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ELECTROCHEMISTRY OF FLAVONOIDS

RELATIONSHIPS BETWEEN REDOX POTENTIALS, INHIBITION OF MITOCHONDRIAL RESPIRATION, AND PRODUCTION OF OXYGEN RADICALS BY FLAVONOIDS

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Abstract—We have investigated the redox behavior of a series of structurally related flavonoids employing cyclic voltammetry under physiological conditions. The flavonoids that auto-oxidized and produced oxygen radicals had oxidation potentials (E_i) significantly lower [−30 to +60 mV vs (SCE)] than those that did not undergo auto-oxidation (+130 to +340 mV vs SCE). The range of E_i values for the auto-oxidizable flavonoids was comparable to the E_i range reported for the optimum quinone induced production of superoxide ($O_2^{\cdot-}$) in mitochondrial NADH–CoQ reductase (complex I). The most potent flavonoid inhibitors of mitochondrial succinate–CoQ reductase (complex II) possessed hydroxyl configurations capable of supporting redox reactions. For a series of 3,5,7-trihydroxyflavones that differed by b-ring hydroxylation it was found that decreasing E_i of the flavonoids was associated with decreasing I_{50} values towards succinoxidase. These findings suggest that the electrochemical properties of the flavonoids may contribute to their biological activity.

In a systematic structure–activity study of the inhibition of mitochondrial electron transport by flavonoids, we reported that four of fifteen flavonoids tested, namely, quercetagetin, myricetin, quercetin and delphinidin chloride, produce substrate-independent cyanide-insensitive respiration in isolated mitochondria and auto-oxidize in solution [1]. The auto-oxidation and mitochondrial respiratory burst result in the production of superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) [1] and hydroxyl radical [2].

Flavonoids have been reported to exhibit other diverse biological activities [3–7], including inhibition of chloroplast electron transport [6] and antioxidant [5–7], mutagenic [5, 7–10], carcinogenic [4, 5, 8], anticarcinogenic [3], cytotoxic [3–6], and antineoplastic [5, 11] activities.

Flavonoids have also been shown to inhibit a broad spectrum of enzymes including those involved in arachadonic acid metabolism (i.e. cyclooxygenase, lipoxygenase, etc.) [7], human neutrophil NADPH-oxidase [12], aldose reductase [5, 6] and several other oxido-reductases [6]. Many of these enzyme systems are redox active, and some of the biological activities may be dependent upon the redox activity of the flavonoids [1, 6, 10]. Accordingly, we have investigated the redox behavior of fifteen representative and structurally related flavonoids using cyclic voltammetry on a glassy carbon electrode in aqueous solutions approximating physiological conditions to better understand the relationship between the redox behavior and the biological activity of flavonoids.

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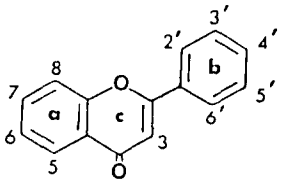
MATERIALS AND METHODS

All cyclic voltammograms were recorded at 25 ± 0.1° in 0.1 M phosphate buffer (pH 7.5) containing a final concentration of 2.5% (v/v) of dimethyl sulfoxide using an EG & G PAR (Princeton, NJ 08540) electrochemistry apparatus consisting of a model RE 0073 recorder, a model 173 potentiostat, and a model 175 universal programmer. A three electrode system with instrumental IR compensation was used throughout. The working electrode, a glassy carbon disk (BAS MF-2012, Bioanalytical System, Inc., West Lafayette, IN 47906), 3.2 mm in diameter, was polished before each experiment with a BAS PK-1 (Bioanalytical Systems, Inc.) polishing kit. The counter electrode was a platinum wire. The reference was a saturated calomel electrode (SCE), separated from the test solution by a salt bridge containing a Vycor plug and filled with the same supporting electrolyte as the test solution. To prevent possible air-oxidation of flavonoids in the physiological pH range, oxygen-free nitrogen was used to thoroughly purge and blanket the solutions being analyzed, before and during recording of the voltammograms respectively. The flavonoids were first dissolved in spectroscopic grade dimethyl sulfoxide (Burdick & Jackson Labs, Inc., Muskegon, MI 49442), 0.02 M, and then diluted with the phosphate buffer to a final concentration of 5×10^{-4} M.

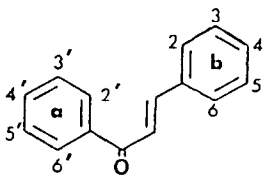
Oxygen consumption was monitored as described in Ref. 1; other experimental details are outlined in the figure legends.

The flavonoids were obtained from Roth (Ato-

Table 1. Cyclic voltammetry data of the flavonoids investigated



flavones



chalcones

Name	Class	Hydroxylation pattern	$E_{\frac{1}{2}}$† (V vs SCE)	ΔEp§ (mV, ν = 100 mV·sec^{-1})	Ip_a (μA, ν = 100 mV·sec^{-1})
Galangin	Flavonol	3,5,7	0.34[a]		4.6[b]
Kaempferol	Flavonol	3,5,7,4′	0.17	61[c]	5.9[b]
Morin	Flavonol	3,5,7,2′,4′	0.14	61[c]	14.7
Quercetin	Flavonol	3,5,7,3′,4′	0.06 −0.03[d]	34	18.6
Myricetin	Flavonol	3,5,7,3′,4′,5′	0.06[a]	55	26.5
Delphinidin Chloride	Anthocyanidin	3,5,7,3′,4′,5′	e		
Fisetin	Flavonol	3,7,3′,4′	0.14	58	18.0
Fustin	(−) Dihydroflavonol	3,7,3′,4′	0.15	49	20.4
Taxifolin	(+) Dihydroflavonol	3,5,7,3′,4′	0.15	110	18.3
Catechin	(+) Catechin	3,5,7,3′,4′	0.15	117	14.0
	(±) Catechin	3,5,7,3′,4′	0.13	66	17.0
Cyanidin Chloride	Antho-cyanidin	3,5,7,3′,4′	f		11.2
Quercetagetin	Flavonol	3,5,6,7,3′,4′	0.06[d] 0.25[a]	44	22.4
Luteolin	Flavone	5,7,3′,4′	0.18	55	16.4
Butein	Chalcone	3,4,2′,4′	0.13	28	36.0

† Midpoint potential, $(Ep_a + EP_c)/2$.
§ Peak potential separation, $EP_a - Ep_c$.
[a] Ep_a only (ν = 100 mV·sec^{-1}).
[b] The solubility is less than 5×10^{-4} M; a saturated solution was analyzed.
[c] At ν = 500 mV·sec^{-1}.
[d] The most prominent oxidation process.
[e] Not electroactive in the investigated range (see text for explanation).
[f] Ill-defined oxidations.

mergic Chemetals Corp., Plainview, NY 11803) and Pfaltz & Bauer, Inc. (Waterbury, CT 06708) and were used as received. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The results of the cyclic voltammetry experiments are summarized in Table 1. Interpretation of the experimental data is rendered somewhat difficult because of the following observations. On successive scans, the peak currents dropped significantly with each scan, distorting the positions of peak maxima, until ultimately the current stopped. This situation is indicative of the formation of an insulating film which coats the electrode [13]. The experiments can, as previously suggested,* be improved by working with a low sample concentration and/or by the addition of a nonaqueous solvent. For this reason the voltammetry in this work was performed on samples having flavonoid concentrations of 5×10^{-4} M or less in solutions containing 2.5% (v/v) dimethyl sulfoxide, and the glassy carbon electrode was polished before each experiment. The film formed on the electrode is probably polymeric in nature since it is known that oxidation of some flavonols in alkaline medium leads, via quinoid structures, to colored polymers [14]. The kinetics of film formation can influence the rate of heterogeneous electron transfer, which becomes increasingly important for the reverse reduction scan. This influences the overall shape of the cyclic voltammograms so that any conclusions on the reversibility of the mechanisms of the redox processes should be viewed with caution.

In Fig. 1 are shown the cyclic voltammograms of morin which illustrate some of the possible ambiguities. At a scan rate of 100 mV·sec^{-1} only the oxidation peak exists, whereas at a higher scan rate (500 mV·sec^{-1}) the reverse reduction peak is also

* *CV Notes*, Bioanalytical Systems Inc., P.O. Box 2206, West Lafayette, IN 74906.

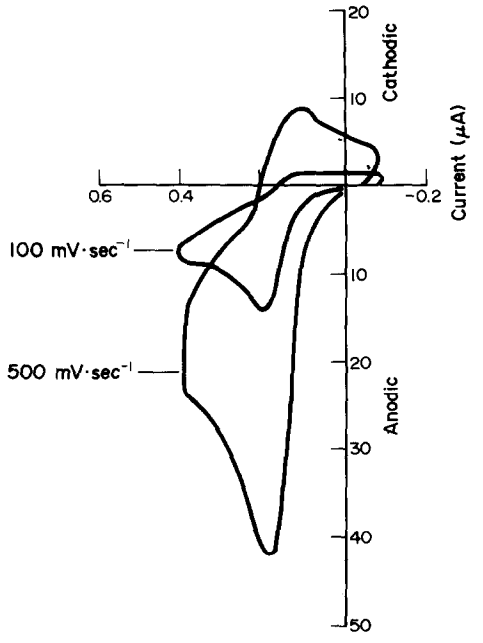


Fig. 1. Cyclic voltammograms for the oxidation of morin. A 5×10^{-4} M solution of morin was analyzed using a glassy carbon electrode in 0.1 M (pH 7.5) phosphate buffer containing 2.5% (v/v) of dimethyl sulfoxide at 25°.

prominent. This, along with the ΔE value of 61 mV, suggests one-electron reversible electrochemistry followed by a rapid chemical process. The disappearance of the reduction peak at low scan rates could be due, therefore, to the instability of the oxidized species and/or to the very rapid film formation on the electrode.

Some of the different types of oxidation processes observed for this series of flavonoids are presented in Fig. 2, where the cyclic voltammograms of luteolin and butein are shown. The oxidation of butein appears to be a $2e^-$ process; both the ΔE value of 24 mV and the high peak currents are indicative of a reversible $2e^-$ oxidation. In contrast, the halved anodic peak current and ΔE value of 55 mV suggest $1e^-$ reversible electrochemistry, i.e. the formation of semiquinone, for luteolin. Quercetin also appears to undergo a $2e^-$ oxidation. However, under identical conditions we detected a semiquinone radical by ESR [15] which suggests that this is really a $1e^-$ process or, more likely, that the 1 and $2e^-$ processes are energetically very close.

As expected, some of the compounds with a number of hydroxyl substituents, for example myricetin and quercetagetin, showed multiple overlapping oxidation processes (when this is the case the potentials given are only approximate). Consistent with this observation is that under the same conditions multiple semiquinone species have been detected by ESR [15].

The behavior of delphinidin chloride and cyanidin chloride, both of which are compounds that contain oxonium ions, is also very interesting. The distorted oxidation wave for cyanidin chloride and the absence of any oxidation, in the potential range accessible with the solvent/electrolyte/electrode system used, for delphinidin chloride are probably due to the enhanced acidities of the positively charged species when compared to the structurally similar neutral molecules. These compounds probably exist at pH 7.5 in their deprotonated forms, which is confirmed by the color change (from purple to green) occurring upon dissolution in the phosphate buffer. It is also known that similar oxonium salts, like pelargonidin for example, when deprotonated go through severe structural changes [16]. Delphinidin chloride, which contains a pyrogallol moiety, should be a stronger acid than cyanidin chloride by analogy to the relative acidities of pyrogallol and catechol [17, 18]. Thus, the former is probably completely deprotonated at pH 7.5 which renders it accessible to possible structural changes and, as a consequence, no oxidation is observed.

The data in Table 1 also show that there was an appreciable difference in the oxidation potentials of the flavonoids investigated (the range is about 340 mV), and that, as expected, extended conjugation and an increasing number of hydroxyl groups lowered the oxidation potential. It is also noteworthy to point out the difference in the oxidation potentials between the racemic mixture of catechin and one of its enantiomers. Also, comparing, for example, quercetin, taxifolin, and luteolin, one can see the relative effects of the extended conjugation and/or the 3-hydroxy group on the oxidation potentials of the respective flavonoids. Other workers have studied the electrochemical behavior of flavonoids [19–21]. However, in these cases the redox potentials were determined in buffers which contained at least 50% (v/v) isopropyl or ethyl alcohol and at various pH values. Since pH and solvent can influence the potentials, they cannot be compared with the potentials reported herein. More importantly, the potentials reported here were determined under conditions (i.e. buffer and pH) that

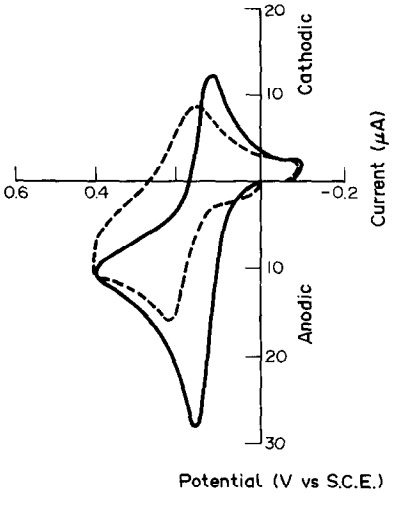


Fig. 2. Cyclic voltammograms for the oxidation of luteolin and butein. Solutions of luteolin (---) and butein (—) at a concentration of 5×10^{-4} M were analyzed using a glassy carbon electrode in 0.1 M (pH 7.5) phosphate buffer containing 2.5% (v/v) of dimethyl sulfoxide at 25°, $\nu = 100$ mV·sec^{-1}.

more closely approximate those encountered in biological systems and the amount of non-aqueous solvent (DMSO) was maintained at a low concentration analogous to that found in many bio-assays.

Four of the fifteen flavonoids tested have been shown to auto-oxidize and produce $O_2^{\cdot-}$ and H_2O_2 [1]. Based on the structural resemblence of quercetagetin, myricetin and delphinidin to pyrogallol, which is known to auto-oxidize with $O_2^{\cdot-}$ being the chain propagating species [22], it was not surprising that these compounds behave in a similar manner [1]. However, quercetin which possesses a catechol moiety also underwent auto-oxidation but was found to have an $E_{\frac{1}{2}}$ identical to that of quercetagetin (0.06 V). Quercetagetin, myricetin and quercetin had the lowest oxidation potentials of the fifteen flavonoids tested. Delphinidin was not electroactive under the experimental conditions, but probably rearranges to quinoid species [16] analogous to pelargonidin, which could be expected to redox cycle with oxygen.

These same four compounds also produced substrate-independent, cyanide-insensitive respiratory bursts in isolated mitochondria [1] which may be due to auto-oxidation and not to any interaction with the mitochondria. However, myricetin is a potent inhibitor of succinoxidase [1] and an auto-oxidation product can draw off reducing equivalents from the respiratory chain (Fig. 3). The observed decreased rate of myricetin-induced oxygen consumption upon addition of superoxide dismutase (SOD) and catalase demonstrates that $O_2^{\cdot-}$ and H_2O_2 were present in the reaction vessel, since both SOD and catalase catalytically generate oxygen. The presence of $O_2^{\cdot-}$ and H_2O_2 is consistent with the production of a free radical intermediate of myricetin formed from auto-oxidation and/or reaction with the respiratory chain. Myricetin can then reduce oxygen non-enzymatically to $O_2^{\cdot-}$ with the concomitant formation of oxidized myricetin. The $O_2^{\cdot-}$ then dismutes to H_2O_2 and O_2. The oxidized myricetin is then able to accept electrons from succinate through the respiratory chain. This is consistent with a redox cycling mechanism involving quinoid and oxygen species.

The auto-oxidation of flavonoids has been reported previously and, in general, has been shown to require the presence of several hydroxyl groups and to proceed only in alkaline solution wherein both a quinoid species and trace amounts of H_2O_2 are produced [14]. An exception is that reported by Hathway and Seakins [23] of the auto-oxidation of neutral aqueous solutions (pH 8) of flavans related to catechin. The four compounds discussed herein auto-oxidized and interacted with isolated mitochondria to produce oxygen radicals at a biological pH which indicates the potential to produce oxidative stress in biological systems.

It is also interesting to note that the range of $E_{\frac{1}{2}}$ values for the auto-oxidizable flavonoids (−30 to +60 mV vs SCE) is comparable to the one-electron reduction potential range for the optimum production of $O_2^{\cdot-}$ in mitochondrial NADH–CoQ reductase (complex I) by quinones (−70 to +30 mV vs the hydrogen electrode at pH 7) [24, 25]. Consistent with this observation is the fact that the NADH–CoQ reductase portion of the electron transport chain redox centers possesses $E_{\frac{1}{2}}$ values from −340 to +100 mV. The succinate CoQ reductase system contains redox centers possessing $E_{\frac{1}{2}}$ of −260 to +120 mV [26]. The oxidation potentials of the flavonoids fall well within this range. Therefore, on electrochemical grounds, they all have the potential to interact with mitochondrial redox centers.

Flavonoids have also been shown to inhibit mitochondrial electron transport [1, 27–30]. We previously reported that for a series of model phenolic compounds [31, 32] and flavonoids [1] the most potent inhibitors of succinoxidase possessed hydroxyl configurations capable of supporting redox reactions (i.e. hydroquinone and catechol). For a series of 3,5,7-trihydroxyflavones which differed in the number and configuration of b-ring hydroxyl groups, it was found that myricetin with a pyrogallol configuration was the most potent inhibitor of succinoxidase followed in decreasing order by quercetin (catechol), morin (resorcinol) and kaempferol (monohydroxy), whereas galangin (unhydroxylated) was inactive. This supports the conclusion that succinoxidase inhibition appears to be linked to redox activity. The most potent succinoxidase inhibitor myricetin had the lowest $E_{\frac{1}{2}}$ (−30 mV) followed by quercetin (+60 mV), morin (+ 140 mV), kaempferol (+ 170 mV) and galangin (+ 340 mV), and their I_{50} values are 45, 715, 730, > 1800 and >

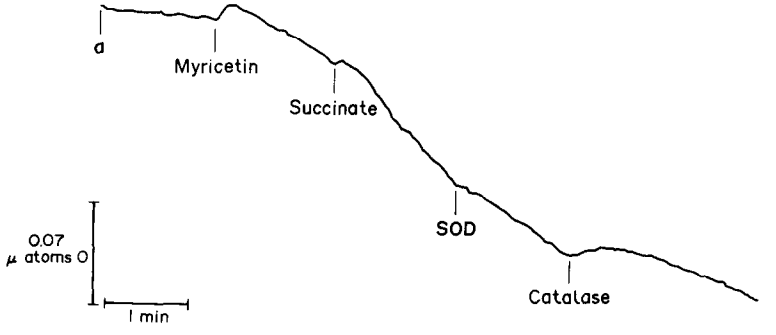


Fig. 3. Redox cycling of myricetin with mitochondrial succinoxidase enzyme system (complex II). (a) 3 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.5, beef heart mitochondria (0.2 mg mitochondrial protein/ml) and antimycin A (1.7 μM final concentration). Myricetin, succinate, SOD and catalase were added to final concentrations of 0.2 mM, 45 mM, 23 μg/ml and 0.4 μg/ml respectively.

1800, nmol/mg mitochondrial protein respectively [1]. Quercetagetin which has the same low $E_{\frac{1}{2}}$ (60 mV) as quercetin is a better inhibitor of succinoxidase, indicating that other parameters in addition to redox potential are important for the inhibitory activity. A similar order of potency for the same series of 3,5,7-trihydroxyflavonoids was observed for inhibition of the NADH oxidase enzyme system with the $E_{\frac{1}{2}}$ values increasing sequentially as hydroxylation of the b ring was diminished which compares with their I_{50} values for NADH oxidase of 35, 145, 430 > 1800 and > 1800 nmol/mg protein respectively [30].

It has been reported that, for a series of naphthoquinones, their $E_{\frac{1}{2}}$ values correlated with their abilities to inhibit succinoxidase, their antineoplastic activities towards Sarcoma 180 in mice [33, 34] and possibly with their abilities to undergo redox cycling and generation of oxygen radicals in isolated mitochondria. By analogy, inhibition of energy-related processes by flavonoids is probably involved in the mechanisms of their cytotoxicity and antineoplastic activity [1, 5]. Also, the ability of some flavonoids to redox cycle and produce toxic oxygen radicals may also contribute to their cytotoxic activities, since it has been shown that the cytotoxic effects of quinones and antineoplastic agents of clinical and research interest that contain a quinone nucleus are probably mediated, at least in part, through the formation of the semiquinone and possibly the subsequent formation of toxic oxygen species. In addition, it has been shown that tumor cells, in general, are deficient in the mechanisms that detoxify oxygen radicals and hence may be more susceptible to pro-oxidant antitumor agents [24].

It has been speculated that other activities of the flavonoids may be dependent on their redox activity [1, 6, 10]. The structural requirements for mutagenicity [10], antioxidant activity [35, 36], the inhibition of lens aldose reductase [37] and neutrophil NADPH-oxidase [12], among several, are similar to the structural requisites we find for the inhibition of mitochondrial respiration and the production of oxygen radicals by flavonoids [1]. This suggests that the electrochemical properties of the flavonoids may contribute to their biological activities.

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