

Purification, Characterization and Kinetic Analysis of a
Flavonol-Ring B O-Glucosyltransferase From
Chrysosplenium americanum

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ABSTRACT

Purification, Characterization and Kinetic Analysis of a Flavonol-Ring B O-Glucosyltransferase From Chrysosplenium americanum.

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A novel flavonol-ring B O-glucosyltransferase, which catalyzes the transfer of glucose from UDP-glucose to positions 2' or 5' of partially methylated flavonols, was isolated from the shoots of C. americanum (Saxifragaceae). It was purified by ammonium sulfate precipitation and successive chromatography on Sephadex G-100, hydroxyapatite, and polybuffer ion exchanger. This glucosyltransferase appeared to be a single polypeptide with an apparent molecular weight of 42,000 daltons, pH optimum of 7.8 and an isoelectric point of 5.1. It had similar kinetic constants for the 2'- and 5'-hydroxylated substrates.

The kinetic patterns obtained for both substrates were identical and were consistent with an ordered Bi Bi mechanism where UDP-glucose is the first substrate to bind to the enzyme and UDP is the final product released.

To our knowledge, this is the first instance where a glucosyltransferase specific to ring B of partially methylated flavonols has been purified and characterized.

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ABBREVIATIONS

UDP	Uridine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
K	Michaelis-Menten constant
V	Maximum velocity
TLC-	Thin layer chromatographic-
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
ME	Mercaptoethanol
EDTA	Ethylenediamine-tetraacetate
PBE	Polybuffer exchanger
pI	Isoelectric point
DEAE-	Diethyl aminoethyl-
DMSO	Dimethyl sulfoxide
SDS	Sodium dodecyl sulfate
pKat	pKatal
DTE	Dithioerythritol
SH-	Sulfhydryl-
o-	<u>Ortho-</u>
p-	<u>Para-</u>
FLAV	Flavonoid substrate
FLAV-GLU	Flavonoid glucoside
d.p.m.	Disintegrations per minute
IUB	International Union of Biochemistry

A. INTRODUCTION

The transfer of the glucosyl moiety from sugar nucleotides to flavonoid acceptors is an important reaction in plant tissues and has been widely studied (1).

Chrysosplenium americanum (Saxifragaceae) contains two 2'- β -O-D-glucosides of partially methylated 2'-hydroxyquercetin and four 5'- β -O-D-glucosides of partially methylated 6-hydroxy- and 6,2'-dihydroxyquercetin (2). Our current interest in the enzymic synthesis of partially methylated flavonols (2-4) prompted us to study the glucosyltransferase(s) involved in their biosynthesis. Whereas flavonol O-glucosyltransferases specific for the 3-, 5-, and 7-positions have been isolated and characterized (5,6), nothing is known of ring-B O-glucosylation. In view of the position specificity exhibited by a number of O-methyltransferases isolated from this tissue (3), it was of interest to find out whether glucosylation of the 2'- and 5'-positions was catalyzed by one or two distinct enzymes.

The kinetic analysis of the 2'- and 5'-glucosylation reactions was used to determine if there was one or two distinct enzymes catalyzing the glucosylation of these two positions.

Two flavonol substrates F₁ (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) and F₂ (5,5'-dihydroxy-

3,6,7,2',4'-pentamethoxyflavone) were used in this study for the 2'- and 5'-glucosylating activities, respectively.

B. LITERATURE REVIEW

B.1. Structural aspects of flavonoids

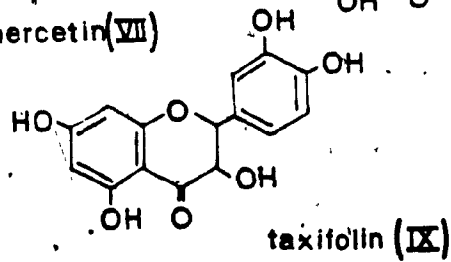
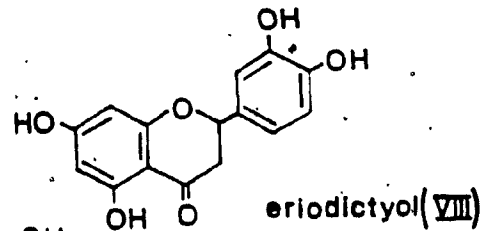
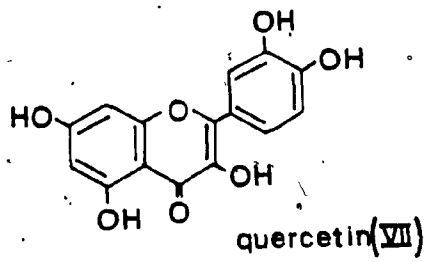
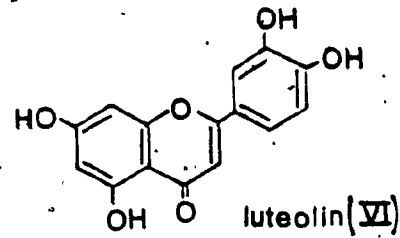
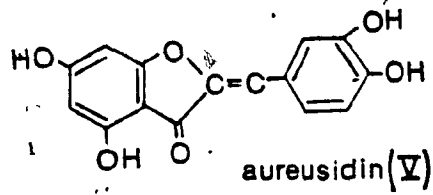
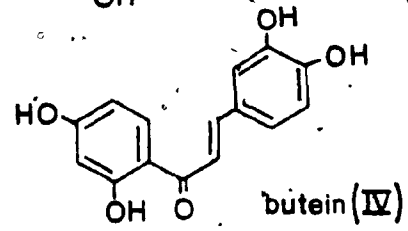
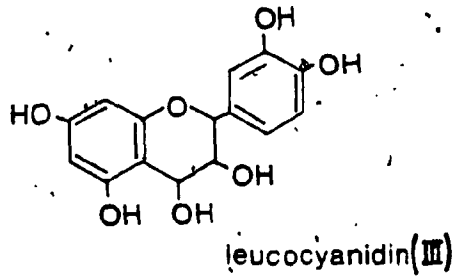
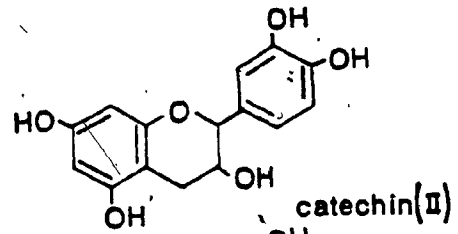
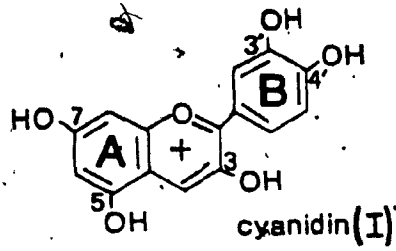
The carbon skeleton of flavonoid compounds is made up of two units: the C₆-C₃ phenylpropanoid fragment which gives rise to ring B and its three carbon side chain and the C₆ fragment (ring A) which is derived from acetate/malonate (7). Condensation of these two units results in the formation of compounds with different oxidation levels of ring C, giving rise to several species of flavonoids (Fig 1).

B.2. Occurrence of flavonoids in Nature

Flavonoid compounds are noted for their wide occurrence in Nature. The synthesis, by the shikimic acid pathway (8), of the phenylpropanoid fragment is one of the fundamental synthetic processes of Nature (9) which results in the formation of the essential amino acids phenylalanine, tyrosine and tryptophan.

Flavonoid compounds are not found in bacteria, fungi, or lichens; since these organisms apparently lack the necessary apparatus for the condensation of rings A and B. Ferns, on the other hand, contain many flavonoid compounds of the type found in flowering plants.

FIGURE 1: Structural formulae of the different classes of flavonoid compounds.



Flavonoid compounds occur in all parts of higher plants: roots, stems, leaves, flowers, pollen, fruit, seeds, wood and bark. However, certain flavonoid types are more characteristic of some tissues than others.

Anthocyanidins (Fig 1, I) occur in highest concentration in flowers, fruits and/or leaves, though they may occur in other parts of the plant. Catechins (II) and leucoanthocyanidins (III) have been isolated from wood and bark more often than from other plant parts. Chalcones (IV) and aurones (V) are largely found in flower petals.

Flavones (VI), flavonols (VII) and their dihydroderivatives (VIII and IX) occur in many parts of the plant and are not characteristic of any one kind of tissue.

B.3. Biosynthesis of flavonoid compounds

Interest in the biosynthesis of flavonoids was first stimulated by studies on genetic aspects of flower color (10,11). Tracer studies were conducted in 1957 with the aim to elucidate the biosynthetic pathways using intact plants or plant tissues. In the course of these tracer experiments it became evident that a more detailed knowledge of the nature and sequence of the individual biosynthetic steps and their regulation could only be gained by investigation of the enzymes involved.

The discovery of the first enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase, by

Koukol and Conn (12) marked the beginning of the enzymatic studies of flavonoids. However, further progress in plant enzymology was limited by problems arising from the accumulation of secondary plant metabolites (alkaloids, flavonoids, etc.). Such difficulties have recently been overcome by the elimination of these metabolites and the use of modern purification techniques.

From feeding experiments with radioactively labelled compounds (13-18), it was elucidated that flavonoids originate from the head-to-tail condensation of three acetate/malonate units (ring A) and a phenylpropanoid intermediate (ring B) that is derived from the shikimic acid pathway (Fig 2). Grisebach (19) then proposed an enzyme-mediated condensation reaction involving the CoA esters of malonic and cinnamic acids (Fig 3).

Different incorporation rates of various substituted cinnamic acids into flavonoids have led to the question of whether the substitution pattern of ring B is determined at the cinnamic acid stage or at the level of a flavonoid intermediate (chalcone or flavanone). Hess (11) suggested that ring B substitution of flavonoids occurred at the cinnamic acid level. Hence, caffeic acid (3,4-dihydroxycinnamic acid) would be the precursor of 3',4'-dihydroxyflavonoids; ferulic acid (3-methoxy-4-hydroxycinnamic acid), of 4'-hydroxy-3'-methoxyflavonoids and so on. On the other hand, evidence from tracer (13-18) and enzymatic (5,20-23) studies seem to indicate that ring

FIGURE 2: Origin of the carbon atoms in flavonoid and iso-
flavonoid compounds.

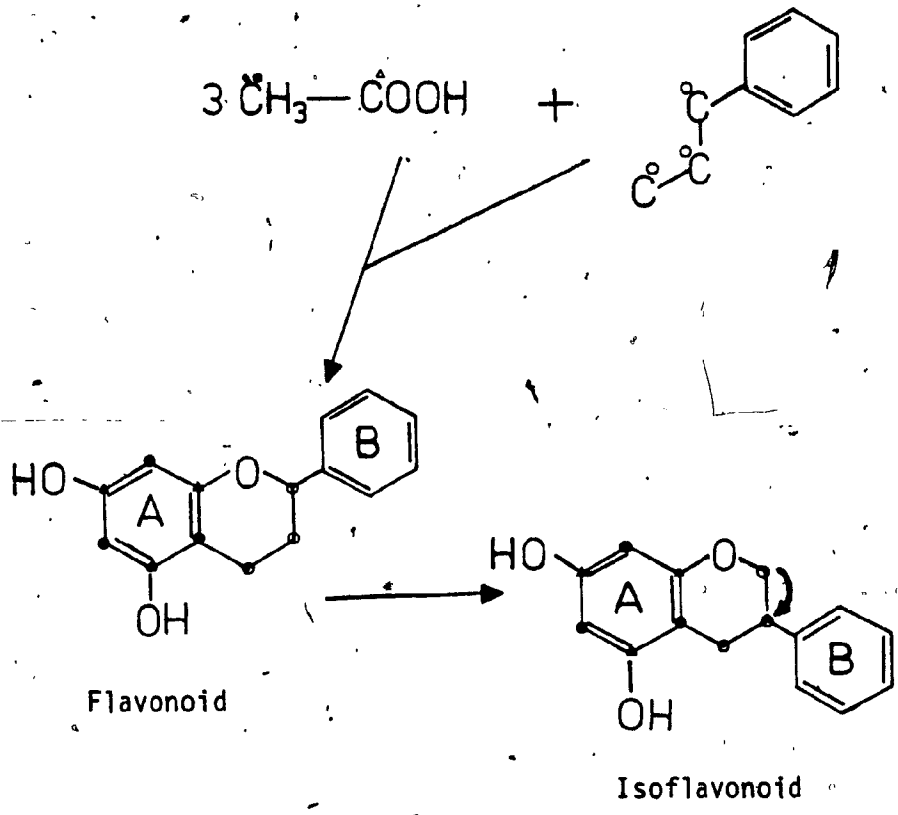
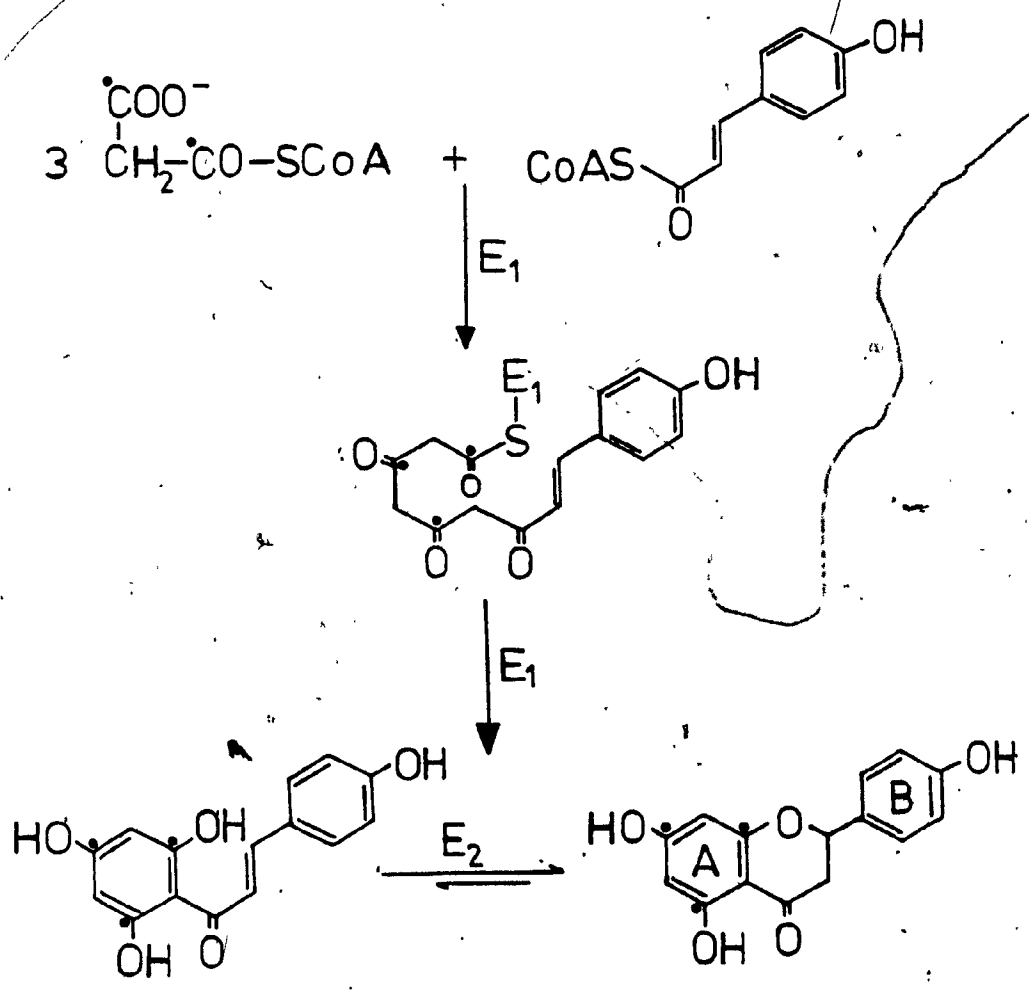


FIGURE 3: Proposed scheme for the enzymic formation of flavonoid compounds from malonyl CoA and substituted cinnamoyl CoA. E₁, chalcone synthetase; E₂, chalcone-flavanone isomerase.

8a.



B substitution may occur at the chalcone/flavanone stage or at a later stage of flavonoid biosynthesis. Obviously, both possibilities may operate in different biosynthetic schemes.

B.4. Properties of flavonoids

B.4.1. Flavonoids as antioxidants or inhibitors of phosphorylative oxidation in mitochondria

Ascorbic acid is a good reductant and is abundant in plant cells of high metabolic activity (24). The oxidation of ascorbate occurs in the presence of O_2 and is catalyzed by metals or by metalloenzymes. Several flavonoids serve as antioxidants for ascorbic acid, apparently by chelating metals from the reaction mixture (25,26). Pratt (27) investigated the antioxidant effect of several flavonoids in leaf slices. He attributed the antioxidant effect to the chelating of metallic ions and accepting free radicals (28). It was, therefore, suggested that a major function of flavonoids is to serve as antioxidants for lipids and polyacetylenes in plant tissues (29). Many flavonoids were found to inhibit ATP formation in isolated plant mitochondria (30,31). Kampferol (3,5,7,4'-tetrahydroxyflavone) was reported to inhibit the oxidative and phosphorylative properties of plant mitochondria (32).

B.4.2. Flavonoids as enzyme inhibitors

Flavonoids react with phenolase and the products formed may inhibit enzymes by non-specific binding, by competing or reacting with the substrate, by oxidation of the sulfhydryl groups controlling the tertiary enzyme structure, or through the formation of complexes with metallic prosthetic groups (33). Wheeler et al (34) found that a number of flavonoids inhibited malate dehydrogenase and glutamate decarboxylase. DeSwardt et al (35) discovered that polymerization of flavonoids caused the release of pectin methylesterase from inhibition. Flavones and flavonols are also known to be potent inhibitors of bovine pancreatic ribonuclease (36).

B.4.3. Flavonoids as phytoalexins

Phytoalexins are secondary metabolites that are absent or present in low amounts in healthy plants and that accumulate in high concentrations in or around cells damaged by different stimuli, particularly infection by pathogenic fungi and bacteria (37,38). Many phytoalexins have been chemically characterized as flavonoids (39-41). The mode of action of these compounds is currently under study. Other phenolic substances have been shown to act as protective agents against fungal or bacterial attacks.

B.4.4. Pharmacological and antibiotic effects of flavonoids

Anthocyanins and phenolic acids were reported to inhibit respiration and reproduction in E. coli and other microorganisms (42). Quercetin, a pentahydroxyflavone, was also reported to inhibit several viruses in vitro (43).

Some flavonoid compounds have been shown to act on malignant cells (44,45); others have been reported to act as anti-inflammatory (46-49) and anti-histaminic (50,51) agents.

B.5. Flavonoids of Chrysosplenium americanum

Chrysosplenium americanum (Saxifragaceae), the experimental tissue used in this study, accumulates a variety of highly O-methylated flavonols. Collins et al (2) isolated six major glucosides of partially methylated flavonols from this tissue. They were identified, by UV-, NMR- and mass spectrometry as two 2'- β -O-D-glucosides of partially methylated 2'-hydroxyquercetin, and four 5'- β -O-D-glucosides of partially methylated 6-hydroxy and 6,2'-dihydroxyquercetin. They also identified trace amounts of the 3-O-arabinosides, 3-O-glucosides, 3-O-rutinosides and 3-O-diglucosides of both kaempferol and quercetin, along with their free aglycones.

B.6. Glucosylation of flavonoids.

The transfer of the glucosyl moiety from sugar nucleotides to flavonoid acceptors has been widely studied (1) and is considered to be a terminal step in flavonoid biosynthesis (52,53). Whereas glucosyltransferases were believed to possess a broad substrate specificity, recent reports tend to indicate their specificity towards the different classes of flavonoids (1).

Enzymes catalyzing the formation of phenolic glucosides were among the first glucosyltransferases that have been studied in plant tissues (54-58). A glucosyltransferase, which catalyzed the synthesis of coniferin from UDP-glucose and coniferyl alcohol was purified and characterized from suspension cell cultures of "Paul's Scarlet" rose (59). A similar glucosyltransferase has been partially purified from lignifying segments of Forsythia ovata (60).

Sutter and Grisebach demonstrated the position specificity of flavonoid glucosyltransferases by separating an enzyme specific for the 3-position from a 7-O-glucosyltransferase from parsley cell cultures (5). Another example of position specificity was reported from the petals of Silene dioica (6), where two distinct enzymes were shown to catalyze the glucosylation of the 3- and 5-positions of cyanidin. In addition, the specificity of glucosyltransferases may be dependent on the type and/or

the substitution pattern of the flavonoid compound. While it is not clear whether glucosylation of flavonols and anthocyanidins is catalyzed by the same enzyme, however, it was recently shown that the glucosyltransferase from soybean cultures did not accept anthocyanidins as substrates (61), whereas that from red cabbage seedlings (62) and Haplopappus gracilis cell cultures (63) utilized both flavonols and anthocyanidins. Flavonoid-specific glucosyltransferases may accept substrates with different substitution patterns though their K and V values may be dissimilar.

Further glucosylation of flavonol glucosides may occur at a different hydroxyl group (eg. flavonol-3,7-diglucoside), or at the level of the glucosyl moiety (eg. flavonol-3-diglucoside). Few glucosyltransferases have been reported to introduce a second or third sugar moiety. Shute et al (64) reported the in vitro synthesis of a flavonol-3-triglucoside by an enzyme preparation from Pisum sativum seedlings. This glucosyltransferase system was resolved into three distinct enzyme activities (65); one accepted the flavonol aglycone, whereas the other two utilized the mono- and di-glucosides as substrates, respectively.

B.7. Physiological role of glucosylation

From a chemical point of view, glucosylated secondary products differ from their free aglycones in that the former show enhanced water solubility and decreased chemical reactivity. This may explain why glucosides, rather than the free aglycones, accumulate in the cellular vacuole of plant tissues (66). Glucosylated products may, therefore, be considered as inert plant storage forms. Plant cell vacuoles contain a variety of hydrolases (67), which may release the sugar moiety and result in the formation of reactive aglycones.

B.8. The glucose group donor

Nucleotide-activated sugars have been shown to be the glucose donors in almost all glucosylation reactions that have been investigated so far. When a "low energy" donor such as α -glucose-1-phosphate was used, it failed to show any activity (23,68-70).

Among the nucleotide sugars known, UDP-glucose has been shown to be the best glucose donor. Exceptions were the glucosylation of quercetin by a mung bean glucosyltransferase (69), of diphenols by a wheat germ transferase (71), among others (23,72-74). The nucleotide sugars of adenine, cytosine or guanine, on the other hand, are poor sugar donors.

C. MATERIALS AND METHODS

C.1. Plant material

Chrysosplenium americanum Schwein ex Hooker

(Saxifragaceae) was collected from Sutton junction, Eastern Townships, Province of Quebec, and was maintained in the greenhouse under conditions simulating its natural habitat with respect to temperature, light and moisture.

C.2. Cinnamic acid incorporation experiments

C.2.1. Pulse experiments

One g young leaves was infiltrated in water for 5 sec, then incubated with 1 μ Ci of cinnamic acid-2- 14 C, 3 mCi/mole (ICN, Irvine, CA) in 1 ml total volume. This was incubated on the top of a lightbox for 30 minutes at room temperature. The leaves were then thoroughly washed with distilled water and extracted with hot 95% methanol. The alcohol extract was evaporated in a Büchi HB-140 Rotavapor-M and the residue was taken up in 50 μ l of methanol. The latter was chromatographed on Polyamid-6 MN TLC plates in two dimensions using toluene-ethylformate-ethanol-water (60:20:19:1); and water-butanol-acetone-dioxane (75:15:10:5) as solvent systems. The plates were then autoradiographed on Kodak No Screen X-Ray film.

C.2.2. Time course experiments

Young leaves were infiltrated in water, then incubated on a lightbox with cinnamic acid- $2-^{14}C$. Leaf samples were taken at 2, 5, 15, 30, and 60 minute-intervals, washed then extracted immediately with hot methanol, and chromatographed as described in the pulse experiments.

C.2.3. Pulse-chase experiments

These were performed in the same manner as described previously except that after washing the label off the leaves, they were chased by incubation with 1mM cold cinnamic acid for two hours. The leaves were then washed and extracted with hot methanol. The extract was chromatographed two-dimensionally and autoradiographed on X-Ray film as per pulse experiments.

C.3. Extraction of glucosyltransferase

Unless stated otherwise, all procedures were carried out at 2-4 C. Shoot tips were frozen in liquid nitrogen, mixed with Polyclar AT, 1:10 w/w (Sigma Chemical Co, St. Louis, MO) and ground to a fine powder. The mixture was homogenized with 0.2 M Tris-HCl buffer, pH 7.8 (1:4 w/v) containing 14 mM 2-mercaptoethanol (ME), 5 mM EDTA and

10 mM diethylammonium diethyldithiocarbamate (buffer A). The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 15,000 g for 15 min. The supernatant was stirred for 10 min with Dowex 1X2, 25% w/v (Bio-Rad, Richmond, CA), which had previously been equilibrated with buffer A, then filtered through glass wool. The filtrate was fractionated with solid ammonium sulfate and the protein fraction that precipitated between 35-70% salt saturation was collected by centrifugation and resuspended in the minimal amount of 25 mM Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol (buffer B). The solubilized protein was chromatographed on a Sephadex G-100 (Pharmacia Fine Chemicals, Dorval, PQ) column which had previously been equilibrated with the same buffer. The eluted protein was monitored using a Gilson Model 111 LC Detector and three-ml fractions were collected (Gilson Micro Fractionator) and assayed for glucosyltransferase activity. Fractions with high enzyme activity were pooled and designated the partially purified enzyme preparation.

C.4. Enzyme purification

C.4.1. Desalting on Sephadex G-25

The 35-70% ammonium sulfate pellet was suspended in the minimal amount of buffer B and was loaded onto a Sephadex G-25 (Pharmacia Fine Chemicals, Dorval, PQ) column

which had previously been equilibrated with the same buffer. The protein was eluted using buffer B.

C.4.2. Chromatography on hydroxyapatite

The partially purified enzyme preparation was applied to a hydroxyapatite, Bio Gel HT (Bio Rad, Richmond, CA) column which was previously equilibrated with 10 mM phosphate buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol (buffer C). After washing the column with buffer C, the bound protein was eluted using a linear gradient (10-100 mM) of potassium phosphate in the same buffer and three-ml fractions were collected for the assay of enzyme activity.

C.4.3. Chromatofocusing

For chromatofocusing, the enzyme was extracted, partially purified and chromatographed on hydroxyapatite in the usual manner, except that 25 mM imidazole-HCl buffer, pH 7.4 containing 10% glycerol and 14 mM 2-ME (buffer D) was used. Fractions with high glucosyltransferase activity were pooled and applied to a column packed with polybuffer ion exchanger, PBE-94 (Pharmacia Fine Chemicals, Dorval, PQ) which had previously been equilibrated with buffer D. Elution of proteins was carried out using polybuffer-HCl, pH 4.0 which generated a linear gradient between pH 7 and

4. The component proteins were thus eluted at their apparent isoelectric points (pI).

C.4.4. Chromatography on DEAE-cellulose

The partially purified enzyme preparation (sect. C.3.) was loaded onto a DEAE-cellulose (Bio-Rad, Richmond, CA) column which had previously been equilibrated with buffer C. The column was washed with three-bed volumes of the same buffer and the bound proteins were eluted using a linear gradient (0-200 mM) of KCl in buffer C. Three-ml fractions were collected for the assay of glucosyltransferase activity.

C.4.5. Chromatography on DEAE-Sephadex A-25

Fractions with glucosyltransferase activity after Sephadex G-100 chromatography were pooled and applied to a DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Dorval, PQ) column which had previously been equilibrated with buffer B. The column was washed with five bed-volumes of buffer B and the bound protein was eluted with 0.5 M KCl in the same buffer. Three-ml fractions were collected and assayed for enzyme activity.

C.4.6. Chromatography on DEAE-Sepharose

For DEAE-Sepharose chromatography, the enzyme was extracted and partially purified, as was previously described (sect. C.3.), before being loaded onto a DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Dorval, PQ) column that was pre-equilibrated with buffer B. The column was washed with three-bed volumes of buffer B and the bound proteins were eluted using a linear gradient (0-500 mM) of KCl in the same buffer.

C.4.7. Affinity chromatography

For affinity chromatography, the partially purified enzyme preparation was loaded onto a UDP-Agarose (Sigma Chemical Co., St. Louis, MO) column pre-equilibrated with buffer B. The column was washed with five-bed volumes of the same buffer and the bound proteins were eluted using 1 M KCl in buffer B. One half-ml fractions were collected and assayed for glucosyltransferase activity.

C.4.8. Chromatography on Phenyl Sepharose.

The partially purified enzyme preparation was loaded onto a Phenyl Sepharose CL-4B (Pharmacia Fine Chemicals, Dorval, PQ) column that had previously been equilibrated with buffer B. The column was then washed with

three-bed volumes of the same buffer and the bound protein was eluted using a linear gradient (0-50% v/v) of ethylene glycol in buffer B. One-ml fractions were collected and assayed for glucosyltransferase activity.

C.5. Preparation of flavonoid substrates

The partially methylated flavonol glucosides were isolated and purified from C. americanum (by M. Jay, Univ. of Lyon I) and were hydrolyzed to their respective aglycones (2). The identity of both glucosides and aglycones was verified by their UV-spectra and Rf values in different solvent systems (2). The concentration of the flavonol substrates was determined using a molar extinction coefficient of 20,000 at 340 nm.

C.6. Glucosyltransferase assay

The standard assay mixture consisted of 15 μ M of the flavonoid substrate (in 10 μ l of 50% DMSO), 1.5 μ M of UDP-[U-¹⁴C]-glucose (Amersham, Oakville, Ont.), containing 0.05 μ Ci, 25 mM Tris-HCl buffer pH 7.8, and 14 mM 2-ME in a total volume of 100 μ l. The reaction was started by addition of the enzyme and the mixture was incubated for 30 min at 30 C. The reaction was stopped by the addition of 10 μ l of 6M HCl and the reaction products were extracted with 250 μ l of ethyl acetate. An aliquot of

the organic phase was transferred to a scintillation vial and counted for radioactivity in a toluene-based scintillation fluid. It should be noted that UDP-glucose is not soluble in ethyl acetate and, therefore, does not enter the organic phase.

C.7. Product identification

The ethyl acetate extracts of several assays were pooled and evaporated to near dryness, then chromatographed on commercial Polyamid-6 MN TLC plates in solvent systems: A, butanone-water (15:85); and B, toluene-ethyl formate-ethanol-water (60:20:19:1). The identity of glucosylated products was verified by co-chromatography with authentic samples (2,75), visualization in UV-light (366 nm) and by autoradiography.

C.8. Molecular weight determination

An estimate of the molecular weight of the purified enzyme was obtained by determining its elution volume from a Sephadex G-100 column which had been calibrated using proteins of known molecular weights (Sigma Chemical Co., St. Louis, MO).

C.9. Disc electrophoresis

Gel electrophoresis of the chromatofocused enzyme preparation was performed using 10% acrylamide according to the method of Weber and Osborne (76), except that the running buffer contained 5 mM 2-ME and 2 mM EDTA.

Electrophoresis was carried out at 6 C. For the determination of enzyme activity, the gels were sliced into 2-mm sections and were disintegrated in 200 μ l of 0.2 M Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME and were incubated overnight at 4 C. The eluates recovered were then assayed for glucosyltransferase activity.

C.10. Definition of enzyme units

The enzyme unit used in this study was expressed in pkat as recommended by the International Union of Biochemistry (IUB, 1973). One pkat is defined as the amount of enzyme activity which is required to convert one pmol of substrate per second under the assay conditions.

C.11. Protein determination

Protein was determined according to the method of Bradford (77) using the Bio-Rad protein reagent and bovine serum albumin as standard.

C.12. Analysis of kinetic data

Reciprocals of velocities were plotted graphically against the reciprocals of substrate concentrations. Since inhibition with the flavonol substrates was observed at concentrations higher than $100 \mu\text{M}$ therefore, lower concentrations were used in subsequent analysis. The curves, thus obtained, were linear and intersected to the left of the y-axis. The data was fitted to equation [1]

$$v = V [S] / (K_m + [S]) \quad [1]$$

where [S] represents the concentration of the varied substrate, the other substrate being present in fixed amounts. The intercepts and slopes obtained from fits to equation [1] were then plotted against the reciprocals of the changing fixed substrate concentration in order to derive the kinetic constants for the reaction. The latter were defined by equation [2] for a sequential mechanism using the nomenclature of Cleland (78-81).

$$v = \frac{V [A][B]}{K_A K_B + K_A [B] + K_B [A] + [A][B]} \quad [2]$$

Inhibition data corresponding to linear competitive and linear non-competitive inhibition were fitted to the appropriate forms of equation [2]. Linear secondary plots of substrate interaction and product inhibition kinetics were obtained in all cases.

C.13. Statistical analysis of kinetic data

Linear regression analysis was performed on the experimentally obtained data. One way analysis of variance was then carried out on the best fit straight lines thus generated. The kinetic patterns obtained were fitted to the appropriate forms of equation [2] with an error of 5-10%.

D. RESULTS

D.1. Cinnamic acid incorporation

A pulse-time course performed with ^{14}C -labelled cinnamic acid indicated that a 30-minute pulse is required to observe sufficient incorporation of label into all the flavonol glucosides of C. americanum. The fact that this tissue was capable of converting cinnamic acid to the final products (Fig 4), indicates that this plant material contains all the enzymes involved in the biosynthesis of partially methylated flavonol glucosides.

D.2. Characteristics of the glucosylation reaction.

Flavonol-ring B O-glucosyltransferase is a novel enzyme that catalyzes the transfer of glucose from UDP-glucose to positions 2' or 5' of partially methylated flavonols. The presence of an SH group protector (10 mM DTE or 14 mM 2-ME) was found to be the only requirement for optimum glucosylation.

Two flavonol substrates, F_1 and F_2 (Fig 5) were used in this study. F_1 (5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone) gave rise to the corresponding 2'-O-glucoside and F_2 (5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone) gave rise to the corresponding 5'-O-glucoside as the only products, respectively (Fig 6).

FIGURE 4: Incorporation of cinnamate-2-¹⁴C into partially methylated flavonol glucosides of *C. americanum*. Leaves were incubated with cinnamate-2-¹⁴C, then chased with cold cinnamic acid. They were extracted in methanol, chromatographed in 2-directions and autoradiographed as described in "Materials and Methods". 1: 5,5'-dihydroxy-3,7,4'-trimethoxyflavone 2'-glucoside; 2: 5-hydroxy-3,7,4',5'-tetramethoxyflavone 2'-glucoside (F₁-Glu); 3: 5,6-dihydroxy-3,7,4'-trimethoxyflavone 5'-glucoside; 4: 5-hydroxy-3,6,7,4'-tetramethoxyflavone 5'-glucoside; 5: 5,2'-dihydroxy-3,6,7,4'-tetramethoxyflavone 5'-glucoside; 6: 5-hydroxy-3,6,7,2',4'-pentamethoxyflavone 5'-glucoside (F₂-Glu). Other labelled spots are unidentified intermediates.



FIGURE 5: O-glucosylation of 2' and 5' positions of the partially O-methylated flavonols F₁ and F₂ in *C. americanum*.



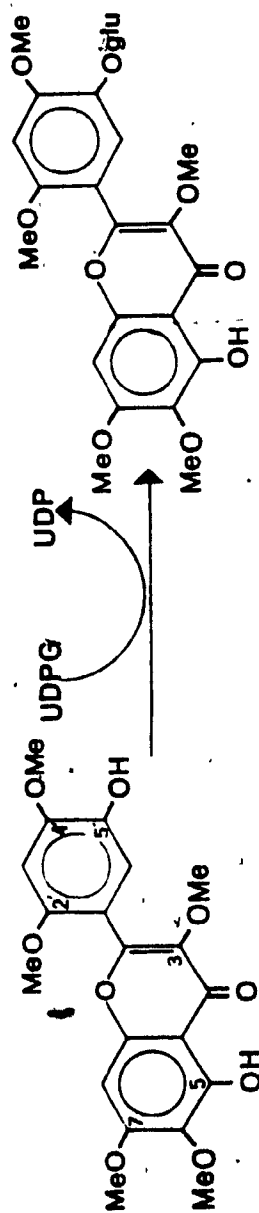
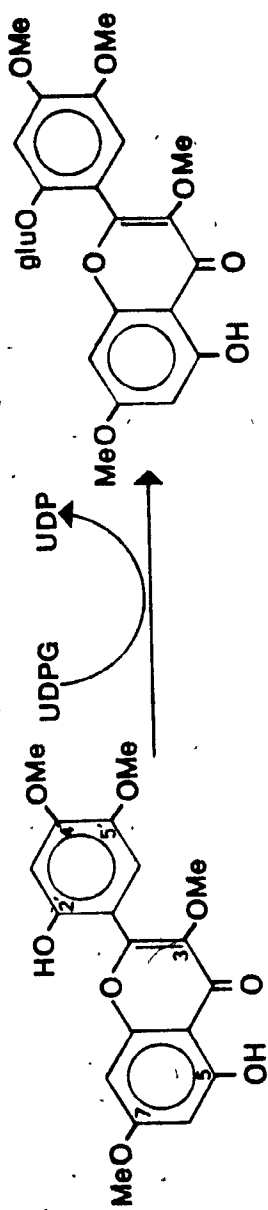


FIGURE 6: Photograph of an autoradiogram of the chromatographed reaction products of O-glucosyltransferase activity, using the Sephadex G-100 enzyme preparation, against the flavonol substrates F_1 and F_2 using the solvent system toluene, ethyl formate, ethanol, water (60:20:19:1). The products formed co-chromatographed with F_1 -Glu and F_2 -Glu, the glucosides of F_1 and F_2 , respectively.

29a.

F₁-Glu



F₂-Glu



origin

^

These products co-chromatographed with authentic samples of F_1 and F_2 glucosides in solvent systems A and B.

D.2.1. Effect of pH

The pH optimum for both the 2'- and 5'-activities, as determined in different buffers, was found to be between 7.5 and 8.0 (Fig 7). Maximum glucosyltransferase activity was observed in imidazole-HCl, Tris-HCl and histidine-HCl, buffers.

D.2.2. Linearity of the glucosylation reaction

At optimum pH (pH 7.8), The reaction rate for both 2'- and 5'-positions was linear for at least 90 minutes (Fig 8) and was proportional to the amount of protein added up to 1 mg/ml (Fig 9) of the Sephadex G-100 enzyme preparation.

D.3. Purification of O-glucosyltransferase

The enzyme was purified by fractional precipitation with ammonium sulfate and successive chromatography on Sephadex G-100 (Fig 10), hydroxyapatite (Fig 11) and polybuffer ion exchanger (Fig 12), columns. The enzyme activity was eluted at pH 5.1 from the latter column. The combined purification steps resulted in an

FIGURE 7: pH optima of the 5' glucosylating activity using the flavonol F_2 as substrate. Twenty μ l of the Sephadex G-100 enzyme protein was added to 100 μ l of 0.2 M buffer adjusted to the indicated pH and assayed for activity. The enzyme activity with F_1 was similar to that of F_2 .

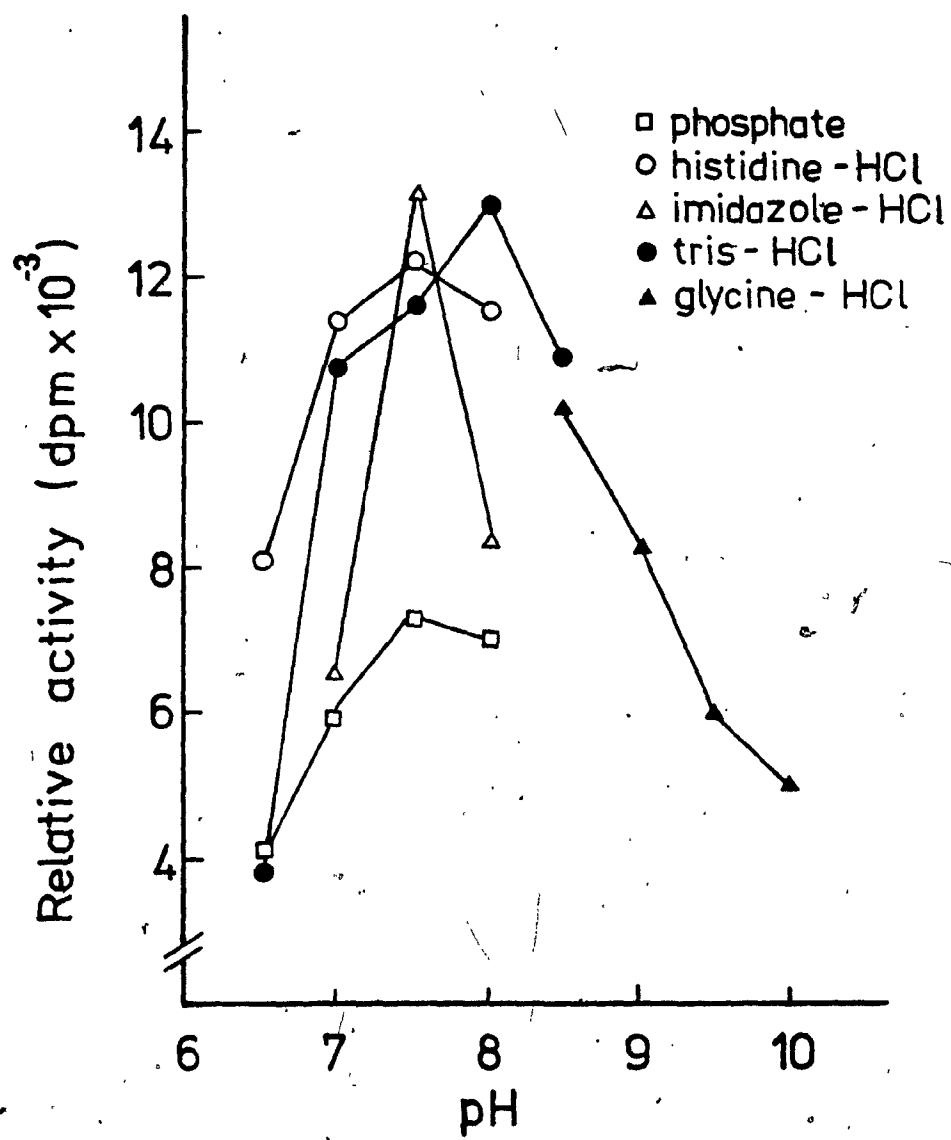


FIGURE 8: Linearity of the glucosylation reaction, using the Sephadex G-100 enzyme preparation against F_2 , with time. The enzyme activity against F_1 was similar to that of F_2 .

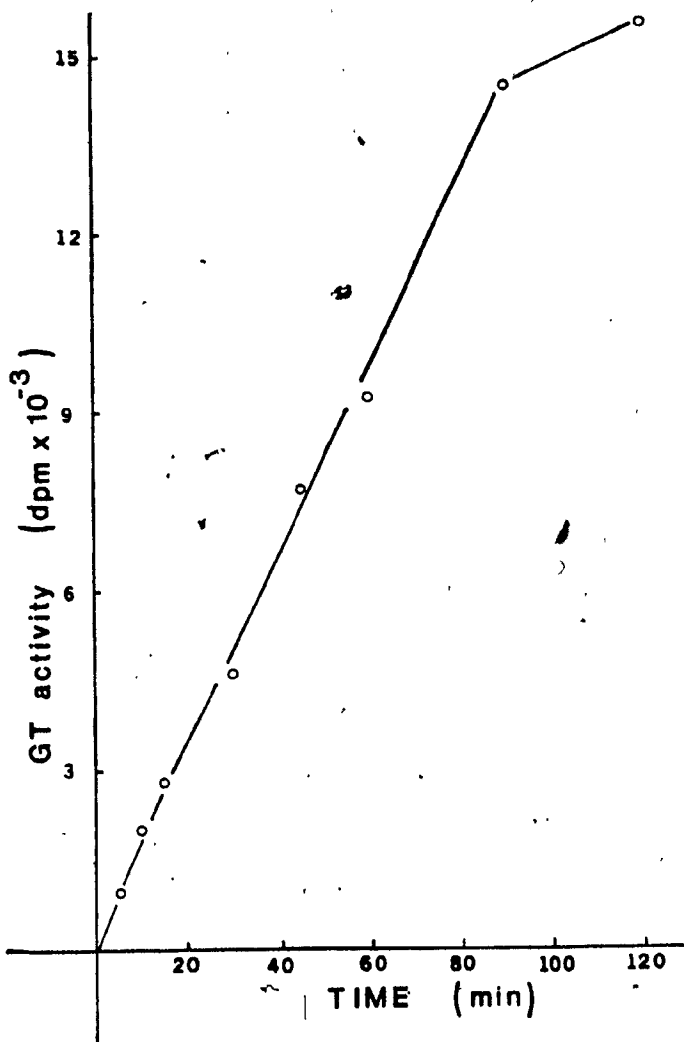


FIGURE 9: Linearity of the glucosylation reaction, using F_2 as substrate, with the amount of the Sephadex G-100, protein added. The enzyme activity against F_1 was similar to that of F_2 .

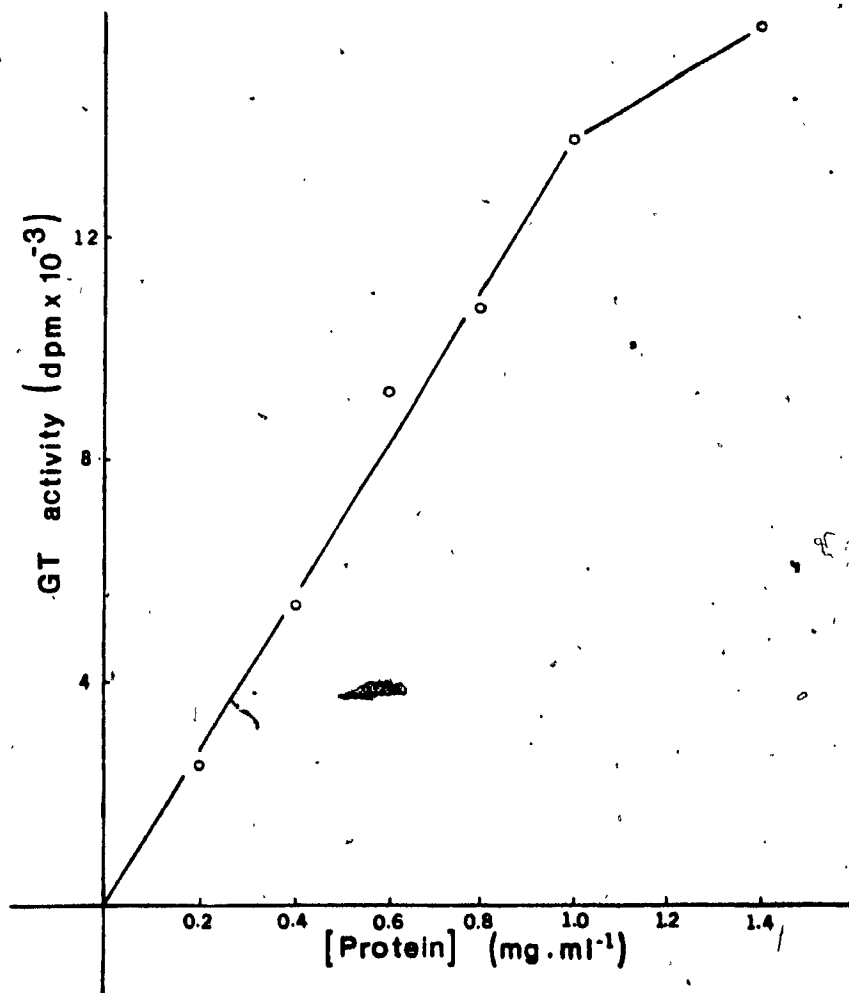


FIGURE 10: Elution profile of glucosyltransferase activity from Sephadex G-100 column using compound F_2 as substrate. The column was pre-equilibrated and the protein eluted with 25 mM Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol. The molecular weight of the enzyme was determined with a similar column which had previously been equilibrated with the indicated proteins. The elution profile of the glucosyltransferase using F_1 as substrate was similar to that using F_2 .
Blue dextr., Blue dextran; GT, glucosyltransferase; Chymotrips., Chymotrypsin; Ribonucl., Ribonuclease.

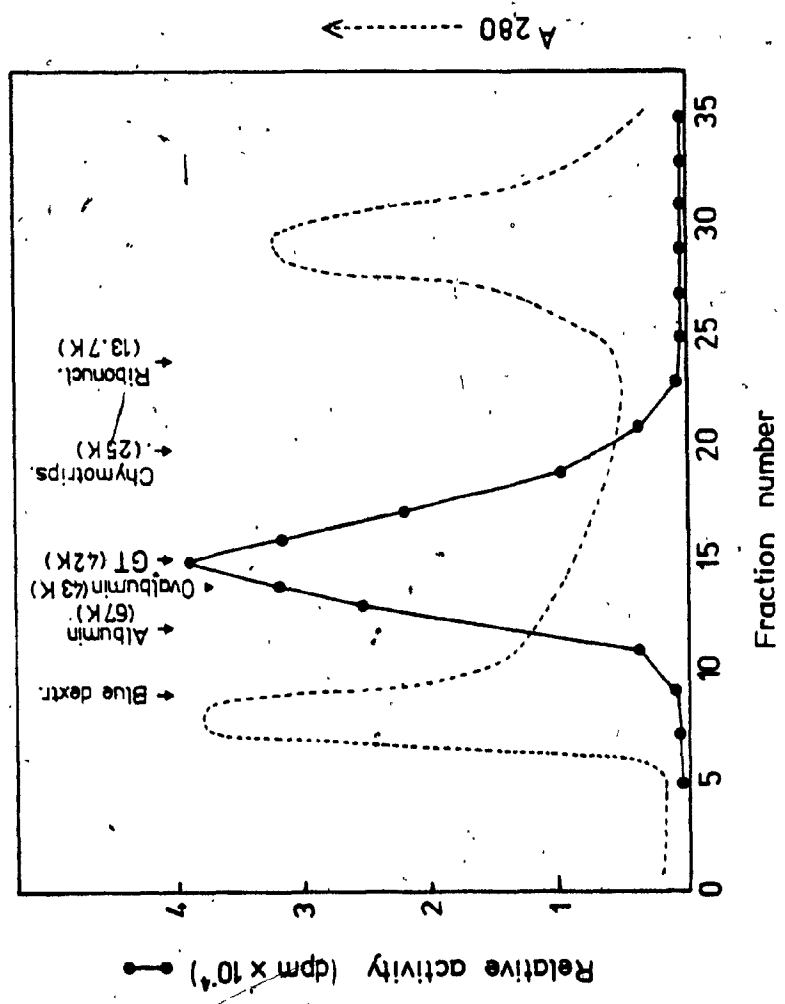


FIGURE 11: Elution profile of glucosylating activity from hydroxyapatite column. Fractions from Sephadex G-100 with high enzyme activity were pooled and applied to the column which had previously been equilibrated with 10 mM phosphate buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol. The protein was eluted with a linear gradient (10-100 mM) phosphate in the same buffer and fractions were assayed for enzyme activity using F_1 and F_2 for 2' and 5' activities, respectively.

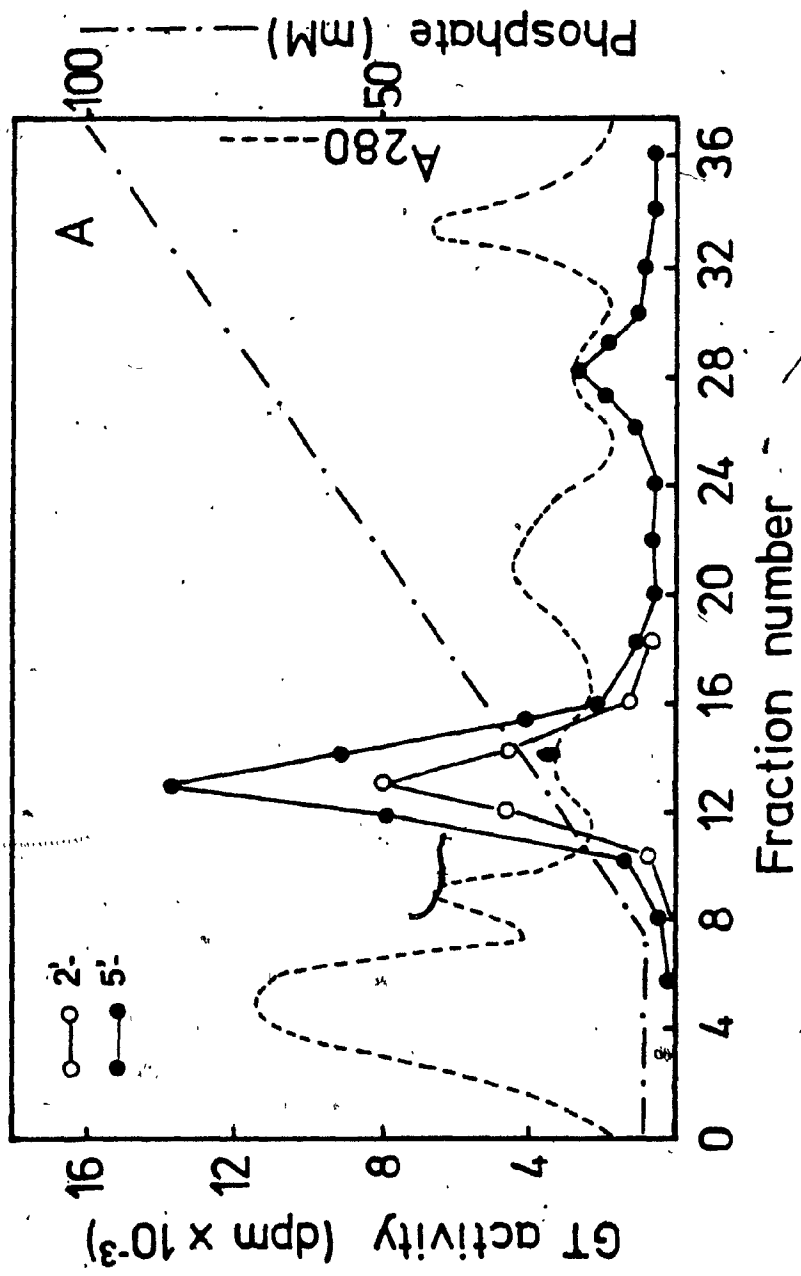
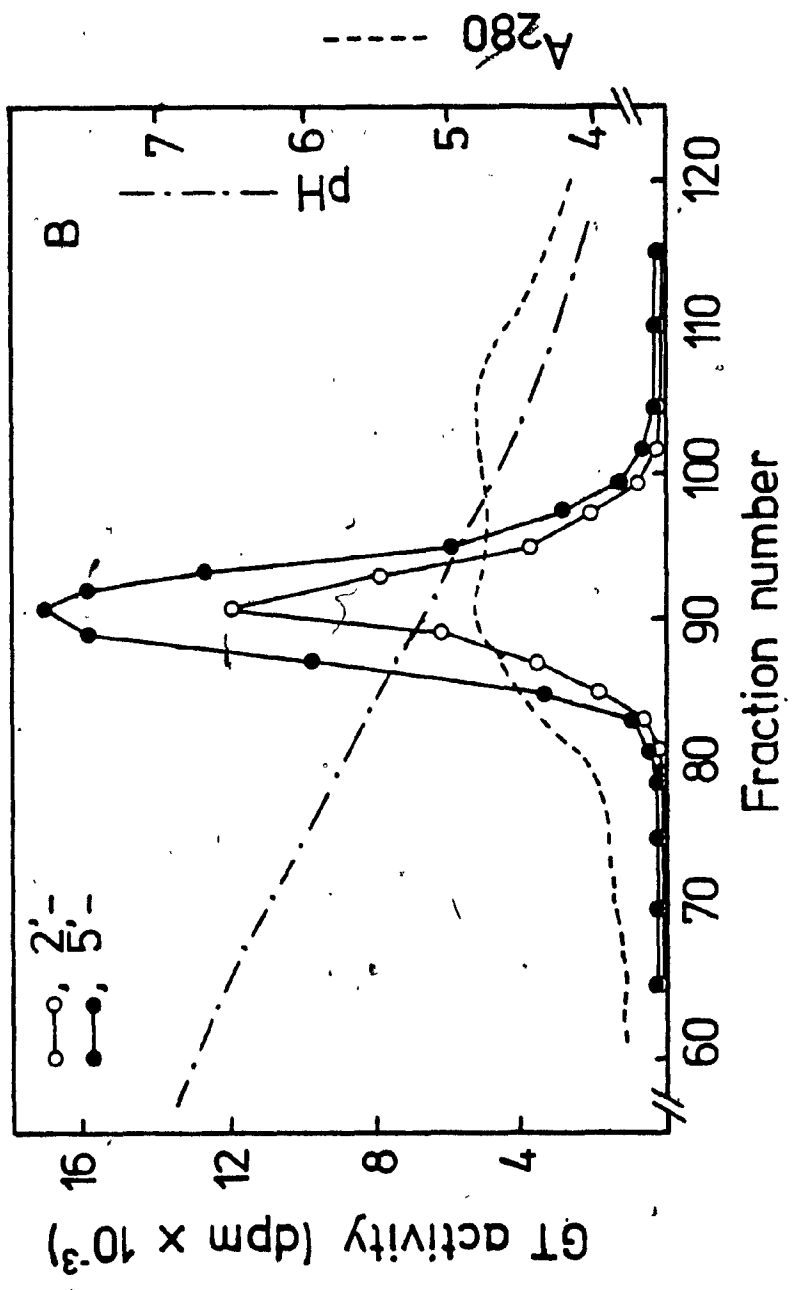


FIGURE 12: Chromatofocusing of glucosyltransferase activity on polybuffer ion exchanger (PBE-94). The enzyme protein was applied to a column pre-equilibrated with 25 mM imidazole buffer, pH 7.4 containing 14 mM 2-ME and 10% glycerol. Elution was carried out using polybuffer-HCl, pH 4.0 which generated a linear gradient between pH 7 and 4. Fractions were assayed against substrates F_1 and F_2 for 2' and 5' activities, respectively.



increase in specific activity of 225-fold as compared with that of the crude enzyme preparation (Table 1) when using either substrates F_1 (2'-activity) or F_2 (5'-activity). Both activities, however, were recovered in the same protein fractions, even when the Sephadex G-100 preparation was further chromatographed on either DEAE-cellulose (Fig 13), DEAE-Sephadex A-25 (Fig 14) or DEAE-Sepharose (Fig 15), columns. The enzyme protein did not bind to UDP-agarose and its activity was lost upon Phenyl Sepharose chromatography under varying conditions.

D.4. Acrylamide gel profile

Figure 16 shows the electrophoretic protein pattern of the purified enzyme preparation. Both the 2'- and 5'-glucosylating activities were found in the same polypeptide band.

D.5. Properties of the purified enzyme

D.5.1. Enzyme stability

In the absence of SH group protectors, the partially purified enzyme lost more than 70 % of its activity against both substrates F_1 and F_2 within 24 hours. Storage of the enzyme preparation in presence of 14 mM 2-ME resulted in 50% loss of activity after 1 week. The

Table 1: Purification of *Chrysoosplenium* 0-glucosyltransferase.

Purification Step	Protein mg	Specific Activity pkat/mg	Purification -fold	Recovery %	2'/5' Act. Ratio
Crude Extract	14.0	0.36		100	0.68
Dowex 1X2	10.5	0.47	1.3	99	0.72
35-70% Amm. Sulfate	3.6	1.44	4.0	103	0.70
Sephadex G-100	0.26	18.0	50	96	0.68
Hydroxyapatite	0.07	44.8	124	62	0.60
Polybuffer ion exchanger	0.007	81.2	225	11	0.70

The standard enzyme assay was used as described in "Materials and Methods". The substrate used was F_2 . 2'/5' represents the ratio of activity using F_1 as compared with F_2 .

FIGURE 13: Elution profile of glucosylating activity from DEAE-cellulose column. The enzyme activity from Sephadex G-100 was applied to a column pre-equilibrated with 10 mM phosphate buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol. The protein was eluted with a linear gradient (0-200 mM) KCl in the same buffer, and fractions were assayed using substrates F_1 and F_2 for 2' and 5' activities, respectively.

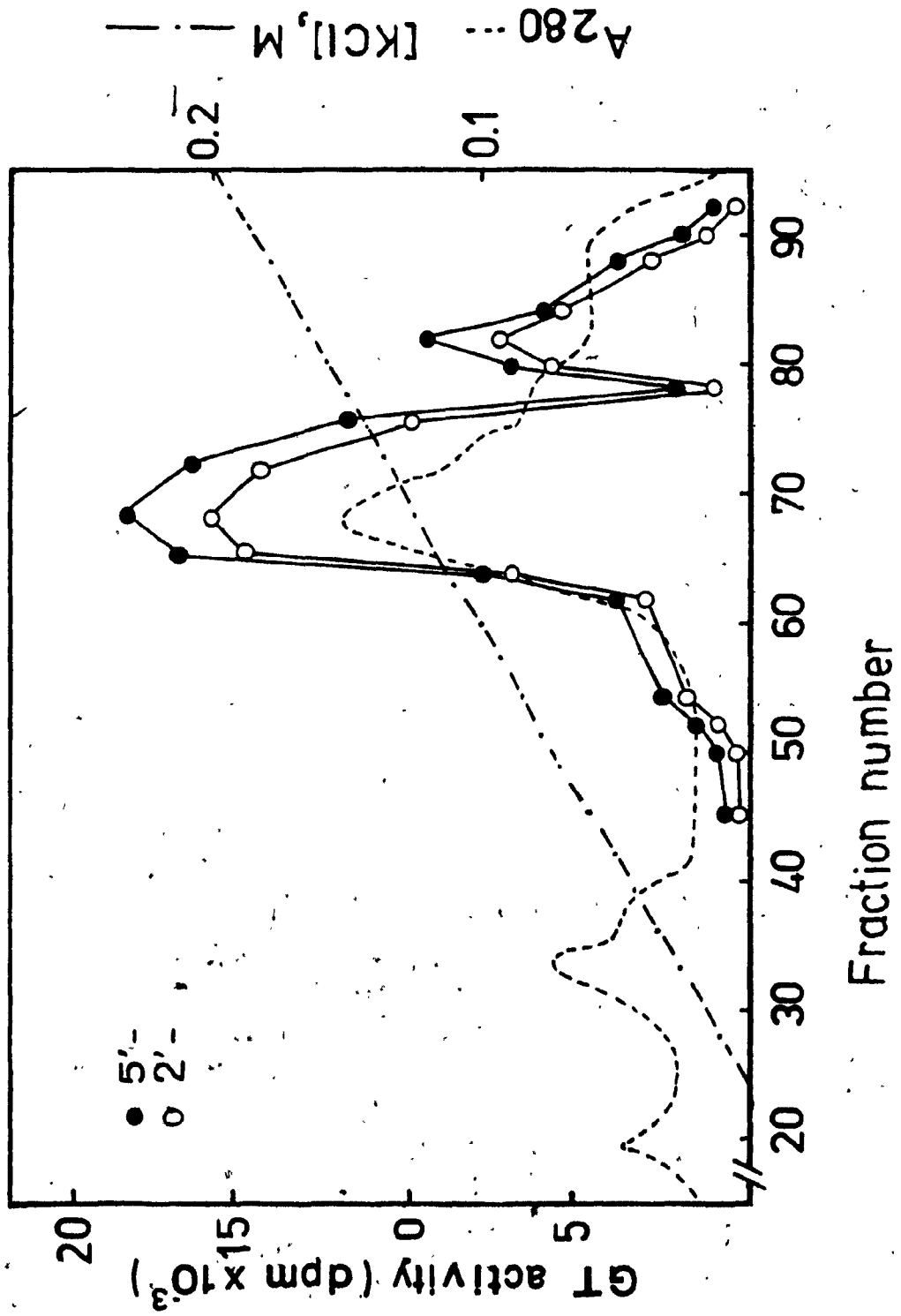


FIGURE 14: Elution profile of glucosyltransferase activity from DEAE-Sephadex A-25 column. The enzyme activity from Sephadex G-100 was applied to a column pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol. The protein was eluted with 0.5 M KCl in the same buffer, and fractions were assayed against substrates F_1 and F_2 , for 2' and 5' activities, respectively.

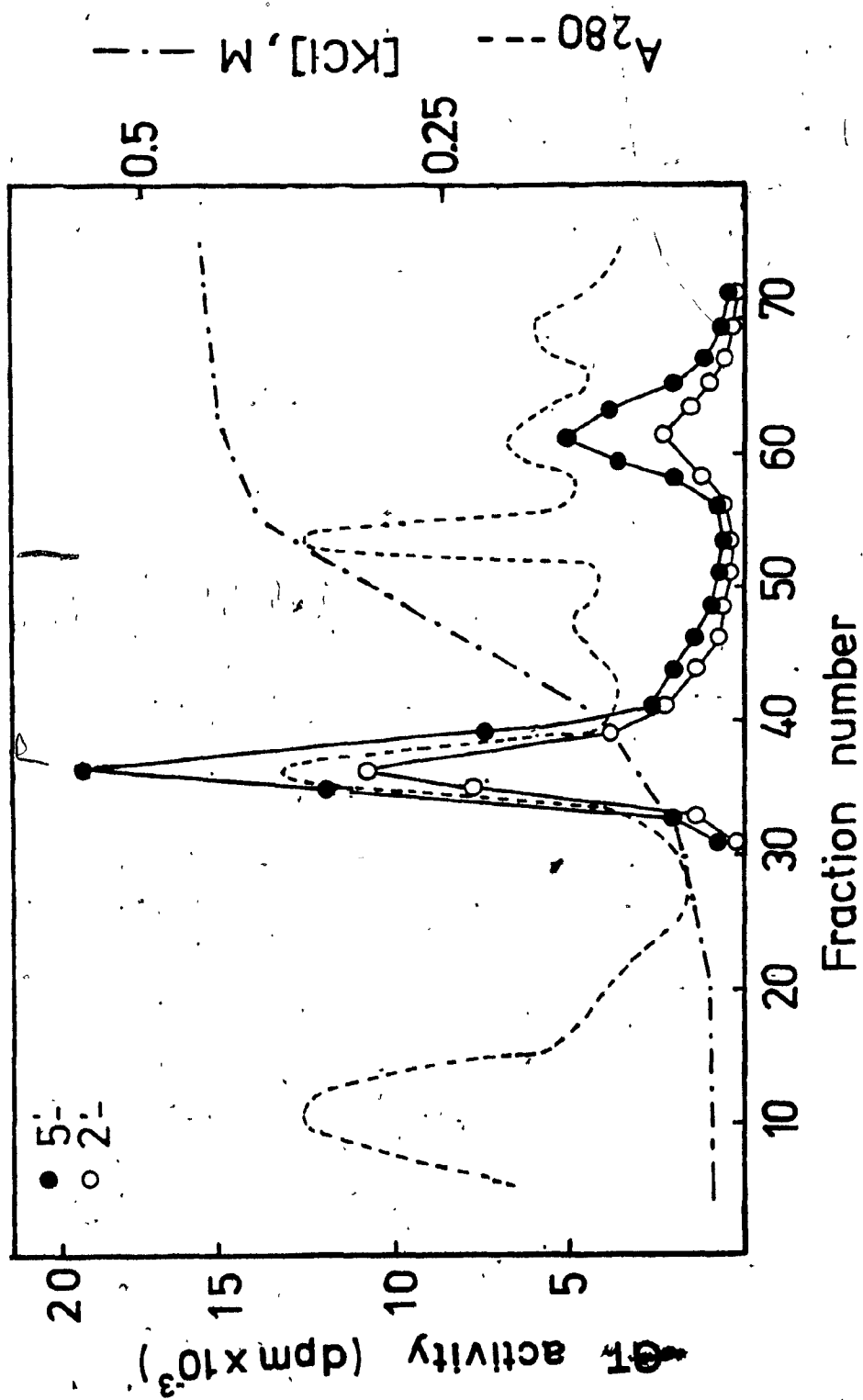


FIGURE 15: Elution profile of glucosyltransferase activity from DEAE-Sepharose column. The enzyme activity from Sephadex G-100 was applied to a column pre-equilibrated with 25 mM Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME and 10% glycerol. The protein was eluted using a gradient (0-500 mM) KCl in the same buffer, and fractions were assayed against substrates F_1 and F_2 , for 2' and 5' activities, respectively.

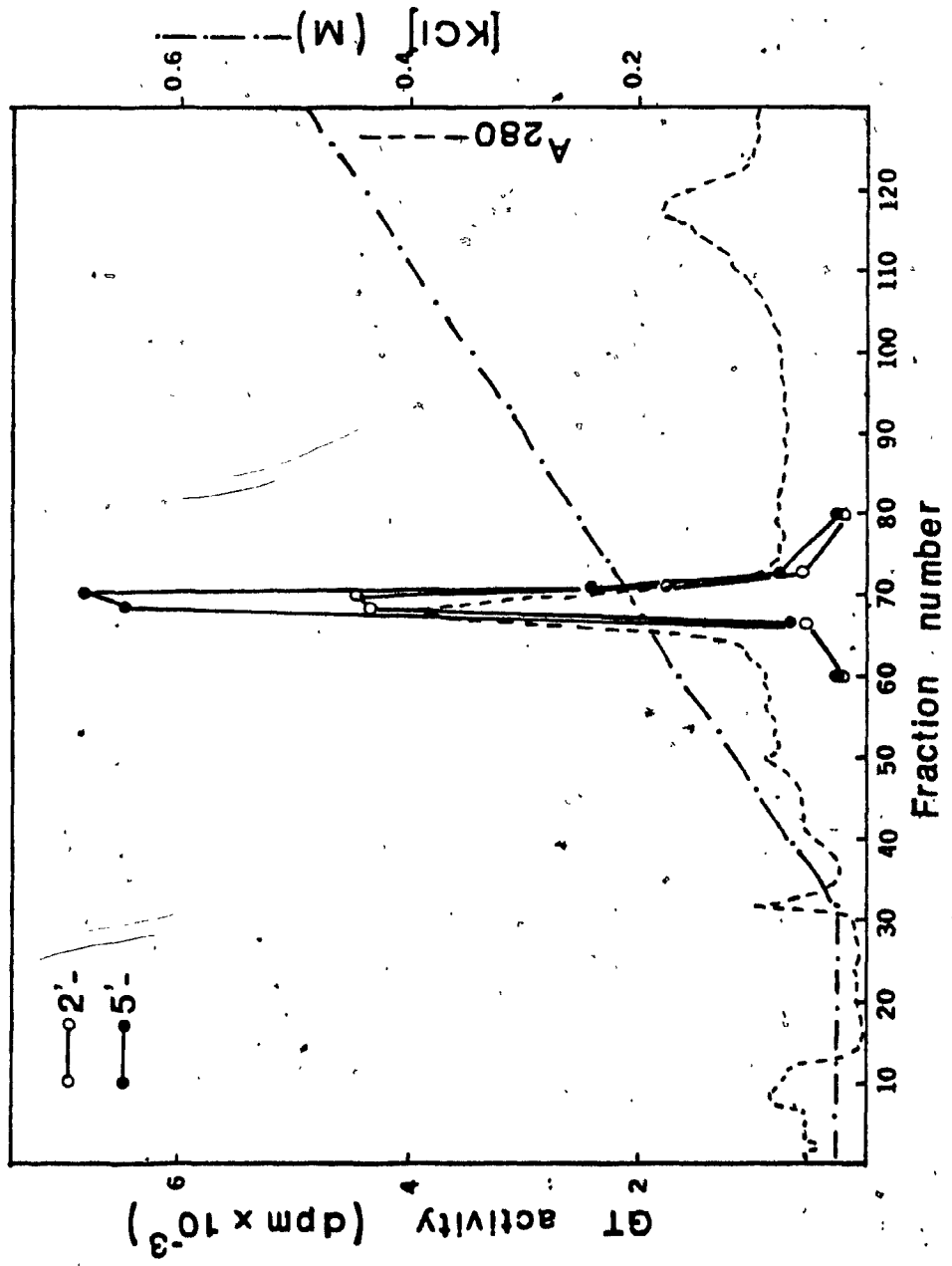
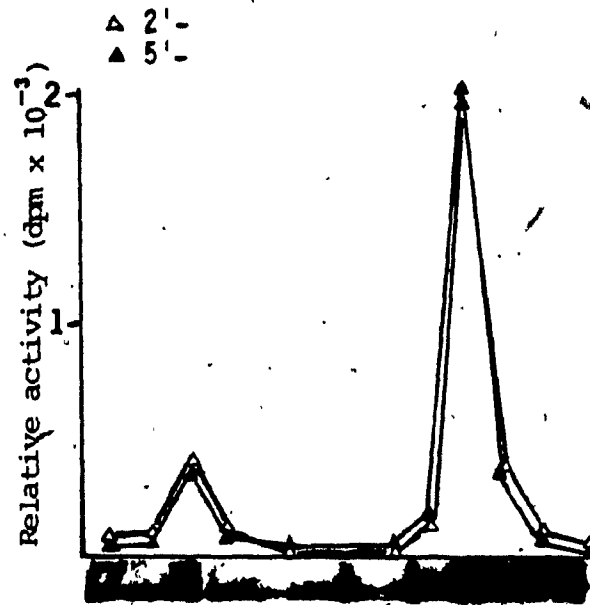


FIGURE 16: Glucosyltransferase activity after acrylamide gel electrophoresis. The purified enzyme was layered on 10% polyacrylamide gels and electrophoresed in a buffer containing 5 mM 2-ME and 2 mM EDTA at 6 C. The gels were sliced into 2-mm sections, disintegrated in Tris-HCl buffer, pH 7.8 containing 14 mM 2-ME, and assayed against substrates F_1 and F_2 for 2' and 5' activities, respectively.

★ -protein band*not necessarily glucosyltransferase enzyme protein.

The minor activity peak may be due to a non-specific glucosyltransferase.



glucosyltransferase activity was lost within 72 hours in the presence of phosphate buffer. The enzyme was stabilized by storage in 25, mM Tris-HCl buffer, pH 7.8 containing 10% glycerol and 10 mM dithioerythritol, under N₂ at -20 C. Such enzyme preparation retained its activity for at least 3 months.

D.5.2. Substrate specificity

Of the various substituted flavonols tested for their glucose acceptor ability, the best substrates were compounds F₁ for the 2'-activity and F₂ for the 5'-activity (Fig 5, Table 2). Compounds F₁ and F₂ gave their respective glucosides as the only products whose identity was confirmed by co-chromatography with authentic samples (2) and by autoradiography (Fig 6). The data on substrate specificity show that substrates with *o*-disubstituted B-ring were poor glucosyl acceptors as compared with those trisubstituted compounds having two *p*-oriented substituents. It seems plausible, therefore, that at least two *p*-oriented substituents are required for catalysis.

D.5.3. Molecular weight

An estimate of the molecular weight of this glucosyltransferase, as determined by chromatography on Sephadex G-100, was found to be 42,000 Daltons (Fig 10).

Table 2: Substrate specificity of *Chrysosplenium*
O-glucosyltransferase.

Substitution		Relative Activity	
Hydroxy-	Methoxy-	2'-	5'-
		%	
5,2'-	3,7,4',5'-	100	
5,5'-	3,6,7,2',4'-		100
5,2',5'-	3,7,4'-	80	20
5,2',5'-	3,6,7,4'-	20	80
5,6,5'-	3,7,4'-		30
5,5'-	3,6,7,4'-		30
3,5,5'-	7,4'-		14
3,5,7,4'-	5'-		12

The standard assay was used with the purified enzyme as described in "materials and methods". Identity of the reaction products was verified by co-chromatography with reference compounds and autoradiography (Fig 6). There was no activity against any of the phenylpropanoid compounds, flavones, dihydroflavonols, or any of the glucosides tested. Concentration of all substrates used was 15 μ M.

D.5.4. Kinetic properties

D.5.4.1. Initial velocity studies

Initial velocity studies were performed with the partially methylated flavonol F₁ or F₂ as the variable substrate in the presence of several, fixed concentrations of UDP-glucose. Inhibition with the flavonoid substrates F₁ (Fig 17) and F₂ (Fig 18) was observed at concentrations higher than 100 μ M. Therefore, lower concentrations were used in subsequent analyses. The patterns, thus obtained (Figs 19,20), gave a family of intersecting straight lines. Figures 21 and 22 show a primary plot of $1/v$ versus UDP-glucose at fixed concentrations of the flavonol substrates F₁ and F₂, respectively using the same data points as those obtained in Figures 19 and 20. Intercept and slope replots versus reciprocal fixed substrate concentrations (inserts of Figs 19-22) generated straight lines. The experimental data may be represented by Equation [2], the rate equation for a sequential bireactant mechanism (78). On the basis of the initial velocity data obtained, the mechanism that best fits is that of a sequential binding. The values of the kinetic parameters were calculated for each reactant as listed in Table 3.

FIGURE 17: Double-reciprocal plots of initial velocities with UDP-glucose as the changing fixed substrate at concentrations of 303 μM (\blacksquare), 103 μM (\circ), 78 μM (\blacktriangle), 45 μM (\square) and 36 μM (\bullet), showing substrate inhibition with respect to the flavonol F_1 .

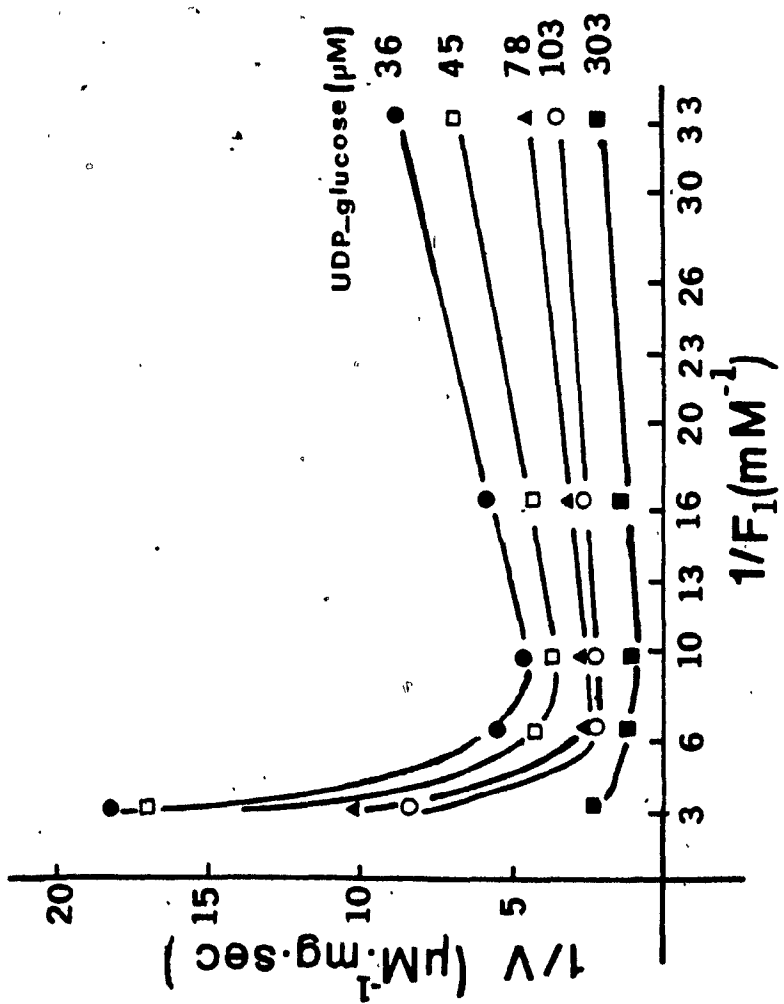


FIGURE 18: Double-reciprocal plots of initial velocities with UDP-glucose as the changing fixed substrate at concentrations of 303 μM (\square), 103 μM (\bullet), 78 μM (\circ), 45 μM (\blacksquare) and 36 μM (\triangle), showing substrate inhibition with respect to the flavonol F_2 .

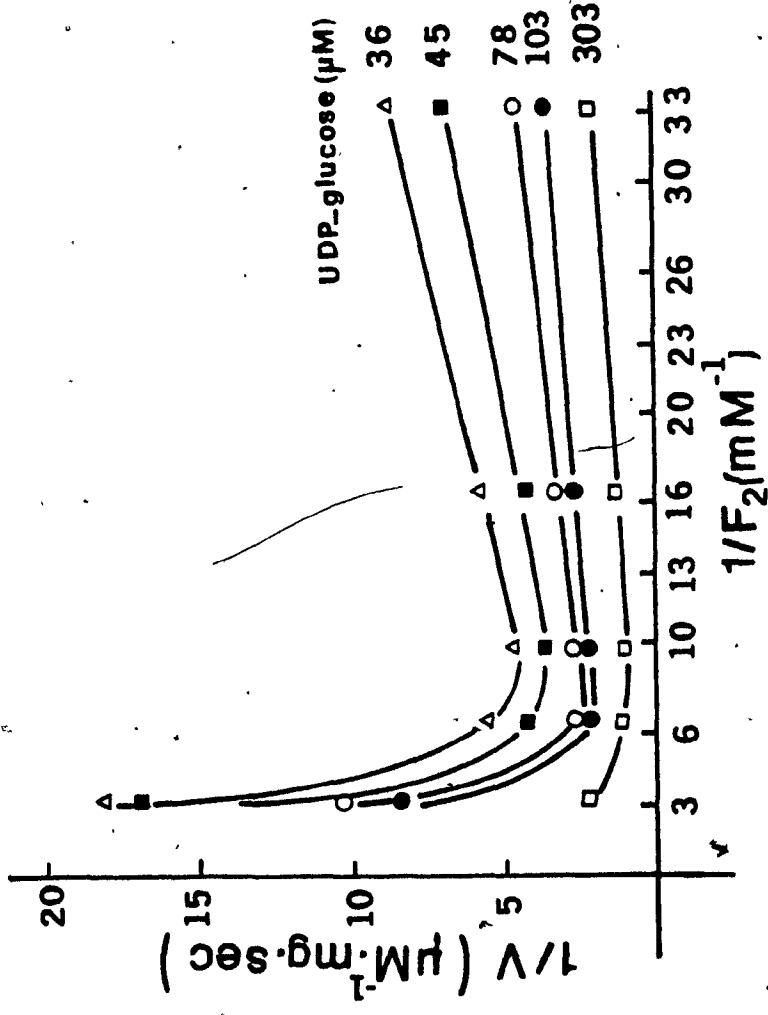


FIGURE 19: Double-reciprocal plots of initial velocities with the flavonol F_1 as the variable substrate and UDP-glucose as the changing fixed substrate at concentrations of 500 μM (\circ), 250 μM (\blacktriangle), 168 μM (\square), 125 μM (\bullet) and 100 μM (\triangle). Insert: slope and intercept replots.

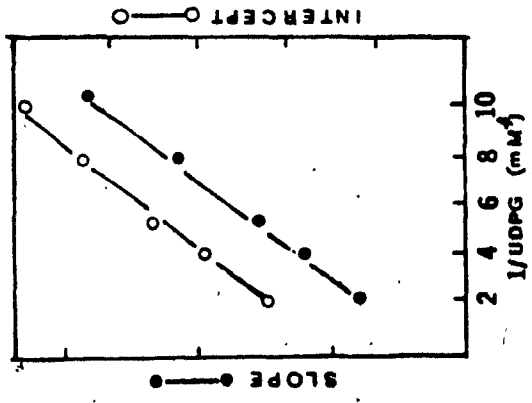
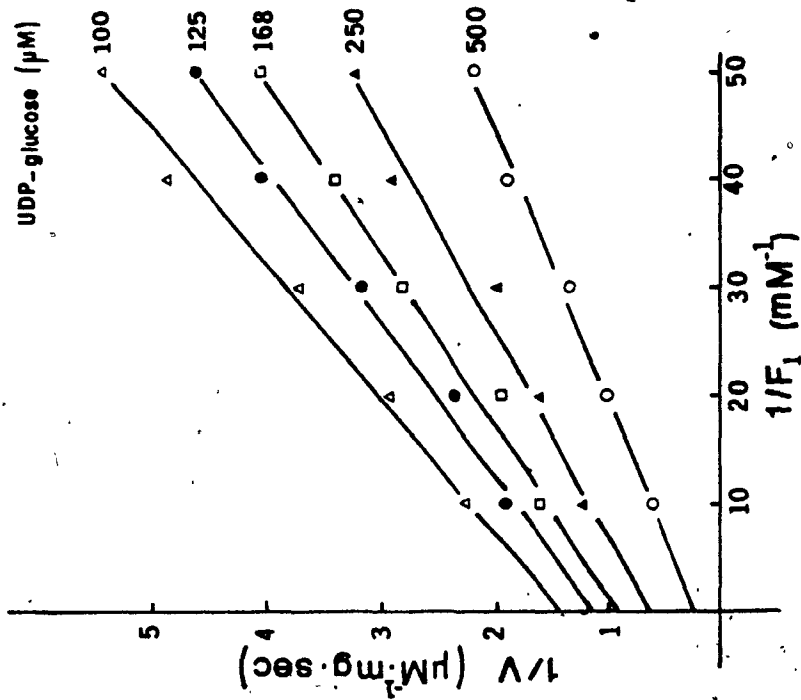


FIGURE 20: Double-reciprocal plots of initial velocities with the flavonol F_2 as the variable substrate and UDP-glucose as the changing fixed substrate at concentrations of 503 μM (\circ), 303 μM (\blacktriangle), 170 μM (\square), 103 μM (\bullet) and 74 μM (\triangle). Insert: slope and intercept replots.

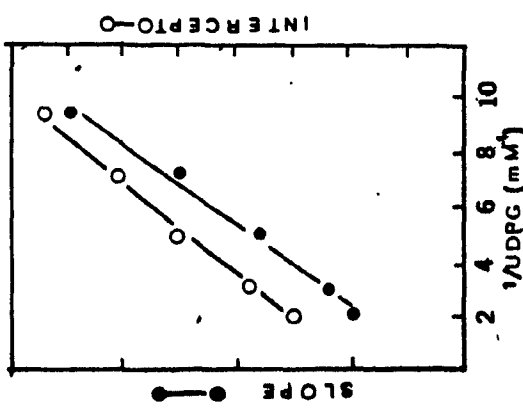
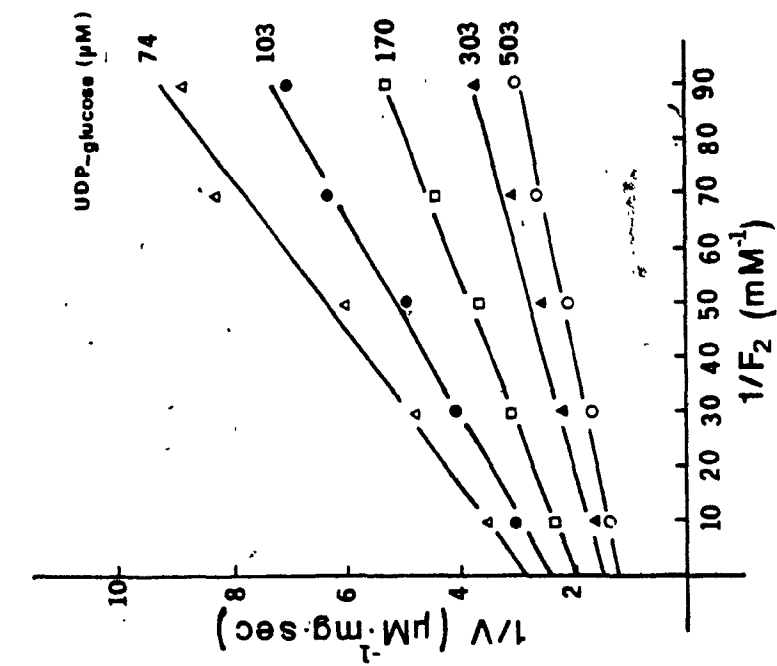


FIGURE 21: Double-reciprocal plots of initial velocities with UDP-glucose as the variable substrate and the flavonol F_1 as the changing fixed substrate at concentrations of 100 μM (\square), 50 μM (\blacktriangle), 33 μM (\circ), 25 μM (\blacksquare) and 20 μM (\triangle). Insert: slope and intercept replots.

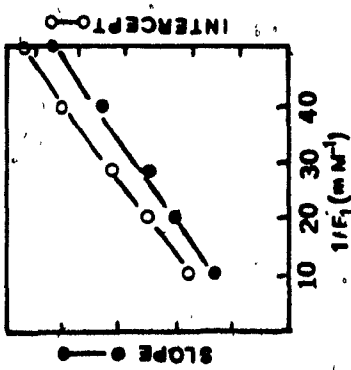
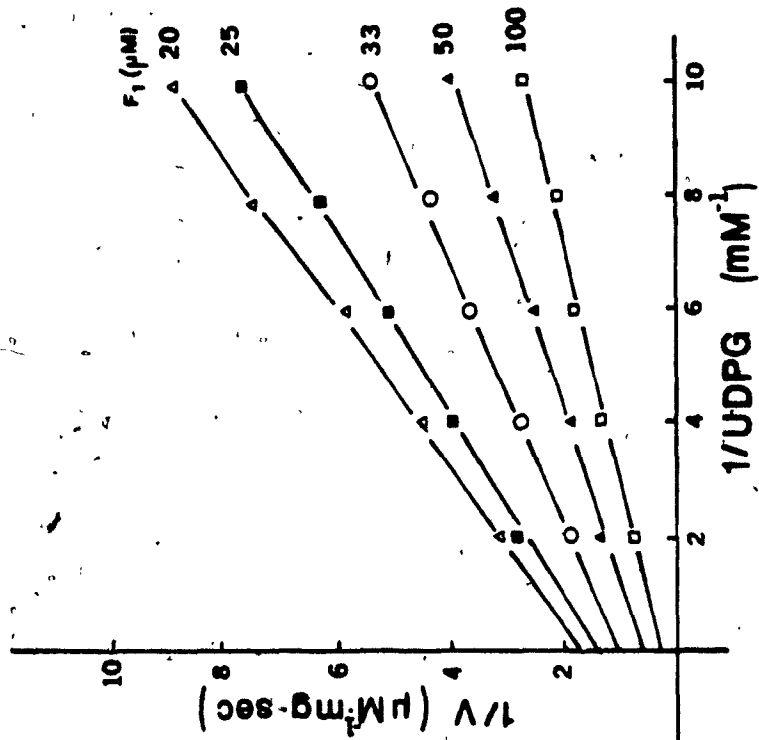


FIGURE 22: Double-reciprocal plots of initial velocities with UDP-glucose as the variable substrate and the flavonol F_2 as the changing fixed substrate at concentrations of 150 μM (\square), 100 μM (\blacktriangle), 33 μM (\circ), 14 μM (\blacklozenge) and 7.7 μM (\triangle). Insert: slope and intercept replots.

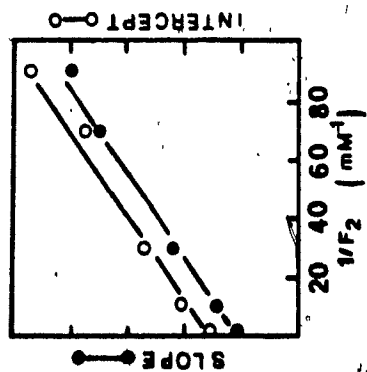
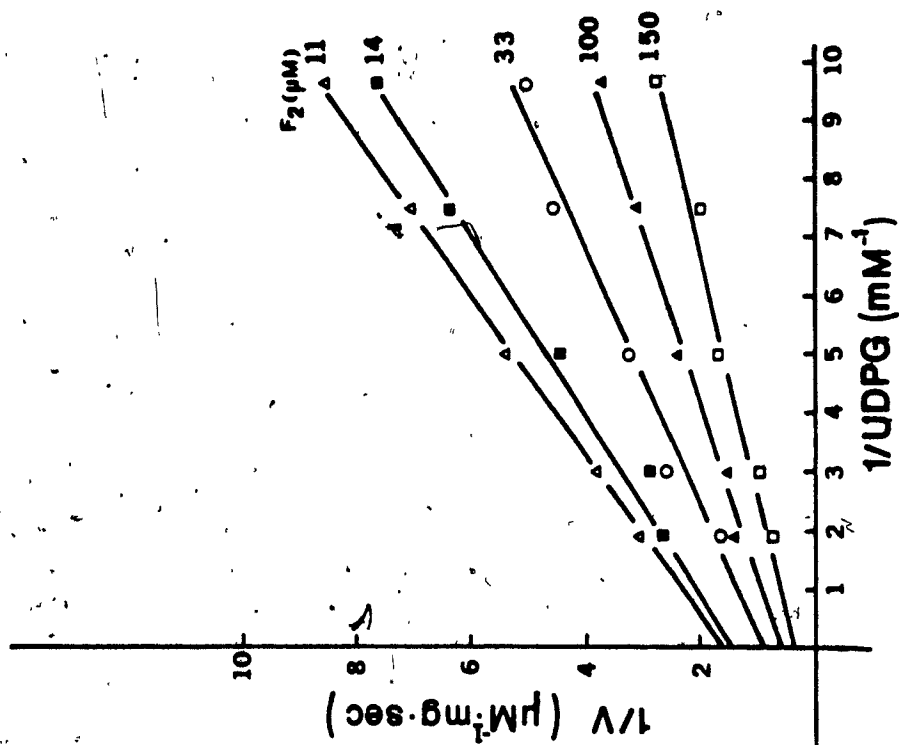


Table 3: Kinetic constants of *Chrysoosplenium* O-glucosyltransferase.

Parameter	F ₁	F ₂
K _A (mM)	0.25	0.25
K _{iQ} (μM)	25	20
K _{iA} (mM)	0.6	0.5
K _B (μM)	5	10
K _{iP} (mM)	1.0	1.0
V _{max} (μM. mg ⁻¹ . sec ⁻¹)	0.5	0.5

The flavonoid substrates used were F₁, 5,2'-dihydroxy-3,7,4',5'-tetramethoxyflavone and F₂, 5,5'-dihydroxy-3,6,7,2',4'-pentamethoxyflavone;

A, UDP-glucose; B, flavonol substrate; P, flavonol glucoside; Q, UDP (following Cleland's nomenclature (78-81)).

D.5.4.2. Product inhibition studies

Further information on the order of substrate binding and product release was obtained from product inhibition studies. The rate equations derived from a Bi Bi mechanism (78-81) predict that a product is a competitive inhibitor with respect to the substrate that binds the same enzyme form. It also predicts a noncompetitive pattern with respect to the other substrate, except when the enzyme forms are separated by an irreversible step, i.e. saturating substrate concentration, in which case an uncompetitive pattern is observed.

The results obtained are in agreement with the theoretical predictions mentioned above. UDP is a competitive inhibitor with respect to UDP-glucose (Figs 23,24) and noncompetitive with respect to the flavonol substrates F_1 (Fig 25) and F_2 (Fig 26). The flavonol glucosides are noncompetitive inhibitors with respect to both UDP-glucose (Figs 27,28) and their respective flavonol substrates (Figs 29,30). These substrate-product relationships show that the kinetic measurements satisfy the expectations of the classical ordered mechanism. Furthermore, they exclude random addition of substrates and/or release of products.

FIGURE 23: Double-reciprocal plots of initial velocities with the flavonol substrate F_1 concentration constant and saturating at 25 μM , UDP-glucose as the variable substrate and UDP as inhibitor at concentrations of 100 μM (\circ), 50 μM (\bullet), 25 μM (Δ) and 0 μM (\blacksquare).
Insert: slope replot.

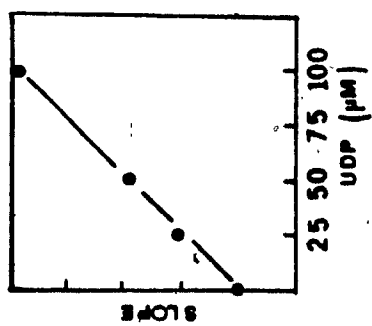
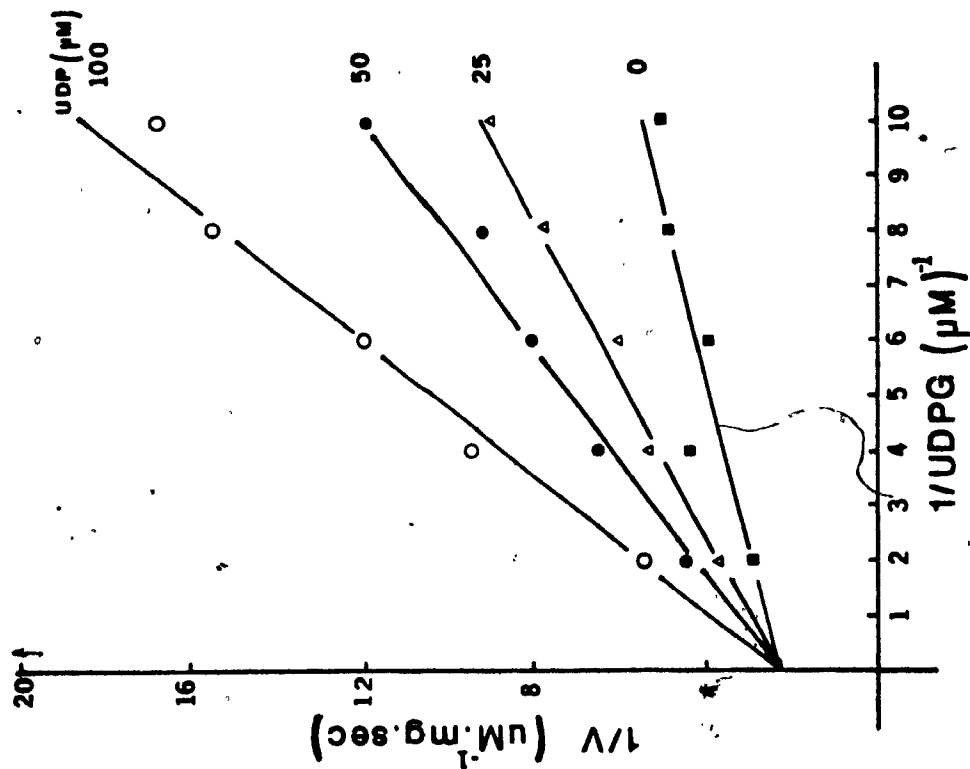


FIGURE 24: Double-reciprocal plots of initial velocities with the flavonol substrate F_2 concentration constant and saturating at $25 \mu\text{M}$, UDP-glucose as the variable substrate and UDP as inhibitor at concentrations of $100 \mu\text{M}$ (\square), $50 \mu\text{M}$ (\bullet), $25 \mu\text{M}$ (Δ) and $0 \mu\text{M}$ (\circ).
Insert: slope replot.

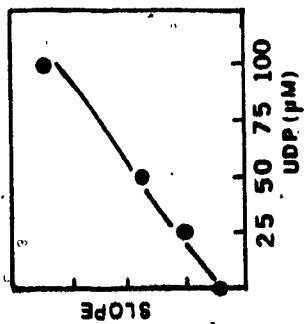
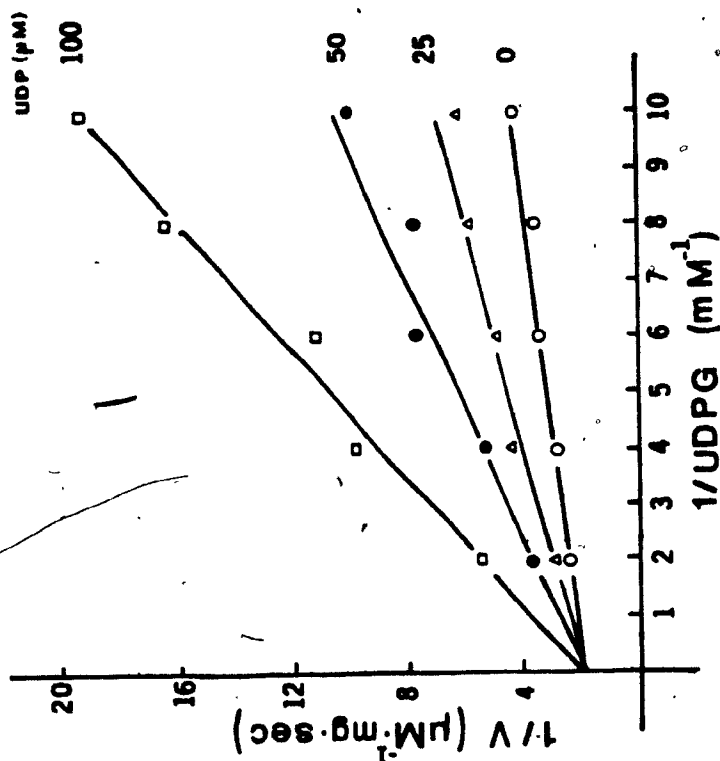


FIGURE 25: Double-reciprocal plots of initial velocities with the UDP-glucose concentration constant and low at $3 \mu\text{M}$, the Flavonol F_7 as the variable substrate and UDP as inhibitor at concentrations of $10 \mu\text{M}$ (\circ), $5 \mu\text{M}$ (\blacksquare), $2.5 \mu\text{M}$ (\triangle), $1 \mu\text{M}$ (\bullet) and $0 \mu\text{M}$ (\square). Insert: slope and intercept replots.

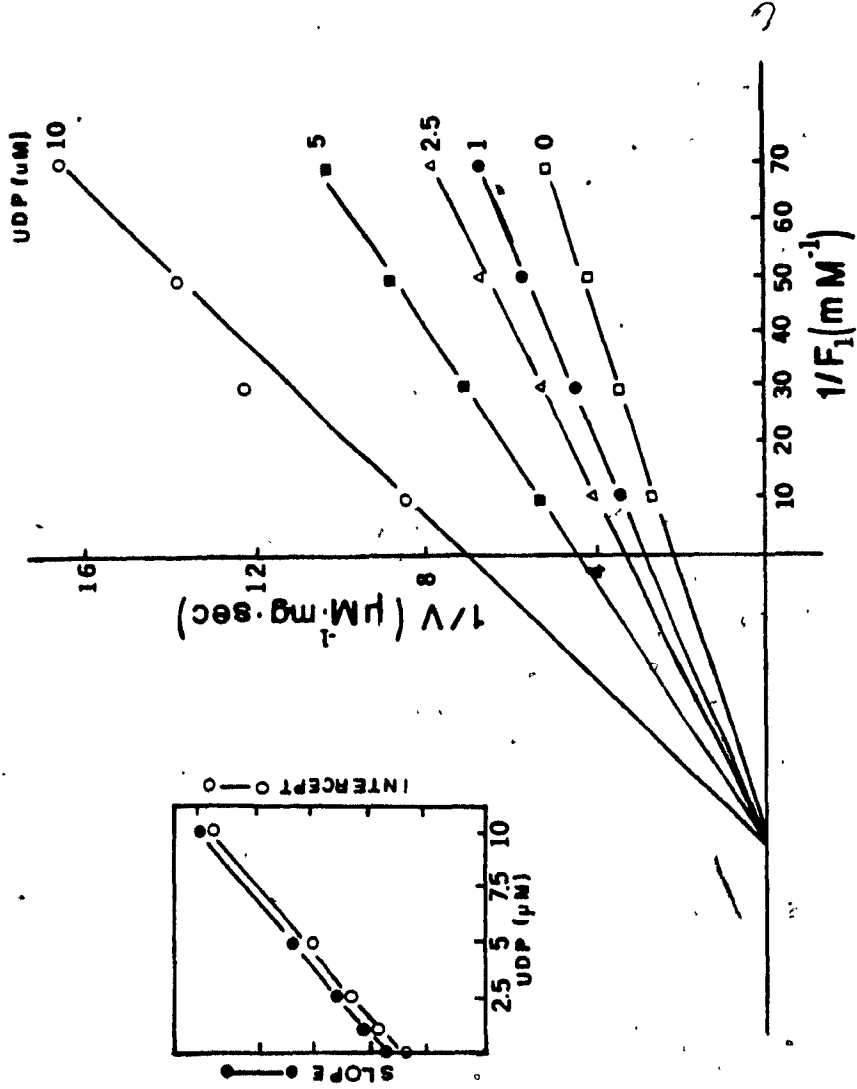


FIGURE 26: Double-reciprocal plots of initial velocities with the UDP-glucose concentration constant and low at $3 \mu\text{M}$. The flavonol F_2 as the variable substrate and UDP as inhibitor at concentrations of $10 \mu\text{M}$ (\circ), $5 \mu\text{M}$ (\blacktriangle), $2.5 \mu\text{M}$ (\square), $1 \mu\text{M}$ (\bullet) and $0 \mu\text{M}$ (\triangle). Insert: slope and intercept replots.

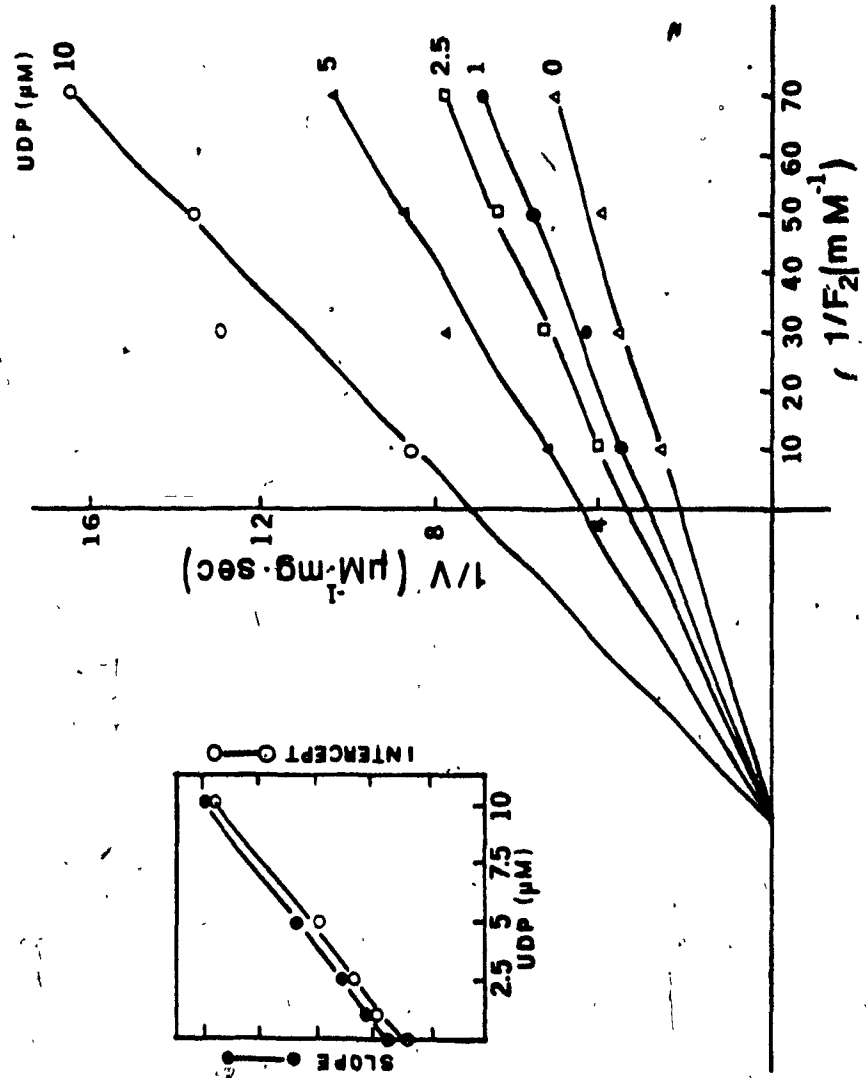


FIGURE 27. Double-reciprocal plots of initial velocities with the flavonol substrate F_1 concentration constant and non-saturating at $1 \mu\text{M}$, UDP-glucose as the variable substrate and the glucoside of F_1 ($F_1\text{-glu}$) as inhibitor at concentrations of 4 mM (\blacktriangle), 2 mM (\square), 1 mM (\bullet) and 0 mM (\circ). Insert: slope and intercept replots.

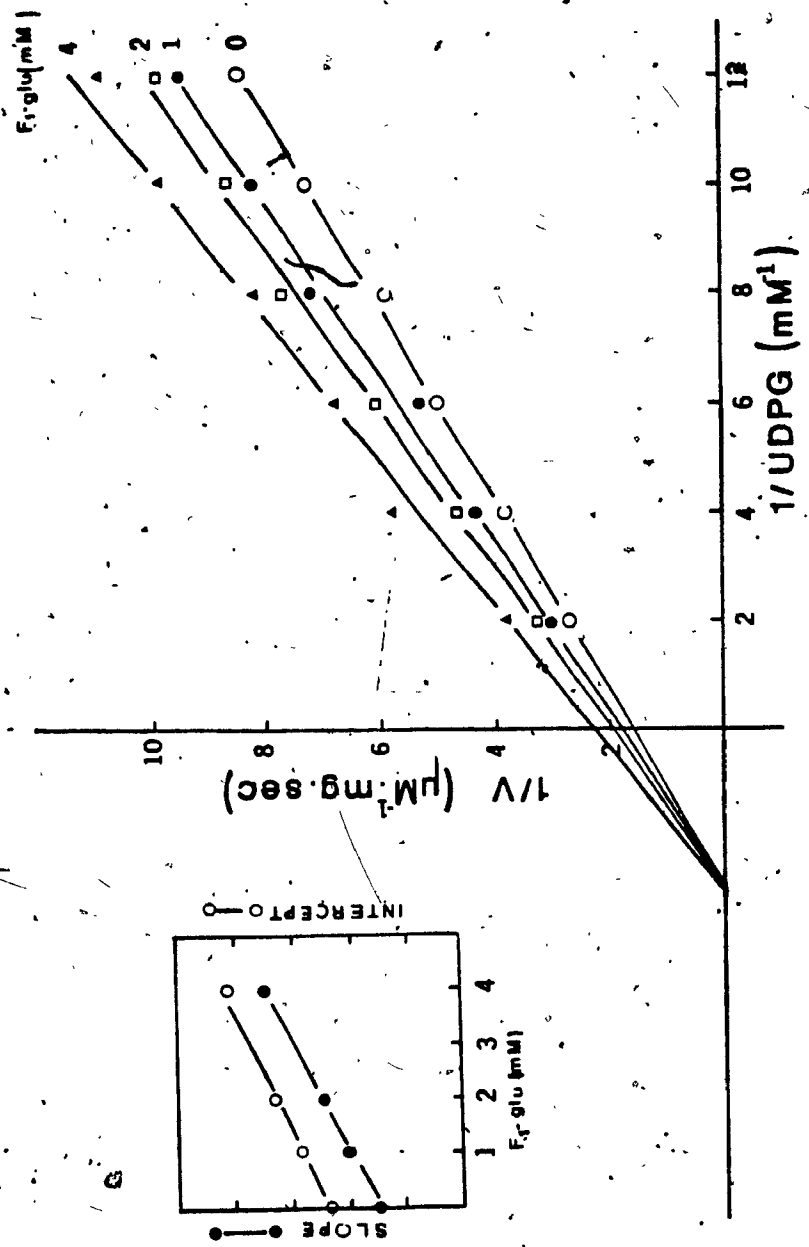


FIGURE 28: Double-reciprocal plots of initial velocities with the flavonol substrate F_2 concentration constant and non-saturating at $1 \mu\text{M}$, UDP-glucose as the variable substrate and the glucoside of F_2 ($F_2\text{-glu}$) as inhibitor at concentrations of 4 mM (\circ), 2.5 mM (\blacktriangle), 2 mM (\square), 1 mM (\bullet) and 0 mM (\triangle). Insert: slope and intercept replots.

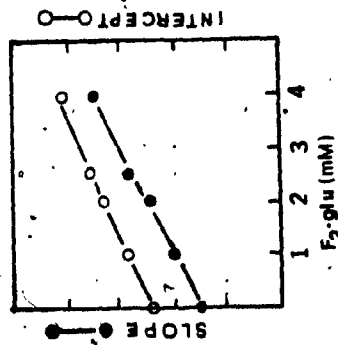
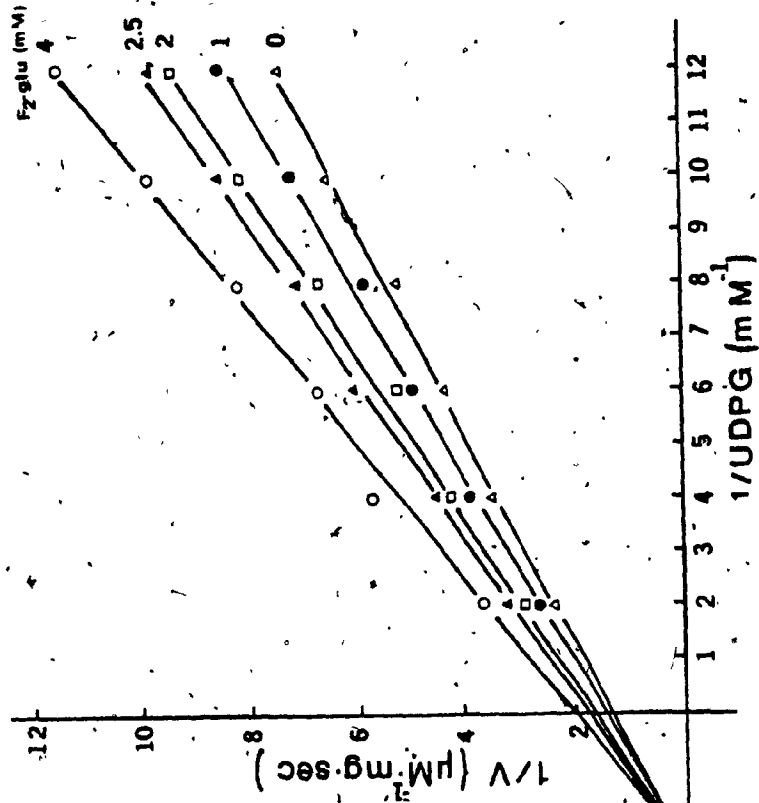


FIGURE 29: Double-reciprocal plots of initial velocities with the UDP-glucose concentration constant and low at $3 \mu\text{M}$, the flavonol F_1 as the variable substrate and the glucoside of F_1 ($F_1\text{-glu}$) as inhibitor at concentrations of 5 mM (\square), 2 mM (\bullet), 1 mM (\circ) and 0 mM (\blacksquare).
Insert: slope and intercept replots.

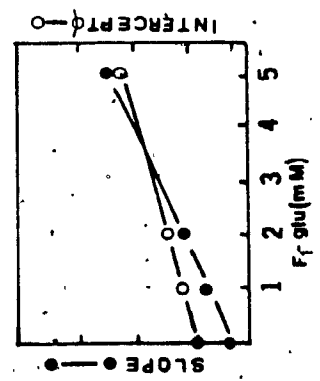
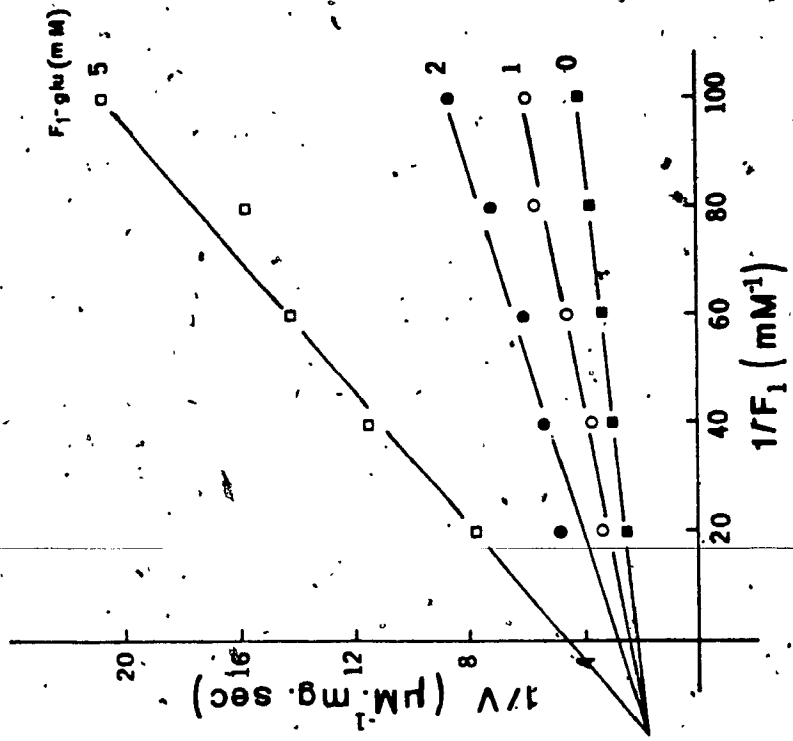
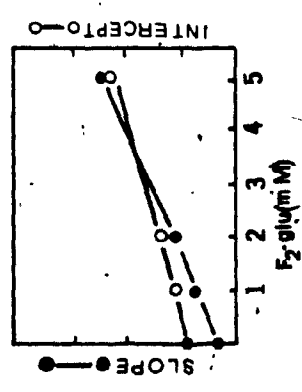
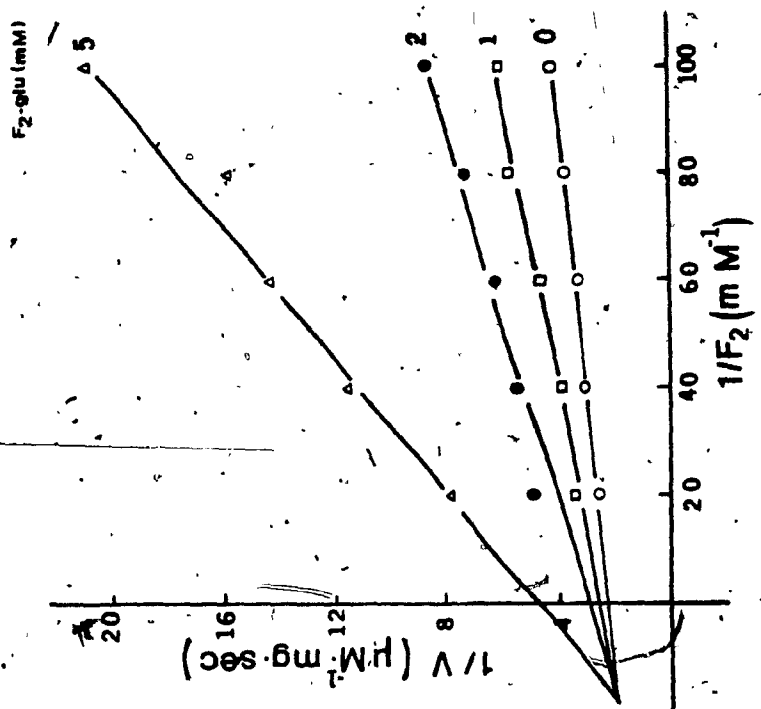


FIGURE 30: Double-reciprocal plots of initial velocities with the UDP-glucose concentration constant and low at $3 \mu\text{M}$, the flavonol F_2 as the variable substrate and the glucoside of F_2 ($F_2\text{-glu}$) as inhibitor at concentrations of 5 mM (Δ), 2 mM (\bullet), 1 mM (\square) and 0 mM (\circ).
Insert: slope and intercept replots.



D.5.4.3. Kinetic constants

K_A , K_B , K_{iP} , K_{iQ} and K_{iA} were calculated from intercept and slope replots (82) of the generated data and are listed in Table 3. A summary of the kinetic patterns obtained is shown in Table 4.

D.5.5. Kinetic mechanism of glucosylation

The kinetic patterns obtained from initial velocity and product inhibition studies of this enzyme (Table 4) are consistent with an ordered Bi Bi mechanism (78-81), whereby UDP-glucose is the first substrate that binds to the enzyme, followed by the flavonol compound F_1 or F_2 . The first product released is the flavonol glucoside, F_1 -glu or F_2 -glu, followed by UDP (Fig 31).

D.5.6. Competition between F_1 and F_2

Initial velocity studies were performed with both substrates F_1 and F_2 , mixed at varying concentrations, as the variable substrates. The reciprocal plot obtained was linear and identical to that obtained with either F_1 or F_2 alone (Fig 32). V_{max} and K_m values were not altered, implying the presence of only one enzyme catalyzing both glucosylation reactions (82).

Table 4: Summary of kinetic patterns of *Chrysosplenium*
O-glucosyltransferase

Variable Substrate	Other Substrate	Inhibitor	Pattern ^a
UDPG	FLAV ^b	-	Int. lines
FLAV	UDPG ^b	-	Int. lines
UDPG	FLAV ^c	UDP	C
UDPG	FLAV ^d	FLAV-GLU	NC
FLAV	UDPG ^d	UDP	NC
FLAV	UDPG ^d	FLAV-GLU	NC

FLAV and FLAV-GLU denote the flavonol substrate and its glucoside, respectively.

^a C, competitive; NC, non-competitive; Int., intersecting

^b Fixed and changing

^c Fixed and saturating

^d Fixed and non-saturating

FIGURE 31: A postulated ordered mechanism of flavonol-ring B
O-glucosyltransferase from *C. americanum*. FLAV,
flavonol substrate; FLAV-GLU, flavonol glucoside.

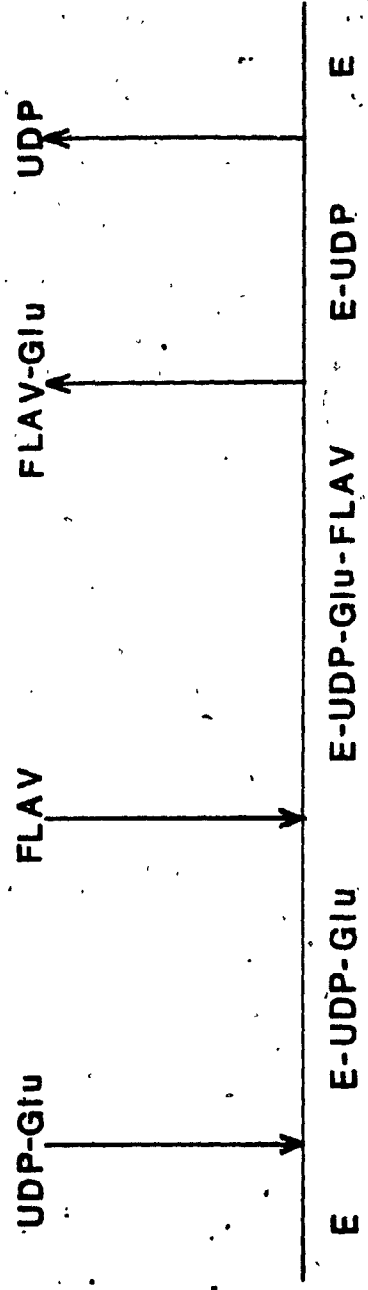
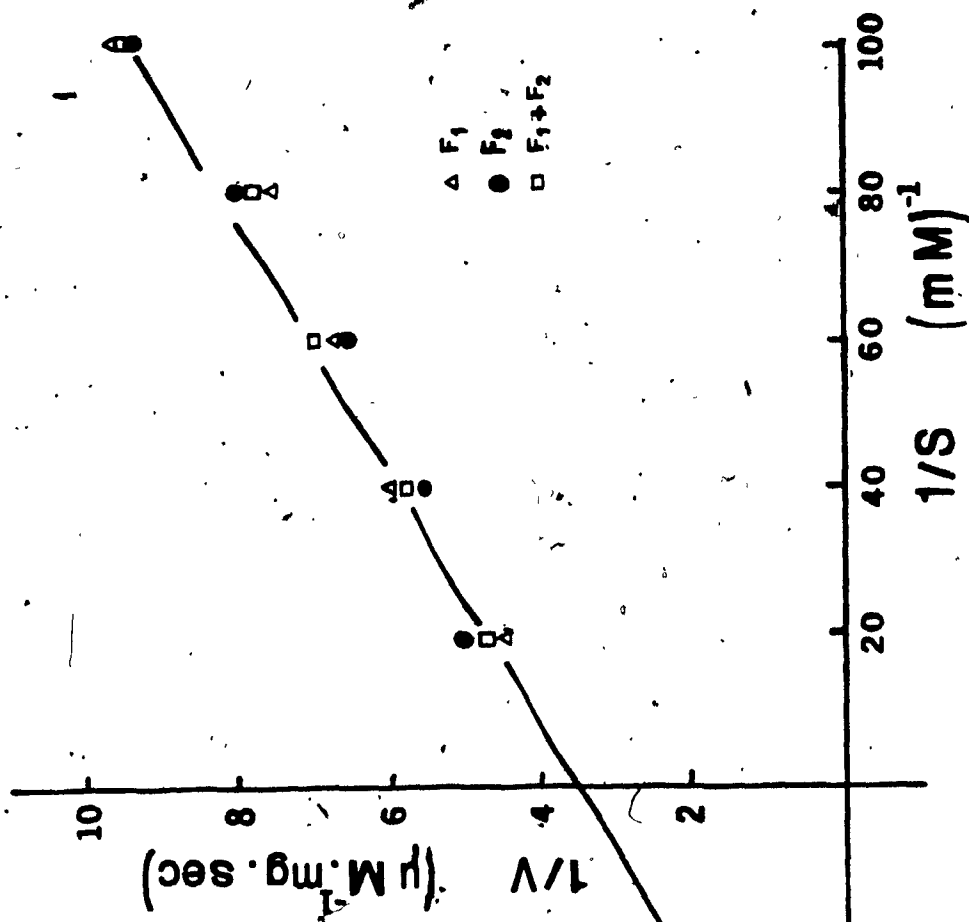


FIGURE 32: Double-reciprocal plots of initial velocities with UDP-glucose concentration constant and low at $3 \mu\text{M}$, the flavonol substrate F_1 (Δ) or F_2 (\bullet) as the variable substrate. The two substrates F_1 and F_2 were mixed (1:1) and the total concentration of the two compounds was varied (\square). S represents the total substrate concentration.



E. DISCUSSION

The experiments described here demonstrate the presence, in C. americanum, of an enzyme that catalyzes the transfer of glucose from UDP-glucose to either 2'- or 5'-hydroxyl groups of its naturally occurring, partially methylated flavonol aglycones (2).

This enzyme was purified (ca. 225-fold) by fractional precipitation with ammonium sulfate and successive chromatography on Sephadex G-100, hydroxyapatite and polybuffer ion exchanger, columns. It had an apparent isoelectric point of 5.1 and a molecular weight of approximately 42 Kdaltons. Its general properties were similar to those of other reported flavonoid glucosyltransferases with respect to pH optimum, molecular weight, requirement for SH groups, and inhibition by one of the reaction products, UDP (23,59,61,65,83-85). This enzyme showed strict substrate specificity towards the 2'- and 5'-positions of partially methylated flavonol aglycones. The substrate specificity expressed by this glucosyltransferase and the low K_A and K_B values obtained, indicate its high affinity for these substrates, as compared with other flavonoid glucosylating enzymes (62,63,65).

Both the 2'- and 5'-glucosylating activities eluted in a single peak from all of the chromatographic columns used. This behavior seems to indicate the presence

of one enzyme that catalyzes the glucosylation of both the 2'- and 5'-positions. This view was supported by the fact that, when both substrates F_1 and F_2 were mixed at near saturating concentrations, no additive effect was observed (Fig 32). This is in agreement with the kinetic theory (86) which demonstrates that, at near saturating substrate concentration, higher activity is observed in the presence of two substrates than with either one alone if the system contains two distinct enzymes mediating the reaction of both substrates.

Further evidence for the presence of one enzyme catalyzing the glucosylation of both positions was obtained from polyacrylamide gel electrophoresis of the purified preparation, in the absence of SDS. Both 2'- and 5'-glucosylating activities were found in the same polypeptide band.

The kinetic data obtained from initial velocity and product inhibition studies of this enzyme is consistent with an ordered Bi Bi mechanism (78-81) where UDP-glucose is the first substrate that binds to the enzyme, followed by the flavonol compound. The first product released is the flavonol glucoside followed by UDP. The fact that inhibition by the flavonol glucoside with respect to its corresponding substrate is noncompetitive clearly indicates that one or more central enzyme complex is present and rules out a Theorell-Chance type mechanism. Figure 31 shows a proposed kinetic mechanism for this glucosyltransferase

based on the kinetic patterns reported in Table 4. To our knowledge, this is the first complete kinetic analysis of a flavonoid glucosyltransferase. However, a similar reaction mechanism has been reported for a coniferyl alcohol glucosyltransferase with respect to the order of substrate binding and product release (87):

Whereas the substrate interaction kinetic studies showed inhibition with the flavonoid compounds, the nature of this inhibition could not be determined due to the interaction of the inhibitor (flavonoid substrate) with the co-substrate (UDP-glucose), as well as with the products of the reaction (82). The fact that all intercept and slope replots were linear, clearly indicates that no multiple combinations of the inhibitor with the enzyme take place.

The K_{iQ} , as determined from replots of product inhibition studies, was found to be 10-fold lower than K_A . This seems to imply that the ratio of UDP to UDP-glucose concentration, and not the concentration of UDP-glucose itself, regulates the glucosylation reaction. The high K_{ip} values obtained, as compared with those of their corresponding K_B , indicate that the reaction is not inhibited by the glucosylated products formed. This, together with the fact that flavonol glucosides were not accepted as substrates for O-methylation (3), supports the view that glucosylation is a later step in the biosynthesis of flavonoid compounds (1,53) and conforms with the accumulation of these glucosides in vivo (2).

The similarity of the K_A and K_B values for the two substrates F_1 and F_2 and the fact that the kinetic mechanism of this enzyme for both substrates was identical further supports the concept of the presence of one enzyme that catalyzes the glucosylation of both the 2'- and 5'-positions.

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