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Relationship of flavonoid oxidation potential and effect on rat hepatic microsomal metabolism of benzene and phenol

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Abstract: The effect of several flavonoids on the benzene hydroxylase and phenol hydroxylase activity of rat hepatic microsomes was determined. The electrochemical characteristics of the flavonoids were also determined. The effect of flavonoids on microsomal phenol hydroxylase activity was found to correlate well with the oxidation potential for flavonoid aglycones. Easily oxidized flavonoids inhibited phenol hydroxylase activity with the extent of inhibition correlated to the ease of oxidation. This inhibition exhibited dose-dependent behaviour, with concentrations below 1 μM having no effect. On the other hand, flavonoids with high oxidation potentials increased phenol hydroxylase activity in a dose-independent manner. Hydroxyl substitution at C-7 was required for inhibition of phenol hydroxylase activity independently of the oxidation potential. Glycosylation at either C-7 or C-3 was found to moderate the inhibition of phenol hydroxylase activity. A linear relation was found between the degree of inhibition and the number of sugar residues for glycosylated flavanoids. There was no correlation between electrochemical properties and effect on microsomal benzene hydroxylase activity.

Keywords: *Flavonoids; phenol hydroxylase inhibition; electrochemical characterization.*

Introduction

Flavonoids are benzo-γ-pyrone derivatives that can be divided into a variety of classes. The general structures of flavones, flavonols and flavonones are shown in Fig. 1. Flavonoids of these classes are common among the naturally occurring flavonoids. Flavonols differ from flavones only in the occurrence of a hydroxyl group at the C-3 position. Flavononones differ from flavones by the lack of the double bond between C-2 and C-3 eliminating conjugation between the A-ring and the B-ring. Flavonoids are widespread in the plant kingdom and prevalent in all photosynthesizing cells. There is high human exposure to flavonoids through dietary intake.

Flavonoids have been shown to interact with NAD(P)H diaphorase [1], aldose reductase [2], human neutrophil NADPH-oxidase [3] and numerous other oxidoreductase systems. Flavonoids are also known to inhibit both enzymatic and non-enzymatic lipid peroxidation [4]. It has been suggested that flavonoids chelate metals that normally would be free to catalyse lipid oxidation [5]. It has also been

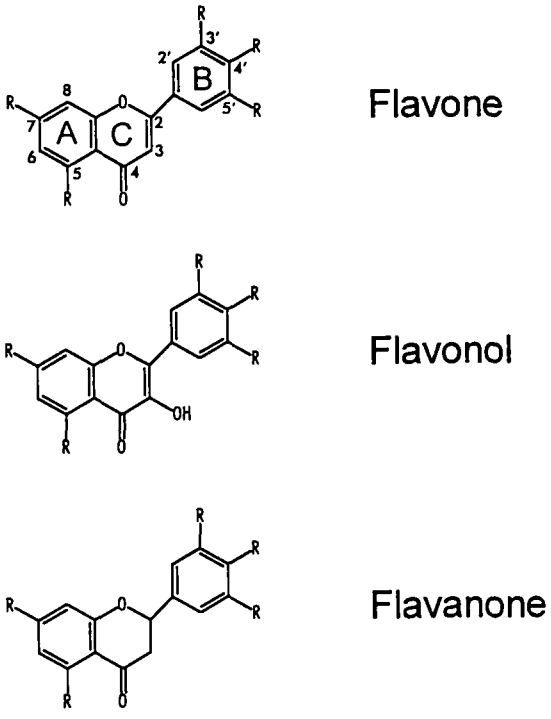

Figure 1
Representative structures of flavonoids. A, flavone; B, flavonol; C, flavanone. R=H, OCH_3, OH, OR, O-Sugar.

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proposed that flavonoids function as radical scavenging antioxidants, interrupting the oxidation of alpha-tocopherol which is an initial step in the enzymatic oxidation of lipids [6]. Beyeler *et al.* have reported that certain flavonoids also inhibit aminopyrine-*N*-demethylation by cytochrome P-450 [7]. This inhibition was shown to depend on the hydroxylation pattern and lipophilicity of the flavonoid.

Beyeler *et al.* have also noted that seemingly small changes in flavonoid structure produce large changes in their pharmacological effects [7]. There is disagreement over the structure–activity relationship governing the pharmacological activities of flavonoids although four properties appear to be significant. These properties are: lipophilicity, hydroxylation, electron transfer capabilities and metal chelation. The oxidation potentials have been determined for some relatively easily oxidized naturally occurring flavonoids [8–11]. The relationship between oxidation potential and biological activity has not been addressed. The tendency of flavonoids to undergo electron transfer may relate directly to many of their known pharmacological activities.

This report describes the electrochemical characterization of several flavonoids and the determination of their biological activity. Inhibition of benzene hydroxylase and phenol hydroxylase activity in rat hepatic microsomes were used as a marker of biological activity. The microsomal metabolites of benzene and phenol have been characterized previously [12–15]. In the liver, benzene is metabolized to phenol that is further metabolized to hydroquinone. The presence of flavonoids can significantly affect the microsomal metabolism of both benzene and phenol. A correlation between the ease of oxidation of the flavonoids and their ability to inhibit microsomal phenol hydroxylase activity is shown. Studies of this type may have utility in defining pharmacological structure–activity relationships.

Experimental

Reagents

Hesperetin, hesperidin, morin, naringenin, quercetin and rutin were purchased from Sigma (St Louis, MO, USA). Apigetrin and apiin were obtained from Carl Roth (Karlsruhe, Germany). Chrysin, 7,8-dihydroxyflavone, fisetin, flavanone, flavone, galangin, 3-hydroxyflavone, 7-hydroxyflavone and α-napthoflavone were purchased from Aldrich (Milwaukee, WI, USA). Apigenin, kaempferid, kaempferol, malvin, myricetin and naringin were obtained from Fluka AG (Ronkonkoma, NY, USA). Quercitrin was purchased from Pfaltz & Bauer (Waterbury, CT, USA) and luteolin was purchased from Plantech UK (England, UK). All flavonoids were used as received. NADPH was purchased from Sigma. All other chemicals were reagent grade.

Apparatus

Cyclic voltammetry experiments were performed with a Bioanalytical Systems (West Lafayette, IN, USA) CV-27 voltammograph. A glassy carbon working electrode, platinum auxiliary electrode and Ag/AgCl reference electrode were used. Solutions were carefully purged with argon prior to each experiment. All potentials are reported versus the Ag/AgCl reference electrode. The scan rate was 200 mV s^{-1} for these experiments.

A packed carbon bed electrolysis flow cell described previously [16] was used to determine the number of electrons transferred in the oxidation (*n*-values). A flow rate of 1.3 ml min^{-1} was used in all experiments.

Liquid chromatography was performed with a Bioanalytical Systems LC-400 liquid chromatograph. Separation of hydroquinone in the phenol incubations was achieved with a Brownlee ODS, 5 μm particle size cartridge column (10 cm × 4.6 mm i.d.) with a 1.5 cm guard column. A mobile phase of methanol–ammonium acetate (pH 4.0, 0.14 M; 30:70, v/v). Separation of phenol in the benzene incubations was achieved as described above except that the mobile phase was methanol–ammonium acetate (pH 4.0, 0.20 M; 50:50, v/v). All mobile phases were prepared from distilled, deionized water and filtered through a 0.2-μm membrane prior to use. A flow rate of 1.0 ml min^{-1} was used. Detection was carried out with an LC-4B amperometric detector (Bioanalytical Systems) using a glassy carbon working electrode operated at a potential of +700 mV for hydroquinone detection and +900 mV for phenol detection and a Ag/AgCl reference electrode. A 200-μl injection loop was used for all experiments.

Electrochemistry

The number of electrons transferred in the

oxidation (n) was calculated from the electrolysis current determined from the flow cell using the Faraday equation. The number of protons involved in the oxidations was determined from the pH dependence of the cyclic voltammetry peak potential.

Microsomal incubations

Hepatic microsomes were isolated from untreated male rats (200–400 g) as previously described [14]. Protein content was determined by the Sigma modification of the procedure of Ohnishi and Barr using micro protein determination kits (No. 690) from Sigma.

Incubations were carried out by a slight modification of the procedure previously described by Lunte and Kissinger [14]. Incubation mixtures contained 1 ml of rat hepatic microsomal protein (4 mg ml^{-1}) in 0.1 M KCl–phosphate buffer (pH 7.4), 100 μl of 140 mM NADPH in 150 mM $MgCl_2$, 100 μl of flavonoid in DMSO, and 10 μl of 200 mM benzene or phenol in acetone for a total volume of 1.21 ml. Reactions were initiated by addition of NADPH. All incubations were carried out at 37°C for 15 min while exposed to the atmosphere. Incubations were quenched by the addition of 200 μl of cold 1 M perchloric acid, centrifuged at 10,000*g* for 10 min and stored frozen (0°C) until analysed. Three types of controls were also run. In the first type of control the incubation mixture was identical to that described above except that the DMSO contained no flavonoid. The second type of control was also identical to the incubations described above except that no benzene or phenol was added. The final type of control was incubations of the flavonoids with either phenol, benzene or hydroquinone without microsomal protein being present. Metabolites were determined by direct injection of the supernatant into the chromatographic column. Results are expressed as the percentage of phenol or hydroquinone formed in the presence of flavonoid relative to its absence. The other controls confirmed the viability of the enzyme preparation and the lack of interferences from the enzyme preparation or metabolism of the flavonoids.

Results and Discussion

Electrochemistry

The electrochemical characterization of the initial oxidation step of the flavonoids is summarized in Table 1. E_p values were determined from cyclic voltammograms obtained at pH 7.0. n-Values were determined from the flow-cell electrolysis results. The number of protons in the reaction were determined from the pH dependence of the oxidation potential. Flavonoid glycones exhibited initial oxidation

Table 1
Electrochemical characteristics of several flavonoids

Flavonoid	E_p*	R-groups	n-Value†	H^+
Flavones				
7,8-Dihydroxyflavone	200r	7,8-OH	2.1 ± 0.1	2.0
Luteolin	200r	5,7,3′,4′-OH	2.0 ± 0.2	2.0
Apigenin	690	5,7,4′-OH	2.0 ± 0.2	1.8
Chrysin	750	5,7-OH	2.0 ± 0.1	2.6
7-Hydroxyflavone	900	7-OH	1.1 ± 0.1	1.0
Flavonols				
Myricetin	30r	3,5,7,3′,4′,5′-OH	2.1 ± 0.1	2.1
Quercetin	80r	3,5,7,3′,4′-OH	2.2 ± 2.0	2.0
Fisetin	110r	3,7,3′,4′-OH	2.5 ± 2.2	2.2
Morin	170	3,5,7,2′,4′-OH	1.7 ± 0.01	1.8
Kaempferol	180	3,5,7,4′-OH	1.2 ± 0.4	1.0
Galangin	370	3,5,7-OH	1.2 ± 0.5	0.8
Kaempferid	390	3,5,7-OH-4′-OCH_3	1.3 ± 0.1	1.2
3-Hydroxyflavone	440	3-OH	1.3 ± 0.5	1.2
Flavanones				
Naringenin	600	5,7,4′-OH	1.8 ± 0.1	1.7
Hesperetin	500	5,7,3′-OH-4′-OCH_3	2.1 ± 0.2	1.0
Malvin	450	7,4′-OH-3′,5′-OCH_3-3,5-glucose	1.0 ± 0.2	1.0

*Cyclic voltammetric peak potentials (mV versus Ag/AgCl) in 0.1 M sodium phosphate buffer (pH 7.0), 50% (v/v) methanol; r indicates a reversible voltammetric wave.

†N-Values were determined using a carbon packed-bed electrolysis flow cell using 0.1 M sodium phosphate buffer (pH 2.5) with 50% (v/v) methanol. Values are mean ±SD, N = 3–4.

behaviour identical to the corresponding aglycone and therefore are not listed separately in Table 1.

Figure 2 shows cyclic voltammograms typical of flavonoids. Figure 2(A) is a voltammogram of luteolin (5,7,3′,4′-OH flavone) obtained at pH 7.0. The voltammogram exhibits two oxidation waves. The first wave corresponds to the reversible oxidation of the 3′,4′-dihydroxy phenolic substituent on the B-ring. This type of electrochemical behaviour is typical of *ortho*- and *para*-substituted dihydroxyphenols (e.g. catechol and hydroquinone). The second oxidation wave corresponds to the irreversible oxidation of the 5,7-dihydroxy substituent on the A-ring. This type of electrochemical behaviour is typical of *meta*-substituted dihydroxyphenols (e.g. resorcinol) or simple phenols. Figure 2(B) is a voltammogram of 3-hydroxyflavone. This voltammogram shows the unique electrochemical behaviour of the 3-hydroxy substituent that is much easier to oxidize than a simple phenol.

The flavonoids exhibit a wide range of electrochemical behaviour. 3′,4′-OH substitution results in easily oxidized compounds that undergo two-electron, two-proton reversible oxidation. Most other substitution patterns do not have conjugated hydroxyl groups that can oxidize to a stable quinone. These flavonoids therefore behave electrochemically like phenol or resorcinol. They are harder to oxidize and undergo one-electron, one-proton irreversible oxidations. The exception is a hydroxyl substitution at C-3 which exhibits unique electrochemical behaviour. The initial oxidation of 3-hydroxyflavone occurs at a much lower potential, E_p = 440 mV, than the initial oxidation of other phenol substitutions (e.g. 7-hydroxyflavone, E_p = 900 mV). This lower oxidation potential is due to the electron donating properties of the ketone at C-4 and the oxygen at C-1 [18].

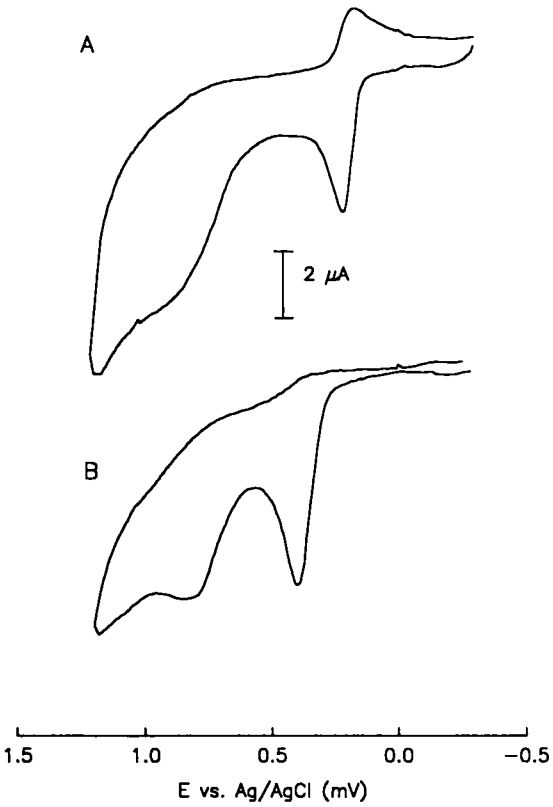

Figure 2
Cyclic voltammograms of luteolin (A) and 3-hydroxyflavone (B) in pH 7.0 buffer at a glassy carbon electrode. The scan rate was 200 mV s^{-1}.

Benzene metabolism

Table 2 lists the degree of inhibition of phenol formation from benzene relative to controls with no flavonoid added in terms of relative activity of benzene hydroxylase. The E_p potential (mV versus Ag/AgCl) for the initial oxidation process of each compound in 0.1 M sodium phosphate (pH 7.0) and 50% (v/v) methanol is also given. There is no apparent correlation between the degree of inhibition and the oxidation potential of the flavonoid for the metabolism of benzene to phenol by rat hepatic microsomes.

Phenol metabolism

Tables 3 and 4 list the relative activity of phenol hydroxylase in the presence of flavone and flavanone aglycones. The oxidation potential correlates well with the inhibition of phenol hydroxylase for flavone and flavone aglycones.

Table 2
Benzene hydroxylase activity* in the presence of flavonoids

Flavonoid	E_p†	% Relative activity
Myricetin	30r	28.0
Quercetin	80r	71.0
Morin	170	27.0
Kaempferol	180	61.0
Hesperidin	490	70.0
Apigenin	690	34.0
Naringin	680	104.0

*Benzene hydroxylase activity was measured by the amount of phenol formed relative to controls which contained no flavonoid.

†Cyclic voltammetric peak potentials (mV versus Ag/AgCl) in 0.1 M sodium phosphate buffer (pH 7.0), 50% (v/v) methanol.

Table 3
Effect of flavone and flavonol aglycones on phenol hydroxylase activity*

Flavonoid	E_p†	Flavonoid concentration			
		1000 μM	100 μM	10 μM	1 μM
Myricetin	30r	7.8 ± 1.8	21.6 ± 5.2	72.3 ± 4.3	9.7 ± 3.6
Quercetin	80r	8.3 ± 2.0	17.7 ± 4.1	70.5 ± 5.8	107.0 ± 9.9
Fisetin	110r	4.9 ± 0.9	19.4 ± 6.1	64.0 ± 18.8	85.7 ± 22.0
Morin	170	12.9 ± 3.5	50.2 ± 9.4	93.1 ± 2.8	101.8 ± 3.0
Kaempferol	180	13.9 ± 2.9	17.1 ± 3.7	52.8 ± 5.6	102.7 ± 10.0
7,8-Dihydroxyflavone	200r	10.0 ± 1.9	42.2 ± 3.0	79.4 ± 12.2	94.5 ± 8.2
Luteolin	200r	16.5 ± 3.5	23.2 ± 2.5	69.2 ± 13.1	95.9 ± 5.4
Kaempferid	390	19.0 ± 9.9	21.5 ± 0.3	29.5 ± 0.5	67.7 ± 1.2
Galangin	370	20.4 ± 8.2	32.6 ± 9.3	55.2 ± 10.2	82.4 ± 5.2
3-Hydroxyflavone	440	98.6 ± 17.8	92.8 ± 7.5	82.1 ± 10.3	83.8 ± 3.2
Chrysin	750	178.2 ± 22.7	109.0 ± 27.8	139.0 ± 7.5	114.0 ± 36.4
7-Hydroxyflavone	900	165.0 ± 22.0	115.0 ± 12.0	169.0 ± 4.2	109.6 ± 5.2
α-Napthoflavone	—‡	184.2 ± 12.2	188.6 ± 11.2	134.9 ± 10.0	99.0 ± 12.2
Flavone	—‡	140.0 ± 38.0	238.3 ± 20.5	178.5 ± 13.0	106.8 ± 9.3

* Phenol hydroxylase activity was measured by the amount of hydroquinone formed after a 15-min incubation relative to controls which contained no flavonoid. Values are means ±SD, N = 3–5.
† Cyclic voltammetric peak potentials (mV versus Ag/AgCl). The scan rate was 200 mV s^{-1}.
‡ Oxidation wave was beyond the potential window of the solvent.

Table 4
Effect of flavanone aglycones on phenol hydroxylase activity*

Flavonoid	E_p†	Flavonoid concentration			
		1000 μM	100 μM	10 μM	1 μM
Narigenin	600	12.8 ± 4.8	34.1 ± 9.5	67.9 ± 10.8	91.5 ± 8.2
Hesperetin	500	11.4 ± 3.9	37.0 ± 11.9	68.9 ± 11.9	93.9 ± 2.6
Malvin	450	24.6 ± 10.7	75.8 ± 19.1	94.2 ± 5.3	92.9 ± 14.7
Flavanone	—‡	252.6 ± 17.6	274.6 ± 33.2	188.7 ± 44.0	116.7 ± 9.5

* Phenol hydroxylase activity was measured by the amount of hydroquinone formed after a 15-min incubation relative to controls which contained no flavonoid. Values are means ±SD, N = 3–5.
† Cyclic voltammetric peak potentials (mV versus Ag/AgCl). The scan rate was 200 mV s^{-1}.
‡ Oxidation wave was beyond the potential window of the solvent.

An increase in enzyme activity is seen for flavanone, flavone, chrysin, α-napthoflavone and 7-hydroxyflavone, all of which have relatively high oxidation potentials or cannot be oxidized within the potential window of the solvent system. Flavonoids that inhibited phenol hydroxylase activity showed clear dose response behaviour whilst those that increased activity showed no dose response relationship. This suggests that inhibition and activation occur by different mechanisms.

Several authors have reported that the type of substitution at the C-3 position is important for the biological interactions of flavonoids. The uniqueness of this position is further borne out by the electrochemistry of 3-hydroxyflavonoids. The oxidation potential of 3-hydroxyflavone is much lower than that of any other mono-hydroxylated flavonoid. Inhibition of phenol hydroxylase activity by 3-hydroxyflavone would be predicted based on oxidation potential, however little effect is actually observed (Table 3). This suggests features in addition to ease of oxidation must be present in order for flavonoids to effectively inhibit phenol hydroxylase activity. Beyeler *et al.* [7] and Ibramhim *et al.* [19] reported that a hydroxyl group at C-7 was required for activation and binding to cytochrome P-450 enzymes. This requirement is confirmed by the results of these studies. The 7-glycones of hesperetin, naringenin and apigenin are much poorer inhibitors relative to the corresponding aglycones containing a free 7-OH (Table 5). However, for those flavonoids with a 7-OH group, the extent of inhibition is directly related to the oxidation potential.

The results in Table 5 show that glycosylation in general affects a flavonoid's ability to inhibit microsomal metabolism of phenol. Increasing the number of sugar units for quercetin and its two glycones, quercitrin and

Table 5
Effect of glycosylation on flavonoid inhibition of phenol hydroxylase activity*

Flavonoid	E_p†	Flavonoid concentration			
		1000 μM	100 μM	10 μM	1 μM
Quercetin	80r	8.3 ± 2.0	17.7 ± 4.1	70.5 ± 5.8	107.0 ± 9.9
Quercitrin (3-O-S‡)	60r	8.8 ± 2.6	44.0 ± 4.8	89.2 ± 5.9	97.2 ± 5.6
Rutin (3-O-S_2‡)	65r	23.0 ± 5.8	70.2 ± 0.2	93.2 ± 0.9	101.0 ± 6.0
Hesperetin	500	11.4 ± 3.9	37.0 ± 11.9	68.9 ± 11.9	93.9 ± 2.6
Hesperidin (7-O-S_2‡)	490	94.9 ± 7.7	98.9 ± 8.8	104.5 ± 12.7	111.2 ± 10.8
Naringenin	600	12.8 ± 4.8	34.1 ± 9.5	67.9 ± 10.8	91.5 ± 8.2
Naringin (7-O-S_2‡)	680	80.2 ± 6.8	104.5 ± 10.5	106.4 ± 8.8	112.3 ± 6.4
Apigenin	690	20.7 ± 3.7	29.5 ± 2.7	53.0 ± 5.4	97.3 ± 5.5
Apigetrin (7-O-S‡)	680	34.9 ± 4.7	57.6 ± 8.0	92.6 ± 4.7	98.2 ± 6.2
Apiin (7-O-S_2‡)	690	48.7 ± 7.6	85.0 ± 4.7	100.0 ± 2.4	106.6 ± 2.2

* Phenol hydroxylase activity was measured by the amount of hydroquinone formed after a 15-min incubation relative to controls which contained no flavonoid. Values are means ±SD, N = 3–5.

†Cyclic voltammetric peak potentials (mV versus Ag/AgCl) in 0.1 M sodium phosphate buffer (pH 7.0), 50% (v/v) methanol; r indicates a reversible voltammetric wave.

‡S represents a sugar residue.

rutin, results in less inhibition of hydroquinone production (Fig. 3). A linear relationship exists between the number of sugar residues and the relative activity fitting the equation $y = 26.5x + 17.6$ ($r = 1.00$). A similar relationship exists for apigenin and its two glycones, apigetrin and apiin, with a fit of $y = 27.8x + 29.6$, $r = 1.00$ (Fig. 3). The correlation of redox potential with inhibition of phenol hydroxylase and the need for a free oxygen at C-7 indicate that both flavonoid-binding and redox potential are important factors in a flavonoid's ability to affect phenol hydroxylase activity.

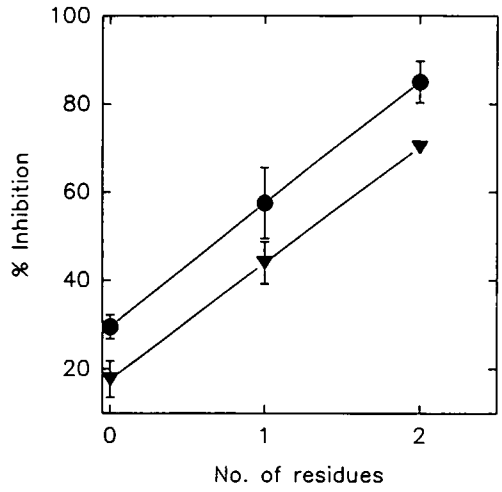

Figure 3
Effect of glycosylation on flavonoid inhibition of phenol hydroxylase activity. Flavonoid concentration was 100 μM in all cases. Symbols: ▼, quercitin; ●, apigenin.

Conclusions

All flavonoids studied with a catechol or pyrogallol structure on the A- or B-ring were strong inhibitors of phenol hydroxylase. These include myricetin, quercetin, fisetin and 7,8-dihydroxyflavone. Hesperetin is also a strong inhibitor even though the substituent at C-4′ is a methoxy group. The oxidation potential of hesperetin is sufficiently low (E_p = 450 mV) to allow inhibition of phenol hydroxylase. The corresponding glycones were far less effective inhibitors, possibly due to the steric hinderance by the sugar residue limiting access to the flavonoid to the active site of the enzyme or due to increased hydrophilicity weakening the interaction at the active site. Because of the low oxidation potential associated with a hydroxyl group at C-3, kaempferol, morin, galangin and kaempferid were all potent inhibitors of phenol hydroxylase. 3-Hydroxyflavone, on the other hand, showed no inhibition of phenol hydroxylase even though its oxidation potential is low (E_p = 440 mV). This is explained by the requirement for a free hydroxyl group at C-7 in order for enzyme–flavonoid binding to occur as reported by Beyeler *et al.* [7] and Ibrahim *et al.* [19]. This requirement is further shown by the low inhibition by 7-glycosylated flavonoids. There is no apparent relationship between the activity of the flavonoids studied here and the number of electrons transferred, protons transferred, or the chemical reversibility of the oxidation. The results presented here indicate that, provided the flavonoid can bind to the active site of the enzyme, the most potent inhibitors of

phenol hydroxylase are those with the lowest oxidation potentials.

We have shown that the oxidation potential at a glassy carbon electrode and the glycosylation of flavonoids are viable indicators of the activity of flavonoids toward the hydroxylation of phenol to hydroquinone by rat hepatic microsomes. Several investigators have suggested the importance of a C2=C3 double bond but have indicated no reason or evidence for its unique properties [20–22]. Data presented in Tables 3 and 4 indicate that the unique properties of the 3-OH position are due to its low oxidation potential. The mechanism behind the activation of phenol hydroxylase by flavonoids with high oxidation potentials is not clear. There was no correlation between flavonoid concentration and phenol hydroxylase activity in these cases.

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