA
4
4.2
4.4
4.6
4.8
5
Log (Molecular Weight)

**Figure 30.** Determination of molecular weight of purified denatured 2'-/5'-O-glucosyltransferases. A 12% SDS-polyacrylamide gel was used.

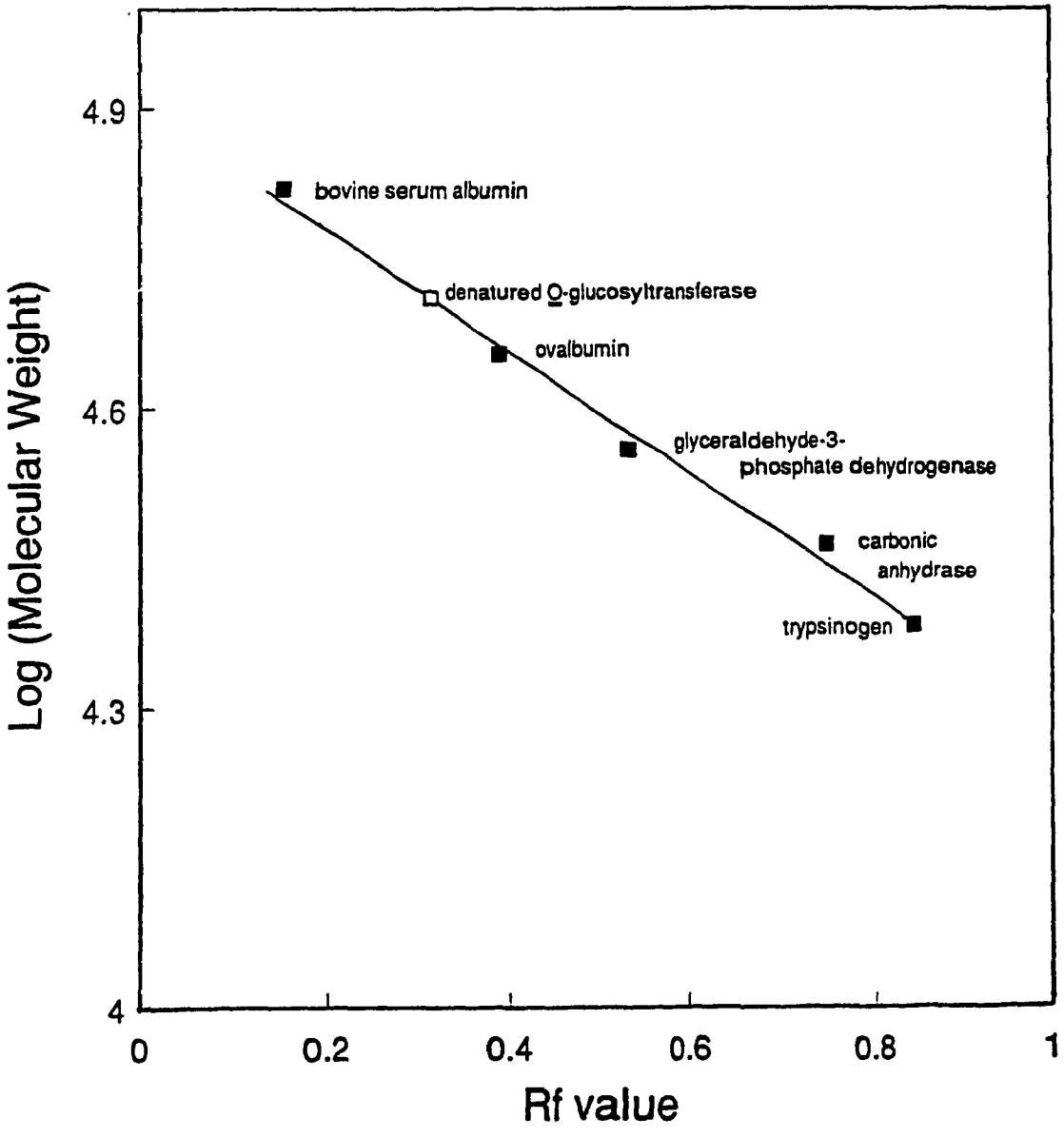

4.9
4.6
4.3
4
Log (Molecular Weight)
bovine serum albumin
denatured O-glucosyltransferase
ovalbumin
glyceraldehyde-3-
phosphate dehydrogenase
carbonic
anhydrase
trypsinogen
0
0.2
0.4
0.6
0.8
1
Rf value

Figure 31. Elution profile of IgGs (▪---▪) from Mono Q column. The column was equilibrated with 20 mM Tris-HCl (pH 7.4) and the bound proteins were eluted using a linear 0–0.5 M sodium chloride salt gradient. One-mL fractions were collected and assayed by enzyme-linked immunosorbent assay (ELISA) for IgGs.

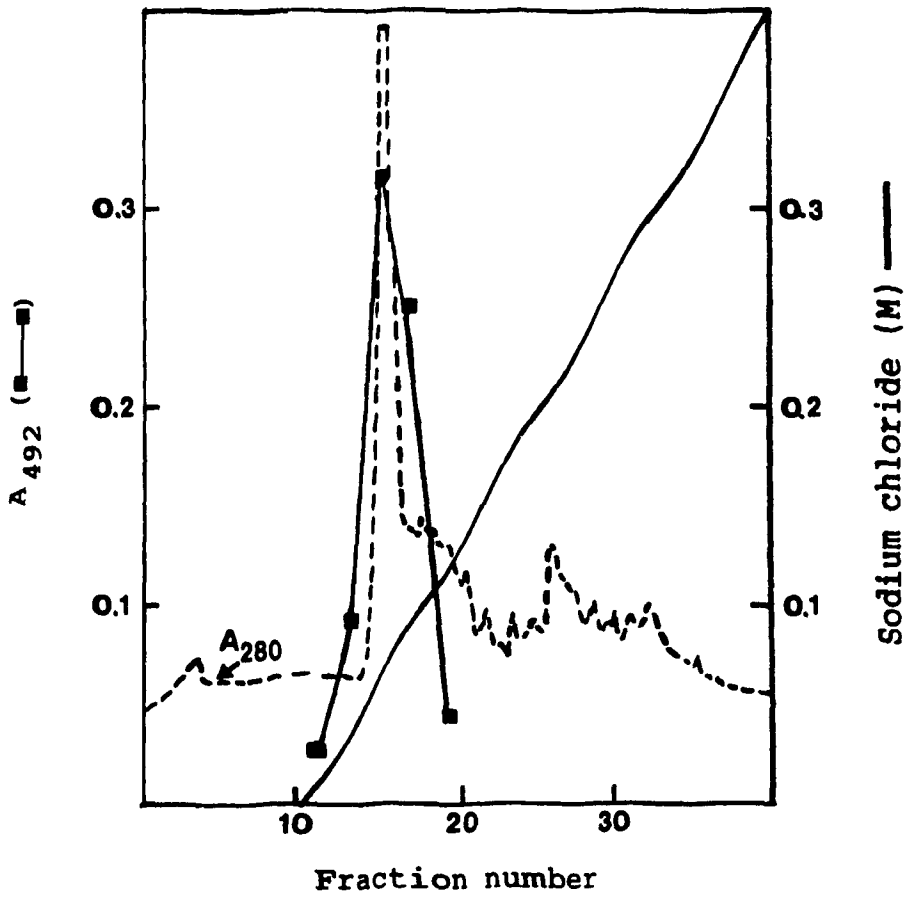
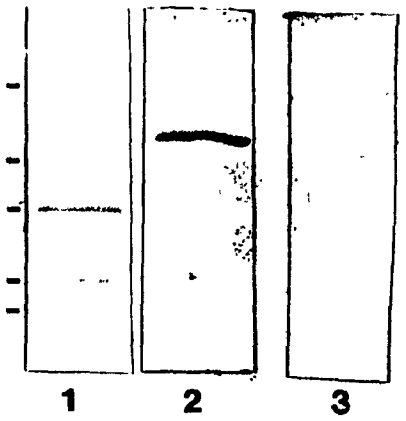

0.3
0.2
0.1
$A_{492}$ (■—■)
$A_{280}$
10
20
30
Fraction number
Sodium chloride (M) ——

Figure 32. Western blot analysis of a partially purified enzyme preparation following 12% SDS-gel electrophoresis. Lane 1, molecular weight standards stained for protein with amido black; lane 2, partially purified enzyme preparation immunodetected using anti-O-glucosyltransferase IgGs preadsorbed with protein impurities; and lane 3, control using preimmune serum IgGs. Antigen-antibody complexes were revealed using goat anti-rabbit IgG-peroxidase.


1
2
3

glucosyltransferases, as well as other cross-reacting bands. However, immunoblotting using impurity-preadsorbed IgGs revealed only one immunoreactive band corresponding to the 2'-/5'-enzymes (Fig. 32). The latter assured the monospecificity of the IgGs which were used for immunocytochemical studies.

## E.4. *In situ* localization of *Chrysosplenium* *O*-glucosyltransferases

### E.4.1. Retention of antigenicity versus ultrastructural preservation

Leaf samples embedded in Lowicryl K4M retained a high degree of protein antigenicity and exhibited a low amount of background labeling (Fig. 33). However, the ultrastructural preservation was poor (Fig. 33,34). On the other hand, post-fixation with osmium tetroxide followed by embedding in LR White, resulted in better preservation of membrane ultrastructure (Fig. 35,36) although protein antigenicity was greatly reduced (Fig. 35). This is not unexpected provided the low abundance of *O*-glucosyltransferases within *Chrysosplenium* cells, along with osmification and heat-curing of the specimens embedded in LR White which are harsh treatments and interfere with retention of protein antigenicity.

### E.4.2. Immunocytochemical localization of *O*-glucosyltransferases

The results shown in Figures 33, 37 and 38, and in Table 7, seem to indicate that the *O*-glucosyltransferases are

Figure 33. A C. americanum leaf section embedded in Lowicryl K4M showing epidermal cells labeled with anti-O-glucosyltransferase Abs followed by secondary Ab-gold (10 nm). Specific labeling of a vesicle-like structure (Ve) is evident. Note the association of gold particles with poorly preserved plasmalemma and accompanying membranes, bar=200 nm. CW, cell wall; Va, vacuole.

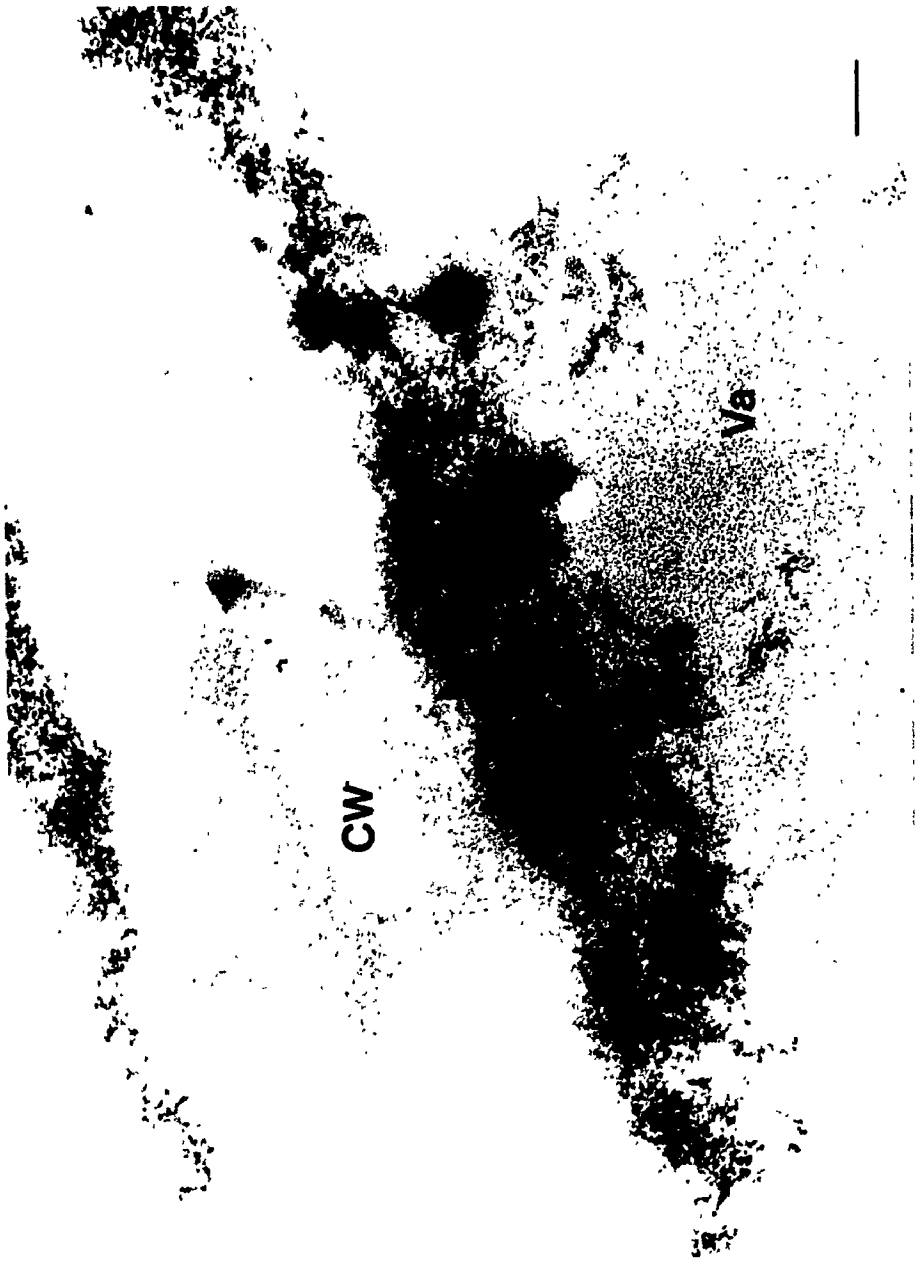

Va
CW

**Figure 34.** Control leaf section embedded in Lowicryl K4M treated with rabbit non-immune serum IgGs followed by secondary Ab-gold (10 nm). Note the poor preservation of the overall membrane ultrastructure, bar=200 nm. CW, cell wall; Va, vacuole.


SB
CW

Figure 35. A leaf section embedded in LR White showing epidermal cells treated with anti-O-glucosyltransferase Abs followed by secondary Ab-gold (20 nm). Note that structural preservation of the LR White-embedded tissue is superior to that of the Lowicryl K4M-embedded tissue (Fig. 33,34), while the density of gold particles is greatly reduced in the LR White section, bar=200 nm. CW, cell wall; Va, vacuole.

Va

**Figure 36.** Control leaf section embedded in LR White and treated with rabbit non-immune serum IgGs followed by secondary Ab-gold (20 nm). Note the better preservation of the membrane ultrastructure of the LR White-embedded section. Also note, a vesicle-like structure (Ve) with accompanying cytoplasmic membranes, bar=200 nm. CW, cell wall; Va, vacuole.


CW

Figure 37. Leaf sections embedded in Lowicryl K4M labeled with anti-O-glucosyltransferase Abs followed by secondary Ab-gold (10 nm). Shown is the specific association of gold particles with vesicle-like structures (Ve) and poorly preserved cytoplasmic membranes. (A) Bar=100 nm. (B) Bar=200 nm. CW, cell wall; Va, vacuole.

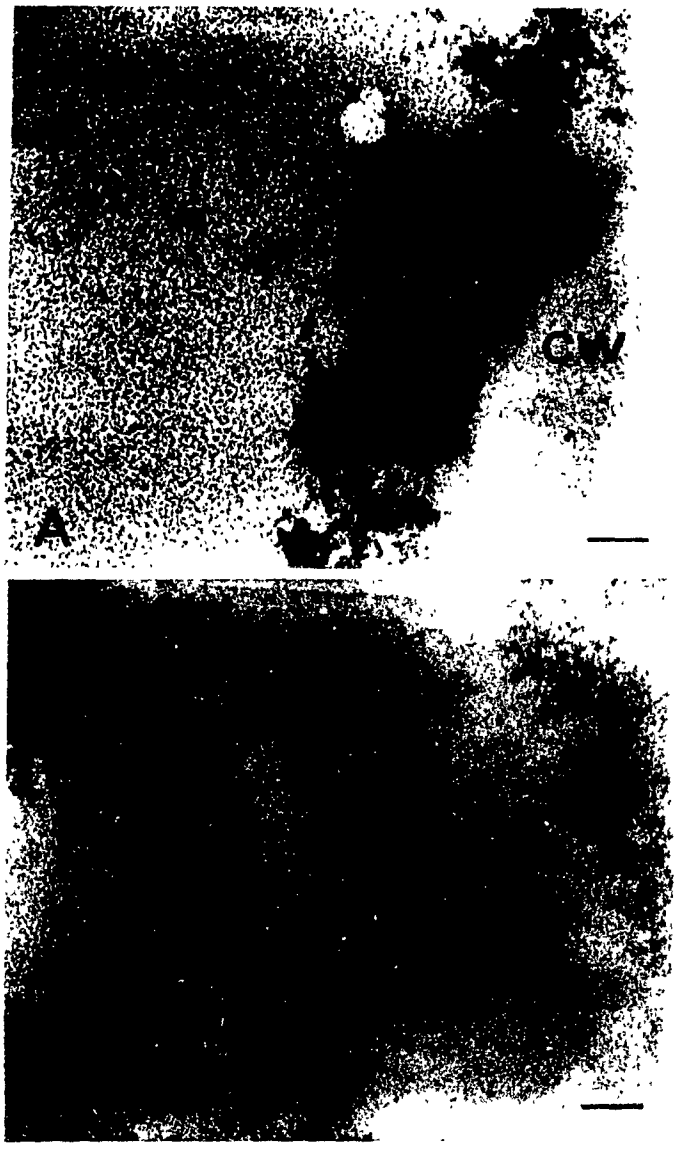

CV
A

Figure 38. Leaf sections embedded in Lowicryl K4M labeled with anti-O-glucosyltransferase Abs followed by secondary Ab-gold (10 nm). Shown is the specific association of gold particles with the plasmalemma (arrows) and accompanying membranes. The cell wall, CW; the Golgi apparatus, G; the mitochondria, M; and the chloroplast, Ch, are free from labeling. (A) Bar=100 nm. (B) Bar=200 nm.

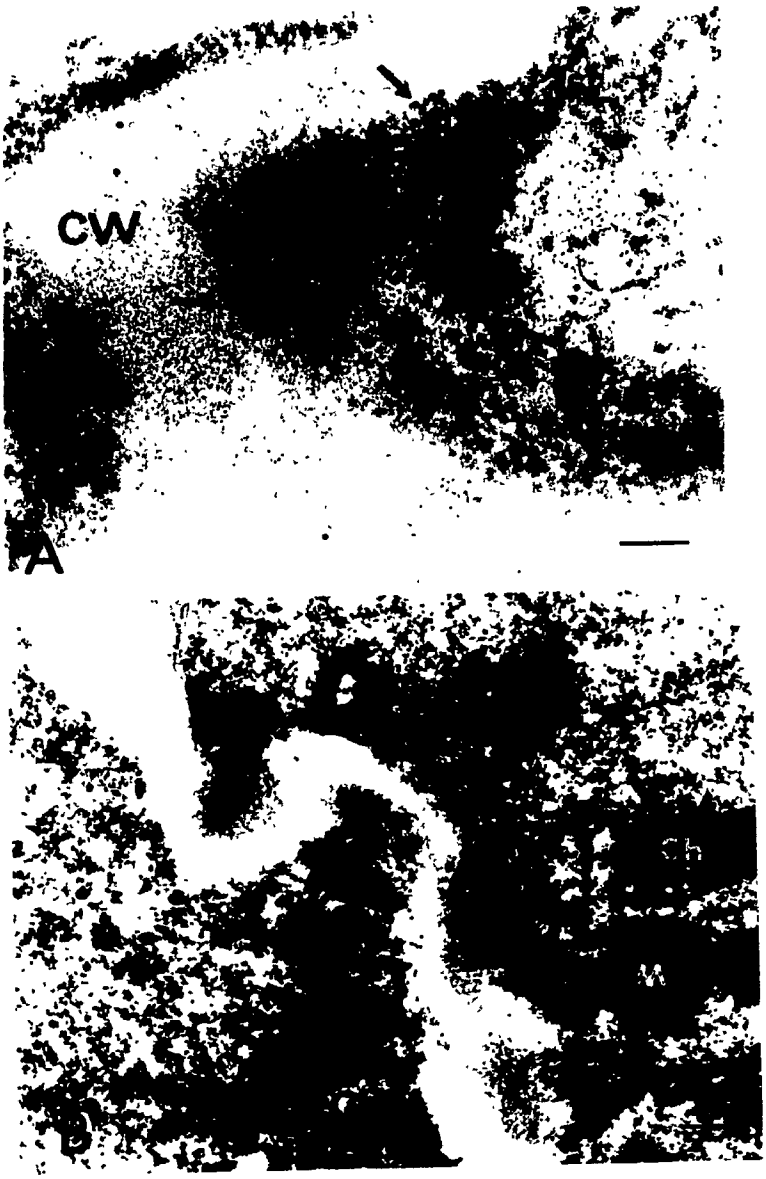

CW
A
B
Ch
M

**Table 7.** Density of immunogold labeling associated with different cellular compartments.[a,b]

| Cellular Compartment | Number of gold particles/$\mu m^2$ (Mean ± S.E.) | |
|---|---|---|
| | Test | Control |
| Plasmalemma and accompanying membranes | 142 ± 12 | 0.91 ± 0.3 |
| Vesicle-like structures | 749 ± 45 | 0.83 ± 0.4 |
| Cell wall | 2.5 ± 1.3 | 0.67 ± 0.4 |
| Vacuole | 3.8 ± 1.4 | 0.85 ± 0.4 |

[a] Using Lowicryl K4M-embedded sections.

[b] Fifteen test and ten control micrographs have been analyzed at x40,000.

associated with poorly preserved plasmalemma and the accompanying cytoplasmic membranes. Association of gold particles with vesicle-like structures is also evident (Fig. 33,37; Table 7). The cell wall, the vacuole, the mitochondria, the Golgi apparatus, and the chloroplast are not labeled (Fig. 38; Table 7).

### E.4.3. Specificity of labeling

Control sections treated with non-immune serum IgGs show negligible background labeling (Fig. 34, Table 7). Furthermore, elimination of the primary Abs, or preadsorption of the IgGs with purified enzyme preparation, resulted in a very low background labeling resembling that of the non-immune serum IgG control (Fig. 34).

## F. DISCUSSION

### F.1. Resolution of 2'- and 5'-O-glucosyltransferases

Previous work in this laboratory (52) indicated that both the 2'- and 5'-O-glucosyltransferase activities were not separable by conventional chromatography on gel filtration, ion-exchange or chromatofocusing columns. In this study, none of the high resolution columns, namely Superose 12 (Fig. 8), Mono Q (Fig. 9), and Mono P (Fig. 10), coupled with the FPLC system, could resolve the 2'- and 5'-enzyme activities. In view of the position specificity of the O-methyltransferases in this pathway (Fig. 4; 12), it is not unexpected that both O-glucosylation reactions are mediated by two distinct enzymes.

Affinity chromatography provides an elegant and efficient approach to the separation of individual enzymes from a complex mixture. Furthermore, the development of specific affinity adsorbents has proved to be a valuable tool for enzyme purification (116,117). Unlike other commonly employed fractionation methods, affinity chromatography makes use of the highly specific binding sites of enzymes, thus allowing their separation according to their ability to reversibly bind to the immobilized ligand. Furthermore, specific elution of the adsorbed enzyme with substrate or inhibitor strongly suggests that real affinity, bioaffinity, is involved in the interaction.

The kinetic mechanism of Chrysosplenium O-glucosyltransferase indicated that UDP-Glc (UDP) binds the free enzyme form (53). This kinetic mechanism, unlike that of the coniferyl alcohol O-glucosyltransferase (118), allowed the binding of the enzyme to the UDP-glucuronic acid-agarose affinity support (119), although it did not bind to UDP-agarose. Both 2'- and 5'-O-glucosyltransferase activities were eluted with a salt gradient in a single peak at approximately 0.26 M potassium chloride (Fig. 11). This step of purification resulted in about 10-fold purification (Table 2). The inability to elute the O-glucosyltransferase from UDP-glucuronic acid-agarose column with specific eluents, such as UDP or UDP-Glc, suggests the non-specific nature of the interaction. On the other hand, additional electrostatic interactions may have been involved. The latter would explain the need of high ionic strength buffer to elute the adsorbed enzyme. It should be noted that success of biospecific elution is dependent on the affinity as well as the concentration of the immobilized ligand (116).

In recent years, some of the widely used ligands in affinity chromatography have been a variety of reactive triazine-based textile dyes immobilized to agarose or other matrices (120-122). The mechanism of the binding of triazine dyes to enzymes is not clearly understood and has been the subject of a number of studies (for review see ref. 120). It has been suggested that these polyanionic aromatic dye

chromophores mimic the overall shape, size, and charge distribution of naturally occurring nucleotides and coenzymes (123-125). Furthermore, both electrostatic and hydrophobic forces have been reported to be involved in binding proteins to these dyes (126,127). This suggested that manipulation of the pH, the ionic strength, and the temperature of eluents is important in order to improve adsorption and subsequent elution of proteins from dye-ligand supports.

Dye-ligand affinity chromatography is increasingly being used for enzyme purification. It has proved to be a powerful tool for the separation of enzymes with closely related chromatographic properties. Schmid and Grisebach (118) reported the separation of coniferyl alcohol O-glucosyltransferase from a second peak of O-glucosyltransferase by chromatography on Matrex Gel red A column. Scawen et al. (128), reported the separation and purification of two bacterial dehydrogenases by chromatography on Procion red H3B-sepharose column. The latter two enzymes were difficult to separate by means of conventional chromatographic procedures (128).

In this study, of the several dye-ligand matrices used, the reactive brown 10-agarose was the only support which allowed binding and separation of the two O-glucosylating activities. This suggested selectivity of the enzymes for the chemical nature of this dye. The change of pH from 6.4 to 8 could effect the separation and elution of bound O-

glucosyltransferase activities (Fig. 13). Similarly, glucokinase, from a gram-negative bacteria, has been released from scarlet MX-G-sepharose column by the stepwise increase in buffer pH from 6 to 7 (129). The poor recovery of Chrysosplenium O-glucosyltransferases from the reactive brown 10-agarose column was partly circumvented by assisting pH elution with a shallow linear salt gradient. Introducing the dye-agarose step in a purification scheme (Table 2) significantly increased the specific activity of the 2'- and the 5'-enzymes. However, drastic changes of pH (from 6.4 to 8), during binding and elution, resulted in highly unstable enzymes with poor recovery of their activities. To further confirm the existence of two distinct O-glucosyltransferases, it seemed important to apply immunochemical studies.

**F.2. Production of an anti-2'-O-glucosyltransferase monoclonal antibody**

In this study, the novel technique of in vitro immunization (105,114,130) for the production of mAbs was applied. Many factors make in vitro immunization of spleen cells preferable to the classical in vivo technique (Fig. 5b; 105,114). These include the requirement for only nanogram quantities of Ag for immunization, short sensitization period (ca. 5 to 7 days), and the possibility to produce Abs to self-Ags which are not produced in vivo due to tolerance. In 1978, Hengartner et al. (131) reported, for the first time, sensitization of spleen cells in vitro followed by their

fusion with myeloma cells to produce specific mAbs. Luben and Mohler (132) have introduced the use of thymus-conditioned medium during *in vitro* sensitization period and found an increase in the number of Ab-secreting hybridomas. Boss (105) has recently developed a more simplified *in vitro* immunization protocol which utilizes a suitable culture medium with the sole addition of a commercially available adjuvant peptide, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (106). The latter is a synthetic analog of a mycobacterial cell wall component having the minimal structural requirement for adjuvant activity, and was used in this study. The mechanism of action of muramyl dipeptide has been the subject of a number of studies (133,134).

The hybridoma technology described by Köhler and Milstein (75,76) for the production of mAbs is particularly well-suited for producing specific Abs against a partially purified protein Ag, specially those which exist in minute quantities. Therefore, the difficulty of purifying the *O*-glucosyltransferases to apparent homogeneity has been circumvented by using a partially purified enzyme preparation (187-fold purification, Table 3) as the source of Ag. Furthermore, since the flavonol *O*-glucosylating proteins are minor components of the partially purified preparation, 2.5 or 12.5 μg of the latter was added per millilitre of the *in vitro* immunization culture medium, although nanogram quantities were reported to be sufficient (132).

The Ig class of the Abs produced by the hybridomas derived from *in vitro* immunization was determined by ELISA (Table 5). The majority of the hybridomas were producing IgM Abs. This result is in agreement with that of Erkman *et al.* (135) who reported the production of Abs only of the IgM class to a human plasma protein by applying the *in vitro* technique followed by fusion with myeloma cells, even though the immunization period was prolonged to over 5 days. This is not unexpected since *in vitro* immunization results in primary immune response where many of the immature, IgM-secreting cells are immortalized upon fusion with myeloma cells (132,135).

ELISA was also used to screen for hybridomas secreting Abs reacting with the partially purified enzyme preparation. However, the usual screening procedures by ELISA (which require pure Ag) to isolate individual cell lines secreting Abs of the required specificity were replaced by inhibition studies using the standard enzyme assay. Thus, in order to demonstrate the specificity of the produced mAbs for 2'- and 5'-*O*-glucosyltransferases, we studied the ability of C3-2 and C7-1 culture supernatants to inhibit enzyme activity. In order to prevent any possible artifacts, the following precautions were taken: (i) to avoid protein stabilization effect by the added mAb or control supernatants on the 2'- and 5'-*O*-glucosylating activities, the enzyme protein was previously stabilized with an optimum concentration of BSA (1.25 mg/mL);

(ii) the protein concentration of the test and control supernatants were made equal; and (iii) the enzyme preparation was used at concentrations low enough to avoid masking of the inhibition produced by the mAbs. Only mAb C3-2 inhibited the 2'-O-glucosylating activity (Fig. 24). Furthermore, neither C3-2 nor C7-1 culture supernatant inhibited the 5'-activity. Direct inhibition of enzyme activity by an Ab may be caused by Ab binding at the active site, steric interference of the Ab with substrate binding, or the Ab preventing conformational changes required for enzyme catalysis (136). Additionally, inhibition of enzyme activity by an Ab indicates that the latter recognizes an epitope which is part of the native structure of the enzyme protein. This seems to be the case with mAb C3-2 which displayed a significant immunoreactive band on Western blots only after nondenaturing-gel electrophoresis. This is further evidence for the recognition by mAb C3-2 of a determinant in the native spatial conformation of the 2'-enzyme, thus implying recognition of an assembled topographic epitope rather than a segmental continuous one (74,90). It should be pointed out that the inability of the mAb C3-2 to inhibit the 5'-enzyme does not exclude the possibility that it may bind to this enzyme species. However, the fact that the highly purified 5'-O-glucosyltransferase, obtained by applying the purification protocol described in Table 2, did not reveal any immunoreactive band upon Western blotting, indicates that mAb

C3-2 neither inhibits nor binds to this form of the enzyme. These results further supported the existence of two distinct O-glucosyltransferases in Chrysosplenium tissues, namely the 2'- and the 5'-enzymes.

Attempts to localize the 2'-O-glucosyltransferase in the plant tissue using mAb C3-2 and an immunogold labeling technique (91-93) were unsuccessful. In view of the strict single-site specificity of mAbs in general, the inability to localize the 2'-enzyme by immunocytochemistry was attributed to either: (i) alteration of enzyme protein antigenicity during processing of the plant tissue for electron microscopy; (ii) low abundance of the enzyme molecules; and/or (iii) masking of the specific mAb-binding site (epitope) on the 2'-O-glucosyltransferase by protein-protein interactions within the plant cell.

While a mAb provides information about the nature and location of a small portion of an Ag, polyclonal Abs provide information about the Ag as a whole. This is because the specificity of conventional polyclonal antisera is the result of hundreds of different clones or Ab populations. As a result, denaturation of a protein Ag, or partly masking it, usually has less significant effect on Ab-Ag interaction. Therefore, it seemed that the use of polyclonal Abs may be a good alternative to localize Chrysosplenium O-glucosyltransferases in situ by immunocytochemistry. However, the unique epitope specificity of mAbs was indispensable to

prove the distinctness of these greatly similar enzymes.

**F.3. Immunocytochemical localization of Chrysosplenium O-glucosyltransferases**

The next objective was to elucidate the site of localization of flavonol 2'-/5'-O-glucosyltransferases in C. americanum leaves by immunocytochemistry using polyclonal Abs. Production of polyclonal Abs requires pure protein Ag. Initially we intended to produce two polyclonal antisera, an anti-2'- and an anti-5'-O-glucosyltransferase. However, the instability of these two enzyme forms desorbed from the reactive brown 10-agarose column prompted the development of an alternative purification protocol (Table 6). Although the latter did not separate the two enzyme forms, it resulted in an apparently homogeneous protein (Fig. 27) which could be used as the source of Ag to raise polyclonal anti-2'/5'-O-glucosyltransferase Abs. The two O-glucosyltransferases displayed the same molecular weight upon gel filtration on a calibrated Superose 12 column (Fig. 28). Hence, it is not unexpected that both appear as a single band on denaturing-polyacrylamide gels (Fig. 27).

Although an apparently homogeneous protein was used as the source of Ag to raise polyclonal Abs, the antiserum produced displayed cross-reactions with other contaminating proteins on immunoblots. Monospecificity of Abs is a prerequisite for immunocytochemical work. Hence, the anti-O-glucosyltransferase IgGs were adsorbed against the

contaminating Ags cut out and eluted from SDS-polyacrylamide gels (137). Specificity of the preadsorbed IgGs was then verified by immunoblotting (Fig. 32).

The most important criteria for successful immunocytochemistry is a fixation-embedding protocol that not only provides good structural preservation of the tissue, but also retains intact antigenic sites within that tissue. The success of any one method also depends on the properties of the Ag under investigation. Properties such as the relative abundance of the Ag, and its ability to retain its antigenicity following tissue processing for immunocytochemistry (39,70,71,92). The hydrophilic properties of the embedding resin, Lowicryl K4M, result in excellent Ag retention and consequently in high label density, specificity, and low background (70,138). However, since osmium tetroxide post-fixation is incompatible with Lowicryl K4M embedding, membranes are poorly preserved (70,139). LR White, like Lowicryl K4M, is hydrophilic but it is compatible with osmium tetroxide post-fixation. Therefore, it allows better structural preservation (140).

The proper choice of embedding resin determines the consequent compromises between structural preservation and retention of antigenicity. This was evident in this study, and as shown in Figures 33 through 38, sections embedded in Lowicryl K4M retained a high degree of protein antigenicity, although ultrastructural preservation was relatively poor. On

the other hand, embedding in LR White greatly reduced the density of labeling while membrane ultrastructure was better preserved. Similar results were obtained by Herman (140) during the localization of an oil-body membrane protein in maturing soybean seeds embedded in Lowicryl K4M and LR White resins. Since retention of antigenicity at the expense of good preservation of membrane ultrastructure is an acceptable compromise, the use of Lowicryl K4M was preferred over LR White in this work. However working with LR White (Fig. 35,36) has allowed the visualization of the actual membrane ultrastructure and vesicles in Chrysosplenium tissues and thus, helped in the interpretation of the results obtained with Lowicryl K4M-embedded sections (Fig. 33,34,37,38).

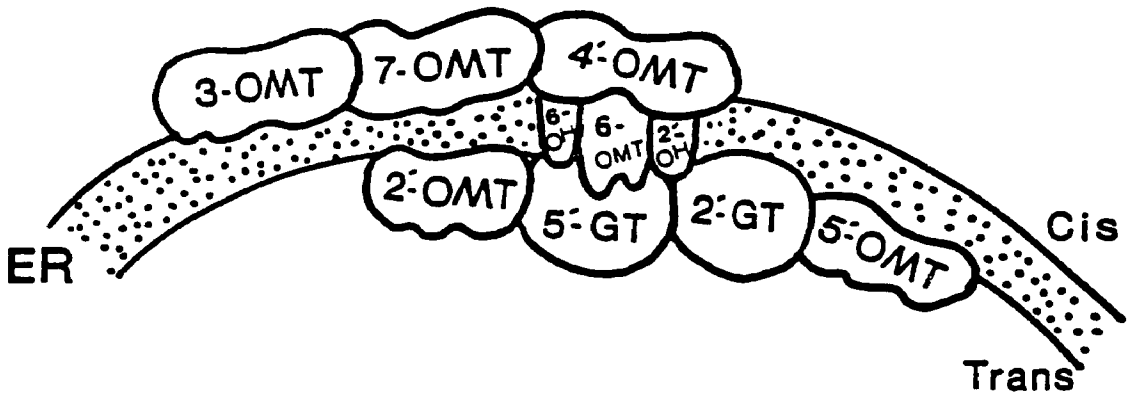
The results obtained in this study revealed that the flavonol O-glucosyltransferases are associated with the plasmalemma and its accompanying membranes, as well as with vesicle-like structures in the vicinity of the cell wall. These membranes most probably originated from the ER since the Golgi apparatus was free from any labeling (Fig. 38). This is not unexpected since previous ultrastructural studies also revealed that the Golgi was not involved in packaging or transport of polymethylated flavonol O-glucosides in the tissues of this plant (141).

**F.4. A proposed model**

Although the flavonol O-glucosyltransferases were always recovered in the cytosolic fraction, previous studies

suggested that they may be easily solubilized, rather than inherently soluble, proteins (12). This assumption was based on biosynthetic, enzymatic, and kinetic evidence and was further supported by ultrastructural studies (141). In situ localization of the flavonol O-glucosyltransferases was also carried out in order to provide additional evidence for the above model. This model (Fig. 39) postulated that the biosynthesis of C. americanum flavonoids takes place on the surface of an aggregated, membrane-associated multienzyme system, where the component enzymes are loosely associated or held together by weak non-covalent bonds (9). Such a compartmentation model facilitates the formation of metabolic chains of intermediates and allows for increased catalytic activity. The final metabolites are packaged into vesicles which would bud off the ER membrane. These vesicles would then move towards, and fuse with, the plasmalemma discharging their flavonoid content within the cell wall. In fact, electron microscopic studies of Chrysosplenium leaves, using caffeine as the fixative and visualizing reagent, showed that various electron-dense membrane profiles and associated vesicles were fused with the plasmalemma, indicating the secretory nature of these cells (141). Furthermore, by using an antibody against one of the major flavonoid O-glucosides, it was possible to localize the final metabolites in the walls of leaf epidermal and mesophyll cells by immunofluorescence (142) and immunogold labeling (143).

**Figure 39.** A proposed model for the compartmentation of the enzymes involved in the biosynthesis of polymethylated flavonol glucosides in C. americanum (12). GT, O-glucosyltransferase; OMT; O-methyltransferase; and OH, hydroxylase.


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

The results of immunogold labeling of the O-glucosyltransferases reported here suggest that the site of O-glucosylation in C. americanum is associated with cytoplasmic membranes and vesicles, most likely originating from the ER. In fact, many classical soluble enzymes are now perceived to operate in vivo as weakly bound aggregates which are dissociated during disintegration of cells (144).

To summerize, the results presented here have demonstrated the existence in C. americanum of two position-specific flavonol O-glucosyltransferases. The two enzyme activities could only be resolved by the use of a dye-ligand affinity support. The existence of two forms of Chrysosplenium O-glucosyltransferases was further supported by the production of a mAb which bound to and inhibited only one form of the O-glucosyltransferases. Furthermore, post-embedding immunocytochemical studies, using monospecific polyclonal Abs, demonstrated that the O-glucosyltransferases are associated with vesicle-like structures and cytoplasmic membranes.

## G. CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

The combined use of dye-ligand affinity chromatography and an FPLC system made it possible to separate, for the first time, two flavonol ring B-specific-O-glucosyltransferases from C. americanum shoot tips.

A mAb to the 2'-O-glucosyltransferase from C. americanum was produced by in vitro immunization of spleen cells from Balb/c mice, followed by fusion with mouse myeloma cells. The Ab produced inhibited the 2'- and not the 5'-O-glucosyltransferase. Furthermore, the native form of the 2'-enzyme was essential for binding, suggesting that the Ab recognizes a conformational discontinuous epitope.

The flavonol 2'-/5'-O-glucosyltransferases from C. americanum were co-purified to apparent homogeneity and used to produce polyclonal Abs. The latter were used for in situ localization studies of the enzymes in the plant tissues by applying a post-embedding immunogold labeling technique. The results of this study provided further evidence to a previously proposed model (12) for flavonoid biosynthesis in this plant.

## H. PERSPECTIVE FOR FUTURE WORK

The nature of the interaction of the flavonol O-glucosyltransferases with the reactive brown 10 as well as with other triazine dyes may be investigated. In fact, a number of synthetic dyes have proved very useful in studying the interaction of ligands with proteins. For example, inhibition of enzyme activity by a dye was utilized to determine the site of interaction on a dehydrogenase (145). Similar studies may provide a better understanding of the nature of the site of interaction in closely related proteins.

Unequivocal evidence for the proposed model (12; Fig. 39) requires the in situ localization of the O-glucosyltransferases and O-methyltransferases in Chrysosplenium tissues by applying immunogold double-labeling technique. This necessitates the purification of the individual O-methyltransferases to apparent homogeneity to raise polyclonal Abs. The O-methyltransferases in this plant are easily resolved by chromatofocusing (66). Alternatively, because of their similar molecular weights, it is possible to develop a purification scheme to co-purify the different O-methyltransferases as a pool to be used as the source of Ag to raise polyclonal Abs. Double-labeling (96) with anti-O-glucosyltransferase and anti-O-methyltransferase Abs would reveal the comparable sites of localization of these enzymes, and whether they are indeed closely-associated together and with cytoplasmic membranes and vesicles.

## I. REFERENCES


1. Mann, J. (1980) Secondary Metabolism, pp.1-19. Clarendon Press, Oxford.
2. Street, H.E. and Cockburn, W. (1972) Plant Metabolism, Second Edition, pp. 186-211. Pergamon Press, Oxford.
3. Mothes, K. (1980) In Encyclopedia of Plant Physiology: Secondary Plant Products, (Bell, E.A. and Charlwood, B.V. eds.), Vol. 8, pp. 1-10. Springer-Verlag, Berlin.
4. Geissman, T.A. and Crout, D.H.G. (1969) Organic Chemistry of Secondary Plant Metabolism, Chapter 1. Freeman Cooper and Company, San Francisco.
5. Bell, E.A. (1981) In The Biochemistry of Plants: A Comprehensive Treatise, (Conn, E.E. ed.), Vol. 7, pp. 1-19. Academic Press, New York.
6. Harborne, J.B. (1988) Introduction to Ecological Biochemistry, second edition. Academic Press, London.
7. Vickery, M.L. and Vickery, B. (1981) Secondary Plant Metabolism, pp. 1-19. University Park Press, Baltimore.
8. Luckner, M. (1980) In Encyclopedia of Plant Physiology: Secondary Plant Products, (Bell, E.A. and Charlwood, B.V. eds.), Vol. 8, pp. 23-63. Springer-Verlag, Berlin.
9. Stafford, H.A. (1981) In The Biochemistry of Plants: A Comprehensive Treatise, (Conn, E.E. ed.), Vol. 7, pp. 117-137. Academic Press, New York.
10. Hahlbrock, K. (1981) In The Biochemistry of Plants: A

Comprehensive Treatise, (Conn, E.E. ed.), Vol. 7, pp. 425-456. Academic Press, New York.

11. Ibrahim, R.K., Khouri, H., Brisson, L., Latchinian, L., Barron, D. and Varin, L. (1986) Bull. Liaison Groupe Polyphenols 13:3-14.

12. Ibrahim, R.K., DeLuca, V., Khouri, H., Latchinian, L., Brisson, L. and Charest, P.M. (1987) Phytochemistry 26:1237-1245.

13. Ibrahim, R.K., Latchinian, L. and Brisson, L. (1989) In Plant Cell Wall Polymers: Biogenesis and Biodegradation, (Lewis, N.G. and Paice, M.G. eds.), ACS Symposium Series No. 399, pp. 122-136. American Chemical Society, Washington.

14. Heller, W. and Forkmann, G. (1988) In The Flavonoids: Advances in Research since 1980, (Harborne, J.B. ed.), pp. 399-425. Chapman and Hall, London.

15. Cody, V., Middleton, E., Harborne, J.B. and Beretz, A. (eds.) (1988) Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties. Alan R. Liss, New York.

16. Wollenweber, E. and Jay, M. (1988) In The Flavonoids: Advances in Research since 1980, (Harborne, J.B. ed.), pp. 233-302. Chapman and Hall, London.

17. Harborne, J.B. and Williams, C.A. (1988) In The Flavonoids: Advances in Research since 1980, (Harborne, J.B. ed.), pp. 303-328. Chapman and Hall, London.

18. Chopin, J. and Dellamonica, G. (1988) In The Flavonoids: Advances in Research since 1980, (Harborne, J.B. ed.), pp. 63-97. Chapman and Hall, London.

19. Barron, D., Varin, L., Ibrahim, R.K., Harborne, J.B. and Williams, C.A. (1988) Phytochemistry 27:2375-2395.

20. Harborne, J.B. (1988) In Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular, and Medicinal Properties, (Cody, V., Middleton, E., Harborne, J.B. and Beretz, A. eds.) pp. 17-27. Alan R. Liss, New York.

21. Markham, K.R. (1988) In The Flavonoids: Advances in Research since 1980, (Harborne, J.B. ed.), pp. 427-468. Chapman and Hall, London.

22. Harborne, J.B. (1980) In Encyclopedia of Plant Physiology: Secondary Plant Products, (Bell, E.A. and Charlwood, B.V. eds.), Vol. 8, pp. 329-402. Springer-Verlag, Berlin.

23. Peters, N.K., Frost, J.W. and Long, S.R. (1986) Science 233:977-980.

24. Redmond, J.W., Batley, M., Djordjevic, M.A., Innes, R.W., Kuempel, P.L. and Rolfe, B.G. (1986) Nature 323:632-635.

25. Maxwell, C.A., Hartwig, U.A., Joseph, C.M. and Phillips, D.A. (1989) Plant Physiol. 91:842-847.

26. Peters, N.K. and Long, S.R. (1988) Plant Physiol. 88:396-400.

27. Dixon, R.A. (1986) Biol. Rev. 61:239-291.

28. Ebel, J. (1986) Annu. Rev. Phytopathol. 24:235-264.

29. Jacobs, M. and Rubery, P.H. (1988) Science 241:346-349.

30. Wiermann, R. (1981) In The Biochemistry of Plants: A Comprehensive Treatise, (Conn, E.E. ed.), Vol. 7, pp. 85-116. Academic Press, New york.

31. Schmelzer, E., Jahnen, W. and Hahlbrock, K. (1988) Proc. Natl. Acad. Sci. USA 85:2989-2993.

32. Hahlbrock, K. and Grisebach, H. (1975) In The Flavonoids, (Harborne, J.B., Mabry, T.J. and Mabry, H. eds.), pp. 866-915. Chapman and Hall, London.

33. Ebel, J. and Hahlbrock, K. (1982) In The Flavonoids: Advances in Research, (Harborne, J.B. and Mabry, T.J. eds.), pp. 641-679. Chapman and Hall, London.

34. Hahlbrock, K. and Scheel, D. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:347-369.

35. Hinderer, W. and Seitz, H.U. (1988) In Cell Culture and Somatic Cell Genetics of Plants, (Constabel, F. and Vasil, I.K. eds.) Vol. 5, pp. 23-48. Academic Press, New York.

36. Grisebach, H. (1979) Rec. Adv. Phytochem. 12:221-248.

37. Jensen, R.A. (1985) Physiol. Plant. 66:164-168.

38. Dangl, J.L., Hahlbrock, K. and Schell, J. (1989) In Cell Culture and Somatic Cell Genetics of Plants, (Constabel, F. and Vasil, I.K. eds.) Vol. 6, pp. 155-173. Academic Press, New York.

39. Ibrahim, R.K. (1990) Phytochem. Anal. (in press).

40. Hrazdina, G., Zobel, A.M. and Hoch, H.C. (1987) Proc. Natl. Acad. Sci. USA 84:8966-8970.

41. Beerhues, L., Robenek, H. and Wiermann, R. (1988) Planta 173:544-553.

42. Hösel, W. (1981) In The Biochemistry of Plants: A Comprehensive Treatise, (Conn, E.E. ed.), Vol. 7, pp. 725-753. Academic Press, New York.

43. Sutter, A., Ortmann, R. and Grisebach, H. (1972) Biochim. Biophys. Acta 258:71-87.

44. Grisebach, H. (1965) In Chemistry and Biochemistry of Plant Pigments, (Goodwin, T.W. ed.), pp. 279-308. Academic Press, London.

45. Kreuzaler, F. and Hahlbrock, K. (1973) Phytochemistry 12:1149-1152.

46. Matern, U., Heller, W. and Himmelspach, K. (1983) Eur. J. Biochem. 133:439-448.

47. Koster,J., Strack, D. and Barz, W. (1983) Planta Med. 48:131-135.

48. Kamsteeg, J., van Brederode, J., Hommels, C.H. and van Nigtevecht, G. (1980) Biochem. Physiol. Pflanzen 175:403-411.

49. Matern, U., Potts, J.R.M. and Hahlbrock, K. (1981) Arch. Biochem. Biophys. 208:233-241.

50. Matern, U., Feser, C. and Hammer, D. (1983) Arch. Biochem. Biophys. 226:206-217.

51. Khouri, H.E., Ishikura, N. and Ibrahim, R.K. (1986)

Phytochemistry 25:2475-2479.

52. Bajaj, K.L., DeLuca, V., Khouri, H.E. and Ibrahim, R.K. (1983) Plant Physiol. 72:891-896.

53. Khouri, H.E. and Ibrahim, R.K. (1984) Eur. J. Biochem. 142:559-564.

54. Schulz, M. and Weissenbock, G. (1988) Phytochemistry 27:1261-1267.

55. Lewinsohn, E., Britsch, L., Mazur, Y. and Gresel, J. (1989) Plant Physiol. 91:1323-1328.

56. McIntoch, C.A., Latchinian, L. and Mansell, R.L. (1990) Arch. Biochem. Biophys. (in press).

57. Hrazdina, G. (1988) Biochim. Biophys. Acta 955:301-309.

57a. Dedonder, R.A. (1961) Ann. Rev. Biochem. 30:347-382.

58. Blume, D.E., Jaworski, J.G. McClure, J.W. (1979) Planta 146:199-202.

59. Hrazdina, G., Marx, G.A. and Hoch, H.C. (1982) Plant Physiol. 70:745-748.

60. Wagner, G.J. and Hrazdina, G. (1984) Plant Physiol. 74:901-906.

61. Jay, M. and Voirin, B. (1976) Phytochemistry 15:517-519.

62. Bohm, B.A., Collins, F.W. and Bose, R. (1977) Phytochemistry 16:1205-1209.

63. Bohm, B.A., and Collins, F.W. (1979) Biochem. Syst. Ecol. 7:195-201.

64. Collins, F.W., DeLuca, V., Ibrahim, R.K., Voirin, B.

and Jay, M. (1981) Z. Naturforsch. 36c:730-736.

65. DeLuca, V. and Ibrahim, R.K. (1985) Arch. Biochem. Biophys. 238:596-605.

66. Khouri, H.E. and Ibrahim, R.K. (1987) J. Chromatogr. 407:291-297.

67. Khouri, H.E., DeLuca, V. and Ibrahim, R.K. (1988) Arch. Biochem. Biophys. 265:1-7.

68. DeLuca, V. and Ibrahim, R.K. (1985) Arch. Biochem. Biophys. 238:606-618.

69. Knox, R.B. (1982) In Antibody as a Tool, (Marchalonis, J.J. and Warr, G.W. eds.), pp. 293-346. John Wiley & Sons, New York.

70. Herman, E.M. (1988) Annu. Rev. Plant Physiol. Plant Mol. Biol. 39:139-155.

71. Larsson, L.-I. (1988) Immunocytochemistry: Theory and Practice. CRC Press, Boca Raton, Florida.

72. Atassi, M.Z. Eur. J. Biochem. (1984) 145:1-20.

73. Benjamin, D.C. et al. (1984) Annu. Rev. Immunol. 2:67-101.

74. Barlow, D.J., Edwards, M.S. and Thornton, J.M. (1986) Nature 322:747-748.

75. Köhler, G. and Milstein, C. (1975) Nature 256:495-497.

76. Köhler, G. and Milstein, C. (1976) Eur. J. Immunol. 6:511-519.

77. Garvey, J.S., Cremer, N.E. and Sussdorf, D.H. (eds.) (1977) Methods in Immunology, Third Edition. Benjamin,

W.A. Inc., Reading, Massachusetts.

78. Hurn, B.A.L. and Chantler, S.M. (1980) Methods Enzymol. 70:104-142.

79. Campbell, A.M. (1984) Laboratory Techniques in Biochemistry and Molecular Biology, (Burdon, R.H. and van Knippenberg, P.H. eds.), Vol.13. Elsevier, Amsterdam.

80. Goding, J.W. (ed.) (1986) Monoclonal Antibodies: Principles and Practice, Second Edition. Academic Press, London.

81. Campbell, A.M. Ref. 79, pp. 33-65.

82. Engvall, E. (1980) Methods Enzymol. 70:419-439.

83. Hu, C., Carbonera, D. and van Huystee, R. (1987) Plant Physiol. 85:299-303.

84. Goldberg, I.J., Paterniti, J.R., France, D.S., Martinelli, G. and Cornicelli, J.A. (1986) Biochim. Biophys. Acta 878:168-176.

85. Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76:4350-4354.

86. Warr, G.W. (1982) In Antibody as a Tool, (Marchalonis, J.J. and Warr, G.W. eds.), pp. 59-96. John Wiley & sons, New York.

87. Burchiel, S.W., Billman, J.R. and Alber, T.R. (1984) J. Immunol. Methods 69:33-42.

88. Campbell, A.M. Ref. 79, pp. 1-32.

89. Goding, J.W. Ref. 80, pp. 5-58.

90. Berzofsky, J.A. (1985) Science 229:932-940.

91. Bendayan, M. (1984) J. Electron Microsc. Tech. 1:243-270.

92. Sternberger, L.A. (1974) Immunocytochemistry. Prentice-Hall, New Jersey.

93. Roth, J. (1986) J. Microsc. 143:125-137.

94. McCurdy, D.W. and Pratt, L.H. (1986) J. Cell Biol. 103:2541-2550.

95. Slot, J.W. and Geuze, H.J. (1981) J. Cell Biol. 90:533-536.

96. Bendayan, M. (1982) J. Histochem. Cytochem. 30:81-85.

97. Doerr-Schott, J. and Lichte, C.M. (1986) J. Histochem. Cytochem. 34:1101-1104.

98. Kearney, J.F., Radbruch, A., Liesegang, B. and Rajewsky, K. (1979) J. Immunol. 123:1548-1550.

99. Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121:404-427.

100. Laemmli, U.K. (1970) Nature 227:680-685.

101. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244:4406-4412.

102. Andrews, P. (1965) Biochem. J. 96:595-606.

103. Bradford, M.M (1976) Anal. Biochem. 72:248-254.

104. Recommendations 1978 of the NC-IUB (1979) Eur. J. Biochem. 97:319-320.

105. Boss, B.D. (1986) Methods Enzymol. 121:27-33.

106. Ellouz, F., Adam, A., Ciorbaru, R. and Lederer, E. (1974) Biochem. Biophys. Res. Comm. 59:1317-1325.

107. Lane, R.D., Crissman, R.S. and Ginn, S. (1986) Methods Enzymol. 121:183-192.

108. Carlemalm, E., Garavito, R.M. and Villiger, W. (1982) J. Microsc. 126:123-143.

109. Bendayan, M. and Zollinger, M. (1983) J. Histochem. Cytochem. 31:101-109.

110. Craig, S. and Goodchild, D.J. (1984) Protoplasma 122:35-44.

111. Johnson, D.A., Gantsch, J.W., Sportsman, J.R. and Elder, J.H. (1984) Gene Anal. Tech. 1:3-8.

112. Reynolds, E.S. (1963) J. Cell Biol. 17:208-213.

113. Weibel, E.R. (1973) In Principles and Techniques of Electron Microscopy: Biological Applications, (Hayat, M.A. ed.), Vol. 3, pp. 237-296. Van Nostrand Reinhold, New York.

114. Reading, C.L. (1982) J. Immunol. Methods 53:261-291.

115. Takahashi, M., Fuller, S.A. and Hurrell, J.G.R. (1987) J. Immunol. Methods 96:247-253.

116. Wilchek, M., Miron, T. and Kohn, J. (1984) Methods Enzymol. 104:3-55.

117. Lowe, C.R. (1979) An Introduction to Affinity Chromatography: Laboratory Techniques in Biochemistry and Molecular Biology (Work, T.S. and Work, E. eds.). North-Holland, Amsterdam.

118. Schmid, G. and Grisebach, H. (1982) Eur. J. Biochem. 123:363-370.

119. Singh, O.M.P., Graham, A.B. and Wood, G.C. (1981) Eur. J. Biochem. 116:311-316.

120. Lowe, C.R., Small, D.A.P. and Atkinson, A. (1981) Int. J. Biochem. 13:33-40.

121. Lowe, C.R. and Pearson, J.C. (1984) Methods Enzymol. 104:97-113.

122. Dean, P.D.G. and Watson, D.H. (1979) J. Chromatogr. 165:301-319.

123. Thompson, S.T., Cass, R. and Stellwagen, E. (1976) Anal. Biochem. 72:293-296.

124. Baird, J.K., Sherwood, R.F., Carr, R.J.G. and Atkinson, A. (1976) FEBS Lett. 70:61-66.

125. Edwards, R.A. and Woody, R.W. (1979) Biochemistry 18:5197-5204.

126. Beissner, R.S. and Rudolph, F.B. (1978) J. Chromatogr. 161:127-135.

127. Jankowski W.J., Von Muenchhausen, W., Sulkowski, E. and Carter, W.A. (1976) Biochemistry 15:5182-5187.

128. Scawen, M.D., Darbyshire, J., Harvey, M.J. and Atkinson, T. (1982) Biochem. J. 203:699-705.

129. Scopes, R.K., Testolin, V., Storer, A., Griffiths-Smith, K. and Algar, E.M. (1985) Biochem. J. 228:627-634.

130. Reading, C.L. (1986) Methods Enzymol. 121:18-27.

131. Hengartner, H., Luzzati, A.L. and Schreier, M. (1978) Curr. Top. Microbiol. Immunol. 81:92-99.

132. Luben, R.A. and Mohler, M.A. (1980) Mol. Immunol. 17:635-639.

133. Specter, S., Cimprich, R., Friedman, H. and Chedid, L. (1978) J. Immunol. 120:487-491

134. Watson, J. and Whitlock, C. (1978) J. Immunol. 121:383-389.

135. Erkman, L., Soldati, G., James, R.W. and Kato, A.C. (1987) J. Immunol. Methods 98:43-52.

136. Ball, W.J., Schwartz, A and Lessard, J.L. (1982) Biochim. Biophys. Acta 719:413-423.

137. Mayer, R.J. and Walker, J.H. (1978) In Techniques in the Life Sciences, (Kornberg, H.L., Metcalfe, J.C., Northcote, D.H., Pogson, C.I. and Tipton, K.F. eds.), Vol. B1/II, B119, pp. 1-32. Elsevier/North-Holland Biomedical Press, Amsterdam.

138. Roth, J. (1989) Methods Cell Biol. 31:513-552.

139. Sossountzov, L., Sotta, B., Maldiney, R., Sabbagh, I. and Miginiac, E. (1986) Planta 168:471-481.

140. Herman, E.M. (1987) Planta 172:336-345.

141. Charest, P.M., Brisson, L. and Ibrahim, R.K. (1986) Protoplasma 134:95-101.

142. Brisson, L., Vacha, W.E.K. and Ibrahim, R.K. (1986) Plant Sci. 44:175-181.

143. Marchand, L., Charest, P.M. and Ibrahim, R.K. (1987) J. Plant Physiol. 131:339-348.

144. Roos, W. and Luckner, M. (1986) In Cell Metabolism:

Growth and Environment, (Subramanian, T.A.V. ed.), Vol. 1, pp. 45-73. CRC Press, Boca Raton, Florida.

145. Small, D.A., Lowe, C.R., Atkinson, T. and Bruton, C.J. (1982) Eur. J. Biochem. 128:119-123.