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PURIFICATION AND PROPERTIES OF AN ENZYME FROM CELL SUSPENSION CULTURES OF PARSLEY CATALYZING THE TRANSFER OF D-GLUCOSE FROM UDP-D-GLUCOSE TO FLAVONOIDS

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SUMMARY

1. An enzyme from cell cultures of parsley catalyzing the transfer of the D-glucosyl moiety of UDP-D-glucose to flavonoids to form 7-*O*-β-glucosides has been purified 89-fold by $(NH_4)_2SO_4$ fractionation, DEAE-cellulose and Sephadex chromatography, and isoelectric focusing.

2. A separation from the UDP-apiose:apigenin-7-*O*-glucoside (1→2)-apiosyltransferase which is also present in parsley was achieved by chromatography on DEAE-cellulose, on hydroxylapatite, or by preparative disc electrophoresis.

3. The UDP glucosyltransferase is a soluble enzyme with a pH optimum of pH 7.5. The molecular weight of the enzyme was estimated by the elution volume on a Sephadex G-100 column to be about 50 000.

4. The apparent K_m value for UDP-glucose is $1.2 \cdot 10^{-4}$ M. TDP-glucose can also serve as glucosyl donor ($K_m = 2.6 \cdot 10^{-4}$ M).

5. A number of flavones, flavanones and flavonols can function as glucosyl acceptors, whereas isoflavones, cyanidin, *p*-coumaric acid, and some other phenols were inactive as acceptors. The best acceptors were luteolin, apigenin, chrysoeriol, and naringenin with relative activities of 210:100:63:45. All these flavonoids are present in parsley or are postulated to be biosynthetic intermediates in the flavone-glycoside pathway.

INTRODUCTION

A few enzyme preparations catalyzing the transfer of the sugar moiety of a sugar nucleotide to a flavonoid to form a flavonoid-*O*-glycoside have been described previously.

The formation of 3-quercetin-D-glucoside from TDP-D-glucose or UDP-glucose and quercetin[1] and the transfer of the L-rhamnosyl moiety of TDP-L-rhamnose to 3-quercetin-D-glucoside to form rutin[2] are catalyzed by enzyme preparations from mung beans (*Phaseolus aureus*). An enzyme preparation from leaves of *Leucaena*

glauca catalyzes the formation of quercetin-3-rhamnoside from quercetin and UDP-L-rhamnose[3]. With extracts of the flower petals of *Impatiens balsamina* a number of flavonols were converted to the 3-monoglucosides with UDP-D-glucose as donor[4].

The transfer of D-glucuronic acid from UDP-D-glucuronic acid to quercetin to form a β-glucuronide was demonstrated with an enzyme preparation from French beans (*Phaseolus vulgaris*)[5]. None of these enzymes have been purified to any extent nor well characterized as to their specificity.

In the course of our work on the biosynthesis of flavone glycosides in young parsley plants[6] and in cell suspension cultures of this plant[7], we have described briefly an enzyme which catalyzes the transfer of D-glucose from UDP-D-glucose to apigenin (5,7,4′-trihydroxyflavone) to form apigenin-7-*O*-glucoside. In the present paper we describe the partial purification of this enzyme from cell cultures of parsley and report some of its properties. The UDP-glucose transferase could be separated from the UDP-apiose:apigenin-7-*O*-glucoside (1→2)-apiosyltransferase which had been found previously in this plant[6–8].

MATERIALS AND METHODS

Substrates and reference compounds

Flavonoids and their glycosides were from our laboratory collection.

UDP-D-[$^{14}C_6$]glucose was purchased from Radiochemical Centre Amersham. Radioactive impurities were separated by paper chromatography on Whatman 3 MM with the solvent system ethanol–1 M ammonium acetate (pH 7.5) (5:2, by vol.).

UDP-glucose, TDP-glucose, CDP-glucose, ADP-glucose, UDP-D-glucuronic acid, α-glucose 1-phosphate, UDP, UTP, and reference proteins were purchased from Boehringer, Mannheim. UDP-xylose was obtained from Sigma Chemical Co., St. Louis, Mo. and β-galactosidase from Serva, Heidelberg.

Synthesis of [2-^{14}C]apigenin

95 mg [*carbonyl*-^{14}C]*p*-benzyloxybenzaldehyde[16] (2.2 mC/mmole) were dissolved in 0.97 ml ethanol and added to a solution of 100 mg phloroacetophenone dimethylether in 2.3 ml 30% KOH *plus* 0.97 ml ethanol. The reaction mixture was stirred for 11 h at room temperature. The solution was then adjusted to pH 6 with 6 M HCl and left for 12 h at 4°. The crystals were collected and the chalcone recrystallized from ethanol. Yield: 70 mg (63%) [β-^{14}C]4-benzyloxy-2′-hydroxy-4′,6′-dimethoxychalcone, m.p. 158° (uncorrected)[18].

50 mg of the chalcone was dissolved in 1.6 ml *n*-amyl alcohol and boiled under reflux for 18 h with 0.4 g SeO_2 (ref. 17). After decanting from the SeO_2 5 ml water was added and steam passed through the solution to remove amylalcohol. When the alcohol had been removed the product crystallized in brown flakes. Some material was lost and 32 mg of [2-^{14}C]5,7-dimethoxy-4′-benzyloxyapigenin[18] was isolated.

To remove the protecting groups 32 mg of this product were then dissolved in 2 ml acetanhydride and boiled under reflux for 2 h with 3.3 ml 38% hydroiodic acid. The reaction mixture was then poured on ice and decolourized by addition of dithionite. The precipitated raw product (30 mg) was then purified by paper chromatography in benzene–acetic acid–water (125:72:3, by vol.). Two radioactive zones with R_F-values 0.3 and 0.5 were detected on the chromatogram. The zone with R_F 0.3, which

corresponded to reference apigenin, was eluted with methanol and rechromatographed on paper with Solvent system 3 (see *Analytical methods*). After elution with methanol and removal of the solvent 10.5 mg [2-^{14}C]apigenin with a specific activity of 2.2 mC/mmole were obtained.

[2-^{14}C]Naringenin

5 mg of [β-^{14}C]4,2',4',6'-tetrahydroxychalcone-2'-glucoside[19] (2.2 mC/mmole) and 2.5 mg 4,2',4',6'-tetrahydroxychalcone were dissolved in 6 ml of a mixture of trifluoroacetic acid–methanol–water (2:1.4:2.6, by vol.) and refluxed for 3 h under nitrogen. The solution was then evaporated to dryness in vacuo in a rotary evaporator. The residue was dissolved in methanol and chromatographed on prewashed paper (see *Chromatographic methods*) with Solvent system 1 (R_F naringenin 0.95) and with 10% acetic acid (R_F = 0.36). Yield: 3.4 mg ($\approx$60%).

Chromatographic methods

For descending paper chromatography on Whatman 3 MM, prewashed with methanol, 10% acetic acid and 0.01 M EDTA the following solvent systems were used: 1, butanol–acetic acid–water (20:1:4, by vol.); 2, 15% acetic acid; 3, chloroform–acetic acid–water (50:45:5, by vol.); 4, ethylacetate–acetic acid–boric acid (saturated aqueous solution)–water (20:2:1:1, by vol.).

Thin-layer chromatography was performed on polyamide–cellulose (15:6, by wt. MACHEREY AND NAGEL) with: 5, chloroform–methanol–methyl ethyl ketone–acetyl acetone (66:20:10:2, by vol.); 6, methanol–water–methyl ethyl ketone–acetylacetone (50:45:25:5, by vol.) and on silica gel (Merck, Darmstadt) with: 7, toluene–ethyl formate–formic acid (5:4:2, by vol.).

The R_F values of flavones and its glucosides are recorded in Table I.

TABLE I

R_F VALUES OF FLAVONES AND ITS GLUCOSIDES IN DIFFERENT SOLVENT SYSTEMS

Solvent system	*Apigenin*	*Cosmosiin*	*Luteolin-7-O-glucoside*	*Chrysoeriol-7-O-glucoside*	*Prunin**
1	0.91	0.49			
2	0.10	0.25			
3	0.70	0.42			
4	0.91	0.17			
5	0.50**	0.55**	0.3**	0.65**	0.8**
6	0.05**	0.48**			
7	0.65***	0.13***			

* 5,7,4'-Trihydroxyflavanone-7-*O*-glucoside.
** On polyamide–cellulose.
*** On silica gel.

Cell suspension cultures

Cultivation of cell cultures of *Petroselinum hortense* has been described previously[7]. Enzyme was extracted from cells which had been illuminated for 24 h with white light[7] prior to being harvested 10 days after the cultures were started.

TABLE II

PURIFICATION OF UDP-GLUCOSE:APIGENIN 7-*O*-GLUCOSYLTRANSFERASE FROM CELL CULTURES OF PARSLEY

One enzyme unit is defined as the enzyme quantity which catalyzes the formation of 1 nmole product per min at 30° in the enzyme assay.

Purification step	*Vol. (ml)*	*Protein (mg)*	*Specific activity (units/mg protein)*	*Enzyme purification*	*Yield (%)*
Crude extract	1348	4720	3.6	–	100
Dowex 1-X2	1323	4100	4.6	1	110
Protamine sulfate	1370	2140	8.5	1.9	107
$(NH_4)_2SO_4$ fractionation (0.38–0.63)	42	431	34.5	7.6	88
DEAE-cellulose column	11	138	83.7	18	68
Sephadex G-200 column	6.5	21	275	61	34
Isoelectric focusing	4	1.3	401	89	3

Enzyme purification (Table II)

All steps were carried out at 4°. About 1395 g (wet wt.) of cells were suspended in 725 ml of 0.5 M Tris–HCl buffer (pH 7.5) containing $1.4 \cdot 10^{-2}$ M 2-mercaptoethanol in a 3-l beaker and homogenized for 30 sec with an Ultra Turrax (Janke and Kunkel, Type T 45 N, 10 000 rev./min). After a stop of 1-min homogenization was repeated and the procedure was continued for a total homogenization time of 20 min. Microscopic control showed that most cells were broken after this time. The homogenate was centrifuged for 20 min at 11 000 × *g* (supernatant 1355 ml).

1348 ml of the supernatant were stirred for 30 min with 150 g Dowex 1-X2 (Cl^- form equilibrated with 0.2 M Tris–HCl, pH 7.5) and the Dowex was filtered off. To the filtrate (1323 ml) was added 132 ml of a solution of protamine sulfate (filtrate of a 2% solution of pH 7.5 at 4°) and the solution was stirred for 15 min. The solution was then centrifuged at 11 000 × *g* for 20 min.

The supernatant was subjected to $(NH_4)_2SO_4$ fractionation at pH 7.5 by addition of the solid salt. Protein precipitated between 0–38%, 38–63% and 63–80% saturation was collected by centrifugation at 11 000 × *g* for 20 min, and each precipitate was dissolved in 35 ml 0.1 M Tris–HCl buffer. The protein precipitated between 38–63% saturation contained 88% of the enzymatic activity. The solution of this fraction was chromatographed on a column (3.2 cm × 25 cm) of Sephadex G-25, equilibrated before use with 0.02 M Tris–HCl (pH 7.5) containing $2 \cdot 10^{-3}$ M dithioerythritol.

The filtrate from the G-25 column was centrifuged for 20 min at 50 000 × *g* and the clear supernatant was then chromatographed with a discontinuous gradient (Gradient mixer Ultrograd, LKB Stockholm) of Tris–HCl buffer (pH 7.5) on a column of DEAE-cellulose (3.2 cm × 25 cm), equilibrated with 0.02 M Tris–HCl buffer (pH 7.5) containing $2 \cdot 10^{-3}$ M dithioerythritol at a flow rate of 87 ml/h. Fractions 53–62 were concentrated by filtration through a "Diaflo" concentrator (AMICON, Model 50, ultrafiltration cell) to 11.3 ml.

10.8 ml of this solution were then chromatographed with $2 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5) containing $5 \cdot 10^{-3}$ M 2-mercaptoethanol on a column of Sephadex G-200 (5 cm × 47 cm), equilibrated with the same buffer at a flow rate of 47.5 ml/h.

14.8-ml fractions were collected for 20 h. Fractions 39–42 were pooled and concentrated with the "Diaflo" filter to 6.5 ml.

With 3 ml of this concentrate, isoelectric focusing was carried out on Sephadex G-75 superfine thin-layer plates (20 cm × 20 cm) in the pH range 4–6 (ref. 9). The plates were prepared from 55 ml gel containing 3.5 g Sephadex G-75 superfine, 1% ampholine (LKB, Stockholm), pH 4–6, and $2 \cdot 10^{-3}$ M dithioerythritol. The protein solution was applied to a 4 cm × 18 cm wide zone at the side of the cathode. The electrofocusing was carried out for 7.5 h in a cooled (2°) apparatus for thin-layer electrophoresis (Desaga, Heidelberg), beginning with 9 mA/300 V, ending with 2 mA/500 V. At the end of the separation the gel was scraped off in 0.5-cm zones and each zone mixed with 1 ml of 0.5 M Tris–HCl buffer (pH 7.5). The separation of the gel was achieved by sieve centrifugation[15]. The enzyme activity was concentrated in a 1-cm-broad zone between pH 4.9 and 5.1. The pH was determined in separate samples of the gel after mixing with CO_2-free distilled water using the microelectrode GK 232/C AB and a pH-meter (Radiometer 26, Kopenhagen). To remove dithioerythritol and ampholine before determination of protein concentration the solution was passed in the presence of $2 \cdot 10^{-3}$ M 2-mercaptoethanol through a Sephadex G-25 column, equilibrated with $2 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5).

Enzyme assay

Standard incubation: The incubation mixture consisted of 455 nmoles UDP-glucose, 10 nmoles [2-^{14}C]apigenin (2.2 mC/mmole), 1.6 μmoles 2-mercaptoethanol, 23 μmoles Tris–HCl buffer (pH 7.5) in a total volume of 230 μl. The reaction was started by addition of protein. The mixture was incubated at 30° for 15–50 min. At the end of this period 20 μl of a solution of 7-*O*-glucosylapigenin (0.5 mg in 1 ml of ethyleneglycol monomethylether) were added to the incubation mixture and 150 μl of this solution was applied to a polyamide thin-layer plate (0.3 mm). The plate was developed with Solvent system 6. The zone of glucosyl apigenin was detected under ultraviolet light ($R_F = 0.5$, apigenin $R_F = 0.03$), scraped off and counted in a dioxane scintillation fluid (1 l dioxane, 100 g naphthalene, 5 g PPO) with a Beckman LS 100 liquid scintillation spectrometer (counting yield approx. 75%). Every test was run in duplicate or triplicate.

Incubation for donor specificity: 500 nmoles of each donor was added to the standard incubation. For the separation of the expected transfer product with UDP-glucuronic acid Solvent system 5 was used (R_F apigenin-7-*O*-glucuronide 0.02).

Incubation for acceptor specificity: The general conditions were the same as in the standard incubation. Each incubation contained 25 nmoles of UDP-D-[$^{14}C_6$]glucose (5 mC/mmole) and 30 nmoles of the phenols, except in the case of luteolin, chrysoeriol, naringenin, and apigenin, in which 10, 20 and 30 nmoles of each flavonoid and 275 nmoles of UDP-D-[$^{14}C_6$]glucose (0.8 mC/mmole) were used.

After incubation with cyanidine the reaction mixture was subjected to high-voltage paper electrophoresis (Pherograph, Hormuth and Vetter, Heidelberg) with 0.15 M triethylamine acetate buffer (pH 4.4) for 90 min at 2000 V and 80 mA. Cyanidine does not move from the start under these conditions and UDP-glucose moves with picric acid.

Separation of glucosyl- and apiosyltransferase on hydroxylapatite

Protein from Fractions 57 to 65 from the DEAE-cellulose column (Fig. 1) was precipitated with $(NH_4)_2SO_4$. After centrifugation at 20 000 × *g* for 20 min the precipitate was dissolved in 5 ml $1 \cdot 10^{-2}$ M sodium phosphate buffer (pH 7.0) containing $2 \cdot 10^{-3}$ M dithioerythritol. This solution was chromatographed on a Sephadex G-25 column, equilibrated with the same buffer and 7.5 ml of an enzyme solution

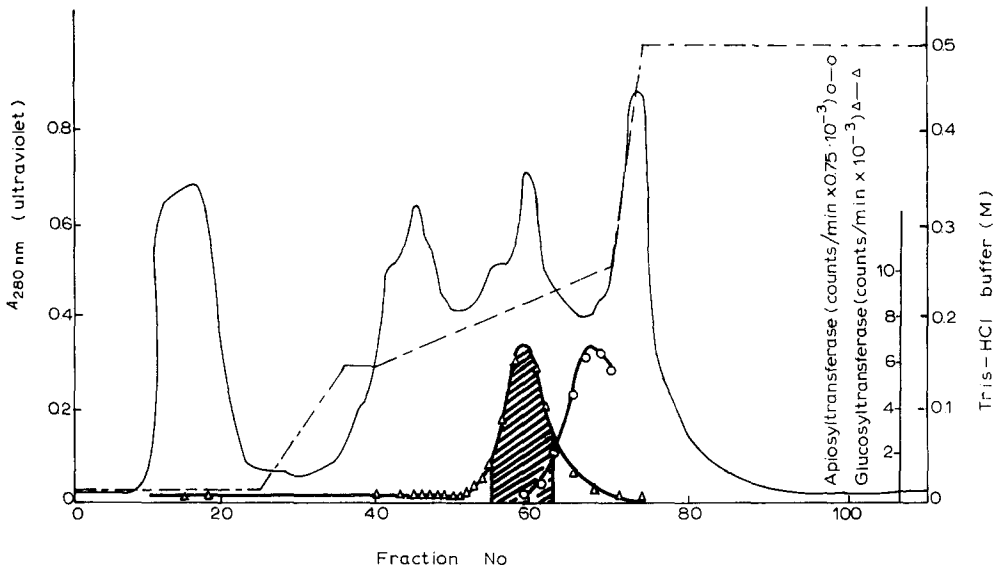


Fig. 1. Chromatography of enzyme after $(NH_4)_2SO_4$ fractionation on a DEAE-cellulose column. ———, protein; — — —, buffer gradient; △—△, glucose transferase; ○—○, apiose transferase.

containing 2 mg protein per ml was obtained. This solution was put on a column (2 cm × 1.5 cm) of hydroxylapatite (Bio Gel HTP, Bio Rad Laboratories, Richmond, Calif.), equilibrated with the above buffer. After the protein which was not adsorbed by the column had been eluted with 15 ml of 0.01 M phosphate buffer the linear buffer gradient was started. The mixing vessel contained 50 ml of 0.01 M sodium phosphate buffer (pH 7.0) with $2 \cdot 10^{-3}$ M dithioerythritol and the reservoir contained 50 ml of $15 \cdot 10^{-2}$ M sodium phosphate buffer (pH 7.0) with $2 \cdot 10^{-3}$ M dithioerythritol. The flow rate was 30 ml/h and Fractions of 3.2 ml were collected.

Incubation of enzymatically synthesized [2-^{14}C]cosmosiin with β-glucosidase

[2-^{14}C]Cosmosiin ($2 \cdot 10^4$ disint./min), 0.5 mg β-glucosidase (4.5 units/mg), and 50 μmoles of sodium acetate buffer (pH 5.5) in a total volume of 0.5 ml were incubated at 30° for 40 min. The chromatography of the reaction mixture was carried out as described in the enzyme assay with Solvent system 7.

Determination of the molecular weight on a Sephadex G-100 column[12]

A column (Pharmacia, Uppsala) with a bed volume of 2.5 cm × 84.5 cm of Sephadex G-100 (40–120 μm) was used. The gel was equilibrated[12] with 0.05 M sodium

phosphate buffer (pH 7.5) containing 0.1 M NaCl. Reference proteins were dissolved in 4.5 ml of the equilibration buffer (1 mg protein per ml). The flow rate was 14 ml/h and the eluate was collected in 3.3-ml fractions. The combined fractions of glucosyl- and apiosyltransferase from the DEAE-cellulose column (2 mg protein per ml) were put on the column. Five runs with different protein mixtures were performed. Figures in parentheses indicate the number of runs for each protein from which the average elution volume (V_e) was calculated and the respective V_e values. Cytochrome *c* (4; 313 ml), chymotrypsinogen (2; 277 ml), bovine albumin (2; 228 ml), egg albumin (3; 199 ml), aldolase (2; 168 ml), dextran blue (4; 142 ml), glucosyltransferase (1; 213 ml), and apiosyltransferase (1; 219 ml). Maximum deviation between two values was 5%.

Preparative disc electrophoresis

This was performed with an apparatus from Shandon (London). The chemicals used were from Canalco, Rockville. Separation was carried out with a stacking gel of 2 mm length and a separating gel (7% acrylamide) of 2.7 cm length. First a layer of 0.7 ml thioglycolate buffer (40 μl thioglycolic acid, 40 ml water adjusted by addition of Tris to pH 8.3) containing 50% sucrose was pipetted on the column followed by a second layer of 2.8 ml enzyme solution (enzyme from Sephadex G-200 with 0.7 mg protein per ml in $2 \cdot 10^{-2}$ M Tris–HCl buffer of pH 7.5) containing 0.5 mg myoglobin and bromophenol blue and 10% sucrose. The buffer of the electrode vessels was $2.5 \cdot 10^{-2}$ M Tris–glycine (pH 8.3) containing $5 \cdot 10^{-3}$ M mercaptoethanol. Electrophoresis was run with a constant voltage of 300 V at 40–50 mA. The protein was eluted with 0.05 M Tris–HCl buffer (pH 7.5) containing $2 \cdot 10^{-3}$ M dithioerythritol. The elution time for myoglobin was 2.25 h under these conditions.

Protein assay

Protein was determined by the biuret method[10] except after the isoelectric focusing, when the method of WARBURG AND CHRISTIAN[11] was used.

RESULTS

Purification of the UDP glucosyltransferase and separation from the UDP apiosyltransferase

We have shown earlier that the extractable activity of the transferases is drastically increased by illuminating the cells with white light and that maximum activity is reached 24 h after the onset of illumination[7]. Therefore the cell cultures were illuminated for 24 h before extraction of the enzymes.

The purification procedure for the UDP-glucose:apigenin 7-*O*-glucosyltransferase is summarized in Table II. An acetone powder of the cells was not suitable for enzyme extraction. An 89-fold purification of the enzyme could be achieved after the isoelectric focusing, based on the activity in the Dowex-treated extract. However, the yield in this last step was poor.

The chromatography of the enzyme on DEAE-cellulose and on Sephadex G-200 is shown in Figs. 1 and 2.

On a 84-cm long Sephadex G-100 column a slight separation of the glucosyltransferase from the UDP-apiose:apigenin-7-*O*-glucoside (1→2)-apiosyltransferase[8] which is also present in the cell cultures and which shows the same kinetic for light

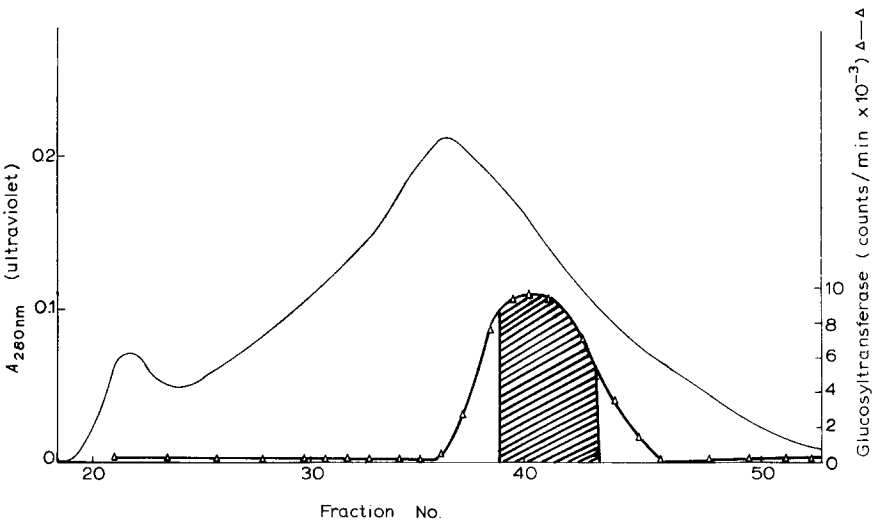


Fig. 2. Chromatography of Fractions 53–62 from DEAE-cellulose (Fig. 1) on Sephadex G-200. ———, protein; △—△, glucose transferase.

activation[7] could be observed. A good separation of the two transferases was obtained by chromatography on DEAE-cellulose with a discontinuous (Fig. 1) or linear gradient of Tris–HCl buffer at pH 7.5.

It was later discovered that a complete separation of the two enzymes is possible by chromatography on hydroxylapatite with a linear gradient of phosphate buffer (pH 7.0) from 10 to 150 mM (Fig. 3) (see MATERIALS AND METHODS).

A separation of the two transferases was also achieved by preparative disc electrophoresis. Fig. 4 shows the best result obtained by this method. However, the

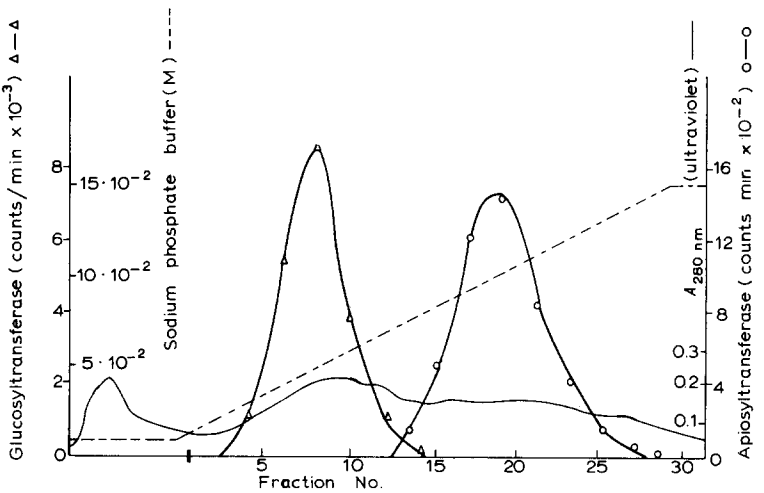


Fig. 3. Separation of glucose transferase (△—△) and apiose transferase (○—○) on hydroxylapatite with a linear gradient of sodiumphosphate buffer (pH 7.0). ———, protein; — — —, gradient.

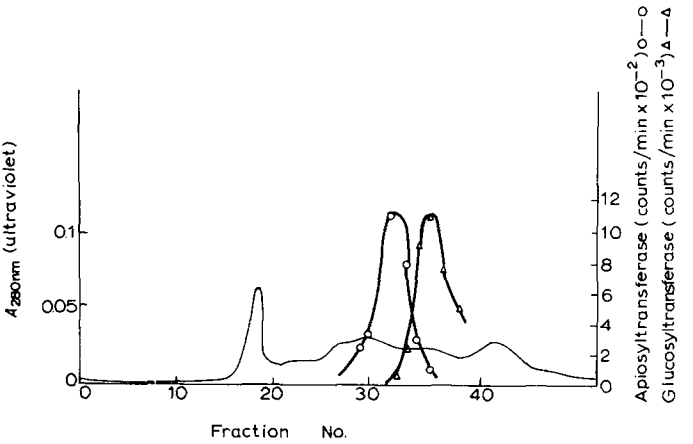


Fig. 4. Separation of glucose transferase (△—△) and apiose transferase (○—○) by preparative disc electrophoresis. ———, protein.

yield of enzyme activities was only about 20% for both transferases. Furthermore a slight separation could be observed by isoelectric focusing in the pH range 3–10 for 8 h, but the isoelectric points of both transferases differ by less than 0.4 pH unit. A typical run for the glucosyltransferase shows Fig. 5. Total enzyme activity is concentrated in a pH range of 0.2 unit around pH 5.0. The peak is symmetrical and no activity of more than 5% of this peak can be detected on the rest of the pherogram.

The 89-fold enriched apigenin 7-*O*-glucosyltransferase was free of apiosyl

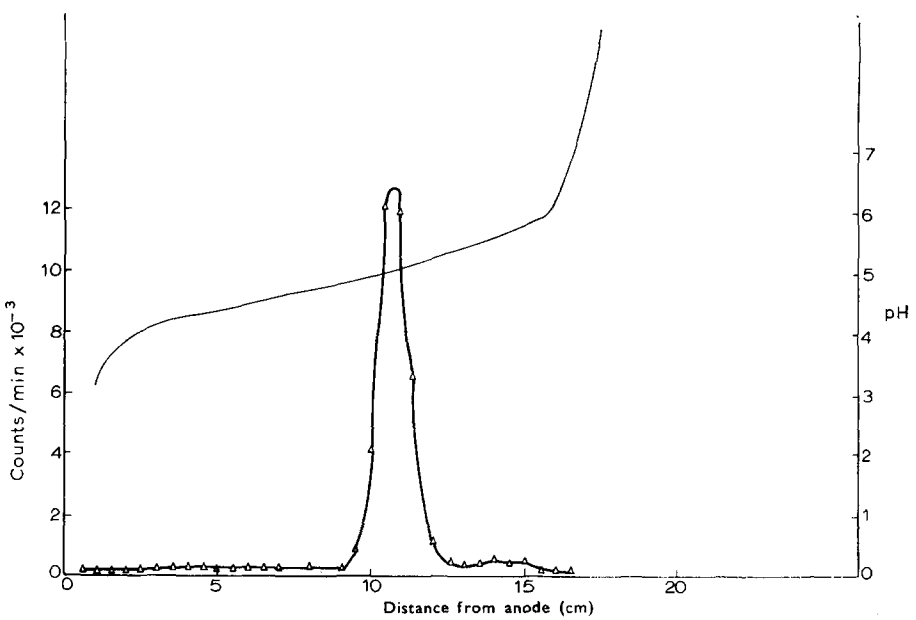
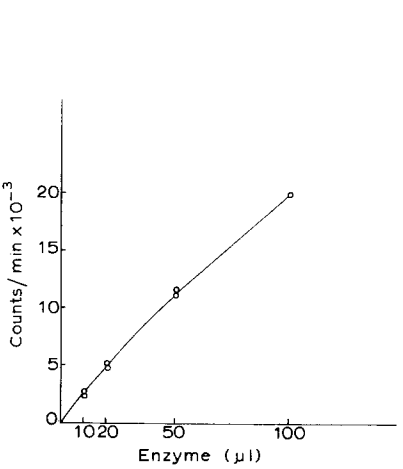


Fig. 5. Isoelectric focusing of glucose transferase from Sephadex G-200 column. ———, pH; △—△, enzyme activity.



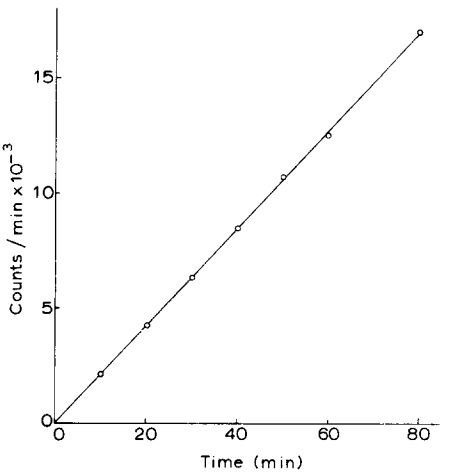
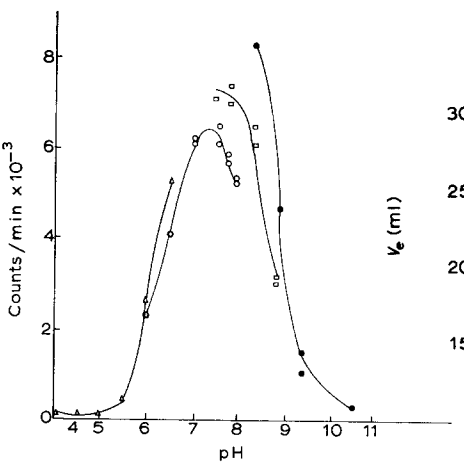


Fig. 6. Dependence of reaction rate on enzyme concentration.

Fig. 7. Dependence of reaction rate on time.

transferase. This is due partly to the separation on the DEAE-cellulose column and partly to the greater stability of the glucosyltransferase at 4°, especially under the conditions of the isoelectric focusing. Analytical electrofocusing of the glucosyltransferase showed approximately 6 protein bands in a range of 0.5 pH unit around pH 5.0 after staining with coomassie blue G-250.



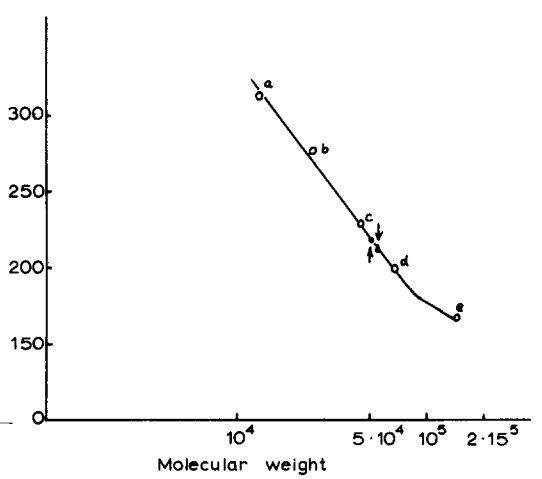


Fig. 8. Dependence of activity on pH. △—△, 0.5 M citrate buffer; ○—○, 0.5 M phosphate buffer; □—□, 0.5 M Tris–HCl buffer; ●—●, 0.5 M glycine buffer.

Fig. 9. Molecular weight determination on Sephadex G-100. a, cytochrome *c* (13 000); b, chymotrypsinogen (25 000); c, albumin (egg) (45 000); d, albumin (bovine) (67 000); e, aldolase (147 000); △, glucosyltransferase; ●, apiosyltransferase.

Properties of the UDP glucosyltransferase

If not stated otherwise the enzyme obtained from the electrofocusing (Table II) was used for the experiments.

Protein and time linearity

The glucosylation of apigenin was linear with protein concentration (Fig. 6) and time (Fig. 7). It can be seen from Fig. 7 that the product of the reaction 7-*O*-glucosylapigenin (cosmosiin) does not inhibit the reaction up to a concentration which corresponds to a saturation of the enzyme with apigenin.

Stability of the enzyme

At 3° and in the presence of $5 \cdot 10^{-2}$ M Tris–HCl buffer (pH 7.5) containing $7 \cdot 10^{-3}$ M mercaptoethanol the enzyme (0.16 mg protein per ml) from Sephadex G-200 or after electrofocusing loses about half of its activity during one month. Under the same conditions the half life of the enzymatic activity in the crude extract is 3 to 4 days. The enzyme is most stable at its pH optimum between pH 7.5 and 8.5. At a pH < 6.5 and > 9.5 the enzyme loses its activity rapidly.

pH optimum

The glucosyltransferase has a pH optimum of about pH 7.5 (Fig. 8).

Influence of SH reagents, ions, and other possible effectors

Mercaptoethanol up to a concentration of $3.5 \cdot 10^{-2}$ M has no effect on the reaction rate. In 1 M Tris–HCl buffer the reaction rate is 70% of that observed in 0.1 M buffer.

Mg^{2+}, Ca^{2+}, and EDTA have no influence on enzymatic activity in the concentration range from 10^{-4} to 10^{-2} M. In the presence of 2% $(NH_4)_2SO_4$ the activity decreases to 90% of the original activity. 10^{-3} M UTP has no influence and 10^{-3} M UDP decreases enzymatic activity by 40% (Sephadex G-200 enzyme). Up to a concentration of $5 \cdot 10^{-4}$ M none of the 7-*O*-glucosides of apigenin, chrysoeriol, and naringenin show any inhibitory effect. Addition of 20 μl ethyleneglycol monomethylether to the standard test decreases the activity by about 35%, addition of 40 μl by about 80%. The extent of the effect of addition of serum albumin (100 μg) depends on the degree of purification of the enzyme. Addition to the crude extract after Sephadex G-25 filtration increases the activity by 20% and addition to the enzyme after Sephadex G-200 chromatography results in a 100% increase in activity. Linearity of the reaction with time and protein concentration results with and without addition of serum albumin.

Localization of the enzyme

No enzyme activity could be detected in the sediment after centrifugation of the crude extract at 15 000 $\times$ *g* for 15 min. The activity in the 15 000 $\times$ *g* supernatant and in the 200 000 $\times$ *g* (2 h) supernatant was the same.

Molecular weight

An estimation of the molecular weights of both the glucosyl- and apiosyltransferase was obtained by determination of the elution volumes on a calibrated

Sephadex G-100 column. The values for both enzymes are about 50 000 to 55 000 (Fig. 9). Determination of molecular weights of globular proteins by this method should be accurate within 10% (ref. 12).

Identification of the reaction product

The identity of the reaction product as 7-*O*-β-glucosylapigenin (cosmosiin) was established by comparison with an authentic reference sample by paper chromatography with Solvent systems 1–4 and by thin-layer chromatography on silica gel (Solvent system 7) and on polyamide (Solvent systems 5 and 6). No other radioactive product was detected on the chromatograms.

Furthermore the ultraviolet spectra in the presence of sodium methylate, $AlCl_3$, $AlCl_3$–HCl, sodium acetate, and sodium acetate–boric acid[13] were identical with those of the reference material. When the labeled cosmosiin obtained from the

TABLE III

DONOR SPECIFICITY OF THE GLUCOSYLTRANSFERASE FROM CELL CULTURES OF PARSLEY
Incubations were carried out in the enzyme assay with 0.5 mmole glucosyl donor.

Substrate	v_{max} *(related to* v_{max} *with UDP-glucose = 100)*	K_m *(M)*
UDP-glucose	100	$1.2 \cdot 10^{-4}$
TDP-glucose	29	$2.6 \cdot 10^{-4}$
CDP-glucose	7	$(36 \cdot 10^{-4})$
GDP-glucose	<0.2	—
ADP-glucose	<1	—
UDP-glucuronic acid	<0.2	—
UDP-xylose	<0.2	—
α-D-Glucose 1-phosphate	<0.2	—

enzyme incubation was treated with β-glucosidase, over 99% of the radioactivity was present in the zone of apigenin after chromatography on silica gel with Solvent system 7.

Donor specificity

Besides UDP-glucose, TDP-glucose and CDP-glucose could also serve to a lesser degree as glucosyl donors, whereas GDP-glucose and ADP-glucose were ineffective. The apparent K_m values and v_{max} shown in Table III were determined in the standard incubation according to Lineweaver and Burk. The plot for UDP-glucose is shown in Fig. 10. The K_m value for CDP-glucose is very inaccurate because the counting rate was quite low. CDP-glucose might also contain a small impurity of UDP-glucose.

No transfer products were detected with α-glucose 1-phosphate, UDP-D-xylose and UDP-D-glucuronic acid as substrates (Table III). A reaction equal to 2% of the reaction in the presence of UDP-glucose could have been detected in the incubations.

Acceptor specificity

From the effect of apigenin on reaction rate the apparent K_m value for this

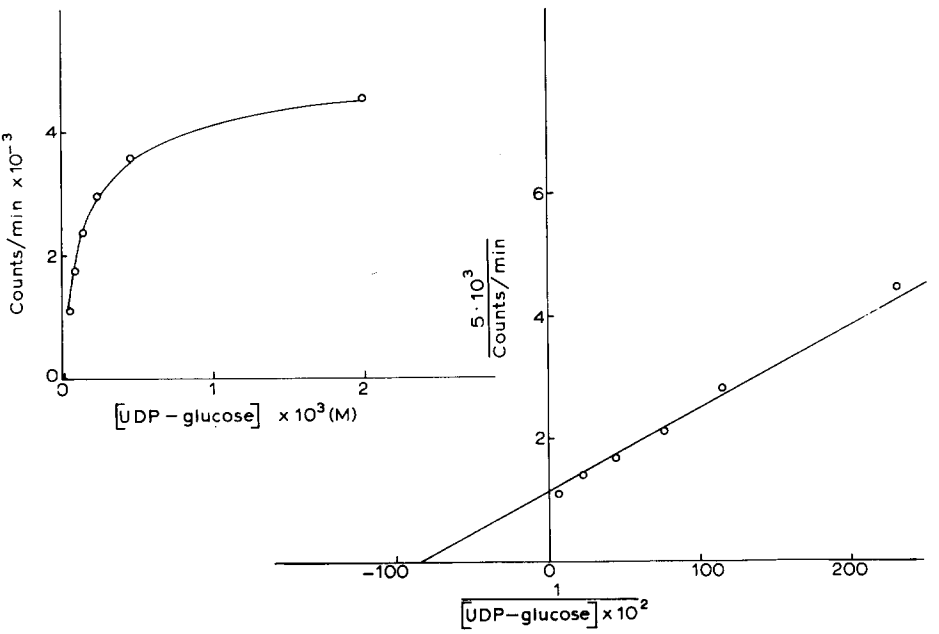


Fig. 10. Effect of UDP-D-glucose concentration on reaction rate.

substrate was determined according to Lineweaver and Burk to be $2.7 \cdot 10^{-6}$ M at pH 7.5 (Fig. 11). At substrate concentration of above $5 \cdot 10^{-5}$ M an inhibition of the reaction rate is observed. In the same manner the apparent K_m value for naringenin was determined to be $1 \cdot 10^{-5}$ M.

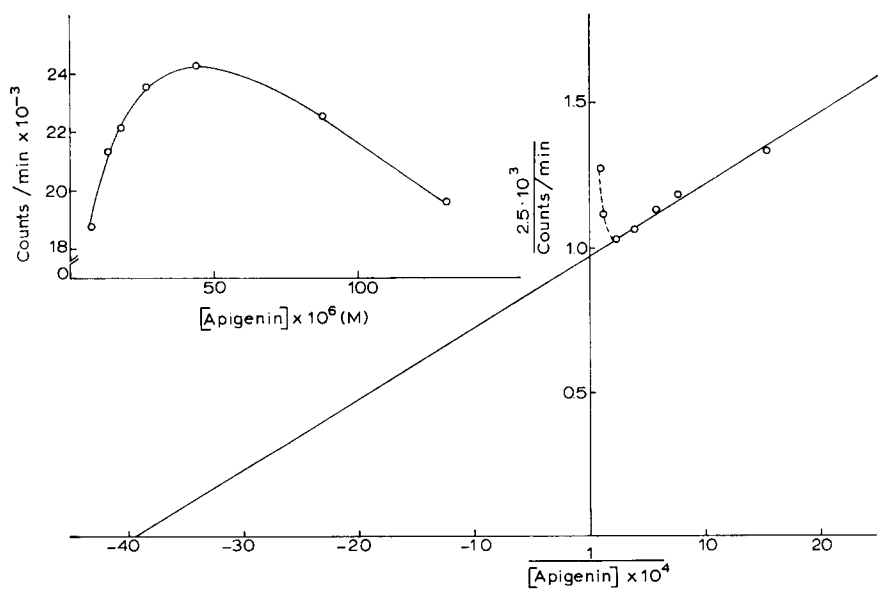


Fig. 11. Effect of apigenin concentration on reaction rate.

TABLE IV

COMPARISON OF VARIOUS FLAVONOIDS AND PHENOLS TO FUNCTION AS ACCEPTOR FOR GLUCOSE WITH THE UDP-GLUCOSE TRANSFERASE FROM CELL CULTURES OF PARSLEY

Incubations were carried out for 120 min in the enzyme assay with the Sephadex G-200 protein. The assay mixture contained 30 nmoles phenol and 25 nmoles UDP-D-[$^{14}C_6$]glucose.

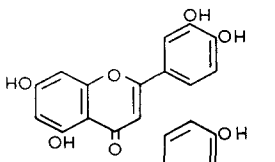
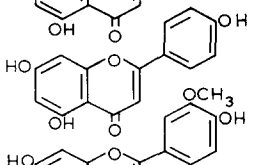
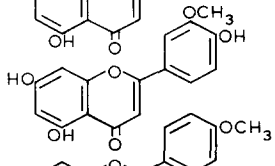
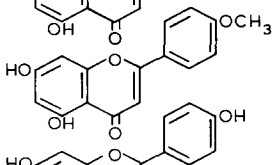
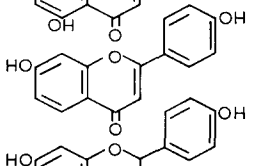
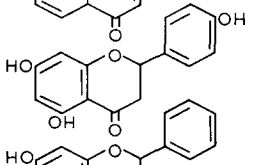
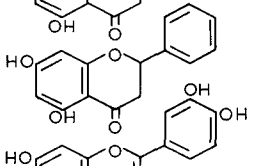
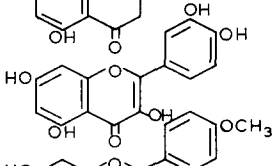
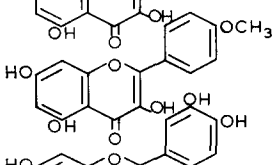
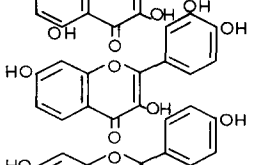
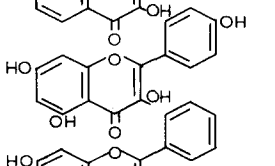
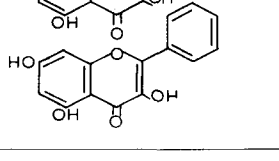
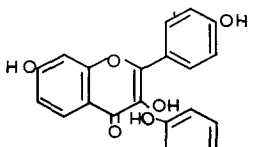
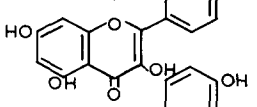
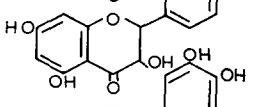
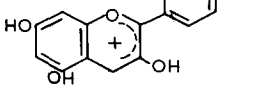
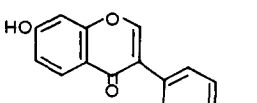
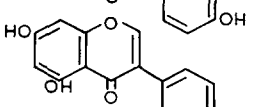
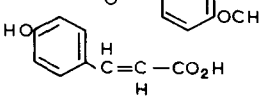
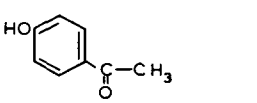
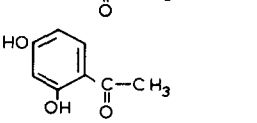
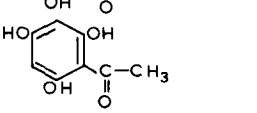
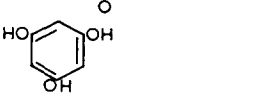
Substrate	*Formula*	v_{max} *(related to* v_{max} *apigenin = 100)*	K_m *(M)*
Luteolin		210	$1.5 \cdot 10^{-6}$*
Apigenin		100	$2.7 \cdot 10^{-6}$
Chrysoeriol		63	
Acacetin		24	
7,4′-Dihydroxyflavone		20	
Naringenin		45	$1 \cdot 10^{-5}$
5,7-Dihydroxyflavanone		15	
Quercetin		16	
Kaempferid		16	
Fisetin		14	
Kaempferol		11	
Galangin		9	

TABLE IV *(continued)*

Substrate	*Formula*	v_{max} *(related to* v_{max} *apigenin = 100)*
3,7,4′-Trihydroxyflavone		8
Datiscetin		4
Dihydrokaempferol		1
Cyanidin		<1
Daidzein		<1
Biochanin A		<1
p-Coumaric acid		<1
p-Hydroxyacetophenone		<1
Resacetophenone		<1
Phloracetophenone		<1
Phloroglucinol		<1

* This value was estimated by a competitive experiment between apigenin and luteolin.

A large number of other flavonoid compounds and phenols were compared with apigenin for their relative acceptor ability. Since only apigenin and naringenin were available in ^{14}C-labeled form, the other tests were run with UDP-D-[$^{14}C_6$]glucose. The results are summarized in Table IV. The flavones luteolin, apigenin and chryso-

eriol are the best acceptors, followed by the flavanone naringenin. 7-*O*-Glucosylluteolin, 7-*O*-glucosylchrysoeriol, and 7-*O*-glucosylnaringenin were identified by chromatographic comparison with authentic substances on polyamide thin layer plates with Solvent system 5.

DISCUSSION

Results presented in this paper prove that the UDP-glucose:apigenin 7-*O*-glucosyltransferase and the UDP-apiose:apigenin-7-*O*-glucoside (1→2)-apiosyltransferase[8] present in young parsley plants[6] and in cell cultures of this plant[7] are two different enzymes. In addition to the fact that the two transferases can be separated by chromatography on DEAE-cellulose (Fig. 1) and hydroxylapatite (Fig. 3) the two enzymes differ in their stability. The apiosyltransferase is more labile than the glucosyltransferase. The purification and properties of the apiosyltransferase will be reported in a separate paper. Although the glucosyltransferase was not purified to homogeneity it can be stated with confidence that the glucosyltransferase we have isolated is only one enzyme. In none of the various analytical separations carried out was any separation of the enzymatic activity for glucose transfer observed.

The specificity of the glucosyltransferase for the sugar donor is quite distinct (Table III). Besides UDP-glucose only TDP-glucose can function as glucose donor, though less efficiently. The slight activity found with CDP-glucose is not certain because this nucleotide might contain a small impurity of UDP-glucose.

The specificity of the transferase for the glucose acceptor is also fairly high (Table IV). Cyanidin, isoflavones, *p*-coumaric acid, and the simpler phenols tested could not function as acceptors. The best acceptors are flavones and the flavanone naringenin. The v_{max} values decrease in the order luteolin, apigenin, chrysoeriol, and naringenin and the K_m values increase in the reverse order (the K_m value for the chrysoeriol was not determined). These four flavonoids can all be postulated to be present in parsley cultures, though only apigenin and chrysoeriol have so far been isolated from these cultures[7].

Naringenin is a good biosynthetic precursor for apigenin in parsley[14], and an *S*-adenosylmethionine:luteolin (or luteolin-7-*O*-glucoside) 3′-*O*-methyltransferase has recently been detected in parsley cell cultures[7] and purified (J. EBEL, unpublished results).

It can, therefore, be assumed that luteolin or its glucoside is the biosynthetic precursor for chrysoeriol or its glucoside in parsley. It follows that the four best acceptors for the transferase are their natural substrates. In all 4 cases the 7-*O*-β-glucosides are formed and no other transfer products could be detected on the various chromatograms.

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