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INHIBITION OF MITOCHONDRIAL RESPIRATION AND PRODUCTION OF TOXIC OXYGEN RADICALS BY FLAVONOIDS

A STRUCTURE–ACTIVITY STUDY

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Abstract—A series of fourteen flavonoids were employed in a systematic structure–activity study to assess their abilities to inhibit succinoxidase and generate toxic oxygen species in beef heart mitochondria. By comparing I_{50} values toward succinoxidase activity, flavonoids with a catechol moiety on the b ring exhibited the following general order of potency: chalcone > flavone > flavonol > dihydroflavonol > anthocyanidin. Catechins were inactive. In a series of 3,5,7-trihydroxyflavones containing various configurations of the b ring hydroxyl groups, it was found that the flavonoids possessing adjacent trihydroxyl (pyrogallol) and b ring ortho-hydroxy(catechol) configurations were the most potent inhibitors of succinoxidase, followed by those with meta-hydroxyl, monohydroxyl and unhydroxylated configurations. Four of the fifteen flavonoids tested exhibited substrate-independent, KCN-insensitive respiration. Two flavonols with a pyrogallol configuration, myricetin and quercetagetin, produced the largest respiratory bursts and were found to auto-oxidize. Evidence is presented that the mitochondrial respiratory bursts induced by both flavonols and their auto-oxidation resulted in the generation of O_2^- and H_2O_2.

Flavonoids have been extensively studied previously and reported to possess widespread biological activities [1–5], including antimicrobial [1–8], antihelminthic [2], mutagenic [3, 5, 9], carcinogenic [2, 3, 9], anticarcinogenic [1, 10–12], antioxidant [4, 5], electron transport [4], antineoplastic [13, 14] and cytotoxic [1–4, 13, 15, 16] properties. Flavonoids have also been shown to inhibit a wide range of enzymes, including phosphodiesterases [3, 4], aldose reductase [3, 4, 17] and ATPases [3–5, 18], and to inhibit tumor cell glycolysis [5, 19], human neutrophil NADPH-oxidase [20] and mitochondrial succinoxidase and NADH oxidase activities [21], in addition to several others. Inhibition of mitochondrial enzymes by flavonoid constituents may contribute to their cytotoxic and antineoplastic activities. Accordingly, in this study we evaluated the ability of a wide range of flavonoids (Fig. 1) to inhibit succinoxidase activity in isolated mitochondria.

Inhibition of succinoxidase by certain compounds appears to be linked to their abilities to participate in oxidation–reduction reactions. Accordingly, a structure–activity study employing a series of model phenolic compounds [22, 23] demonstrated that the most potent succinoxidase inhibitors possessed hydroxyl configurations capable of supporting oxidation–reduction reactions (i.e. hydroquinone and catechol). We therefore extended the range of flavonoids tested in this study to include compounds with hydroxyl configurations analogous to those in the series of model compounds studied previously. In addition, myricetin has been shown previously to cause a substrate-independent, CN-insensitive respiratory burst in isolated mitochondria [21]. Xenobiotic-induced CN-insensitive mitochondrial respiration is often associated with the production of superoxide, H_2O_2 and hydroxyl radical, which may contribute to the overall cytotoxicity. We therefore screened various flavonoids for their abilities to generate a respiratory burst and cytotoxic oxygen radicals, in addition to their abilities to inhibit succinoxidase activity.

METHODS

Heavy beef heart mitochondria (HBHM) were isolated by differential centrifugation and stored as described previously [24]. Ageing of the mitochondria was achieved by repetitious freeze/thaw cycles to uncouple respiration from phosphorylation. Mitochondrial protein was assayed by the method of Lowry *et al.* [25]. The activity of HBHM succinoxidase was determined manometrically in the absence and presence of the various text compounds [23, 26]. The I_{50} (dose required to inhibit activity by 50%) was determined by extrapolation from a titration curve. The number of replicates for each concentration of flavonoid was from 6 to 24, and the specific activity of the succinoxidase controls ranged between 0.3 and 0.9 natoms of oxygen consumed per min per mg protein. The test compounds were added in dimethyl sulfoxide (DMSO), which was main-

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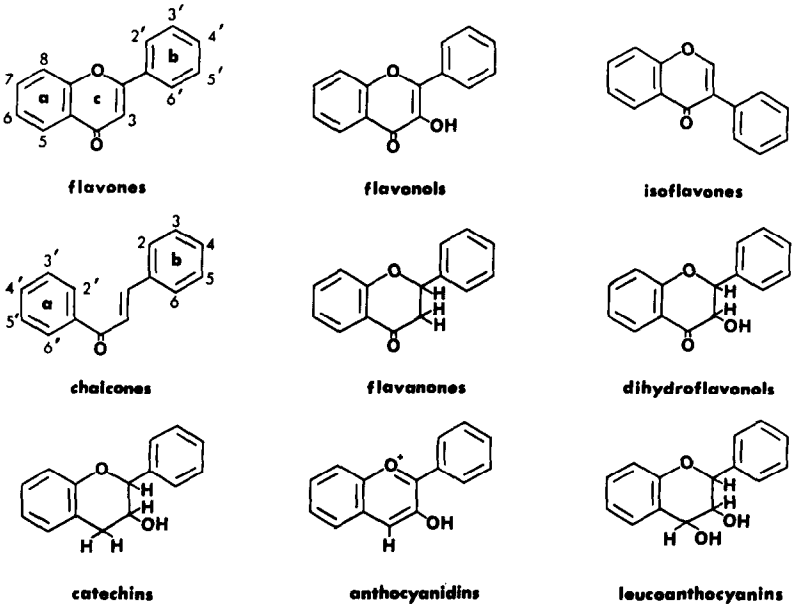


Fig. 1. Classification and position numbering systems of flavonoids.

tained at a constant concentration in all assays (0.1 ml DMSO/3 ml of reaction mixture), a dose that did not affect control rates of respiration. The protein concentration varied between 0.9 and 1.4 mg/flask. In each experiment a 5-min preincubation was employed to ensure thermal equilibrium and depletion of endogenous substrates.

Oxygen consumption was measured polarographically with a YSI model 53 oxygen monitor equipped with a Clark electrode (Yellow Springs Instrument Co., Yellow Springs, OH) in a water-jacketed chamber (Gilson Medical Electronics, Inc., Middleton, WI), magnetic flea stirbar, and a strip chart recorder (Linear 1800, Linear Instruments Corp., Reno, NV) which was calibrated over a 0–100% saturation scale with a 3 mM 3-[*N*-morpholino]propane sulfonic acid (MOPS) buffer, pH 7.5, which was previously air saturated at the experimental temperature of 37°. To the buffer, HBHM (1.9 to 4.1 mg mitochondrial protein/ml) was added, and the chamber was sealed with a glass stopper which had a capillary port for additions. KCN (5 mM) was added subsequently. The flavonoids were then added (0.1 mM) in the absence of substrate. The test compounds were added in DMSO, which was maintained at a constant concentration (0.02 ml DMSO/2 ml of reaction mixture). Controls were conducted in the absence of flavonoids and in the presence of substrate, sodium succinate (50 mM), to provide an inhibited control and to obtain a baseline or inhibited rate of oxygen consumption.

Spectrophotometric assays were conducted on a Hitachi model 100–80 (Hitachi Scientific Instruments, Mountain View, CA) spectrophotometer.

Flavonoids were purchased from Roth (Atomergic Chemical Corp., Plainview, NY) and Pfaltz & Bauer, Inc. (Waterbury, CT). Antimycin A and quercetin were obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade. Antimycin A was dissolved in EtOH/H_2O (1/1, v/v).

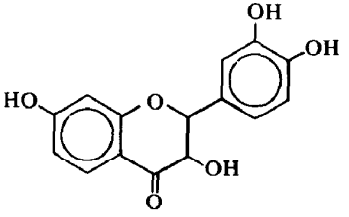
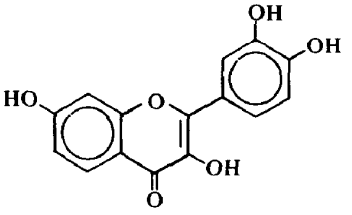
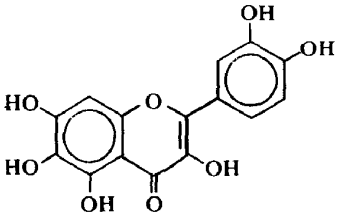
RESULTS AND DISCUSSION

A systematic inhibition study of mitochondrial succinoxidase activity by fourteen structurally related flavonoids was conducted, and the I_{50} values are shown in Table 1. In general, among the limited number of flavonoids tested having a catechol moiety on the b ring, the following order of potency was observed: chalcone > flavone > flavonol > dihydroflavonol > anthocyanidin. Catechins were inactive. Any compound not inhibiting succinoxidase activity by 50% at the initial test dose of 1800 nmoles/mg protein was arbitrarily considered not active and was not tested further. This dose was chosen because of solubility limitations of the test flavonoids in DMSO. The flavonol quercetin appears to be an exception to this order of inhibition in that it was less active than the corresponding dihydroflavonol, taxifolin. This discrepancy is at the present time unexplained and suggests that the number and location of the hydroxyl groups may be more important for succinoxidase inhibition than certain class distinctions among the flavonoids.

The low activity of the anthocyanidin, delphinidin chloride, and the absence of activity for the two catechin stereoisomers suggest that the c ring keto group is essential for activity. The exception is cyanidin chloride, which is the corresponding anthocyanidin to quercetin. Thus, in the case of catechins and anthocyanidin, class distinction between flavonoids may be important.

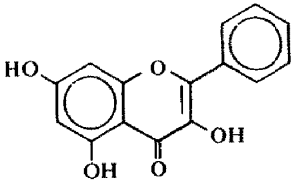
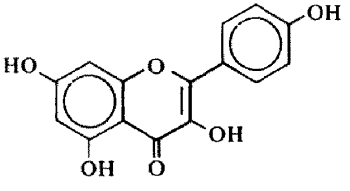
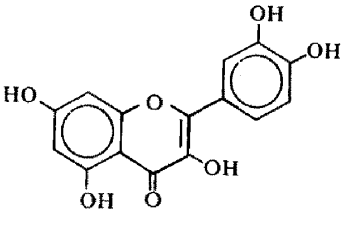
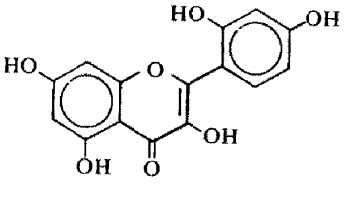
Butein, the chalcone that possesses the same hydroxyl configuration as the flavone luteolin, was the most potent inhibitor of succinoxidase. This finding is not surprising in view of the structural similarity of butein to nor-dihydroguaiaretic acid

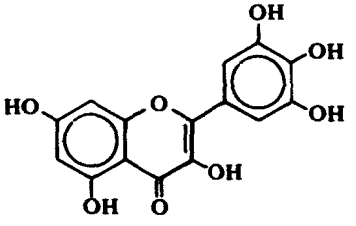
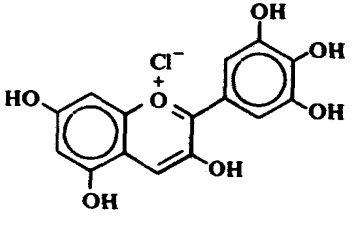
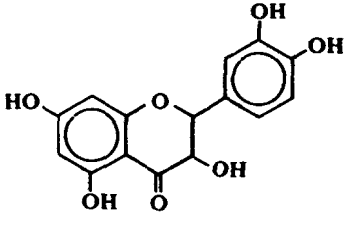
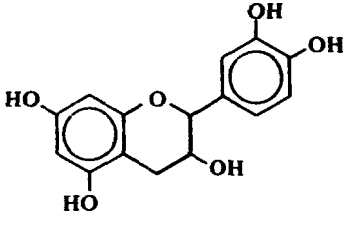
Table 1. Inhibition of mitochondrial succinoxidase by flavonoids

Compounds added	Structures	Class	I_{50} (nmoles/mg protein)	Number of hydroxyl groups
a Ring substitution				
Fustin		Dihydroflavonol	148	4
Fisetin		Flavonol	45	4
Quercetagetin		Flavonol	104	6

(*continued*)

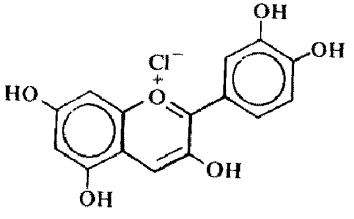
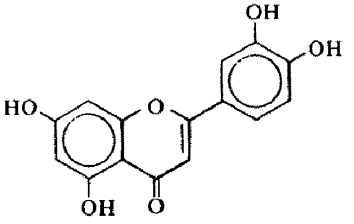
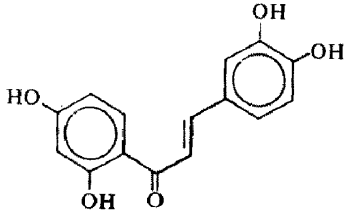
Table 1. (*continued*)

Compounds added	Structures	Class	I_{50} (nmoles/mg protein)	Number of hydroxyl groups
b Ring substitution				
Galangin		Flavonol	N/A	3
Kaempferol		Flavonol	N/A	4
Quercetin		Flavonol	715	5
Morin		Flavonol	730	5

Myricetin		Flavonol	45	6
c Ring substitution				
Delphinidin chloride		Anthocyanidin	740	6
Taxifolin		Dihydroflavonol	220	5
D,L-Catechin		Catechin	N/A	5

(*continued*)

Table 1. (*continued*)

Compounds added	Structures	Class	I_{50} (nmoles/mg protein)	Number of hydroxyl groups
Cyanidin chloride		Anthocyanidin	290	5
Luteolin		Flavone	32	4
Butein		Chalcone	21	4

(NDGA), which has been shown to be a potent inhibitor of mitochondrial electron transport [27].

A similar comparison was conducted on a series of 3,5,7-trihydroxyflavones which differed in the number and configuration of b ring hydroxyl groups. It was found that, in this series, myricetin (pyrogallol), was the most potent inhibitor of succinoxidase followed by catechol (quercetin) and then flavonols with meta-hydroxyl (morin), monohydroxyl (kaempferol) and the unhydroxylated (galangin) configurations respectively. These results are similar to the hydroxyl configurations found inhibitory towards succinoxidase by a series of model phenolic compounds [22, 23]. In addition, the general trend for the order of potency for the total number of flavonoid hydroxyl groups was $4 > 6 > 5 > 3 > 2 > 1 = 0$. The structure–activity relationships herein found for succinoxidase are analogous to those reported for the effect of flavonoids on antigen-induced histamine release from human basophils [28].

It has been reported that a series of naphthoquinones that inhibited succinoxidase activity also possessed antineoplastic activity towards intraper-

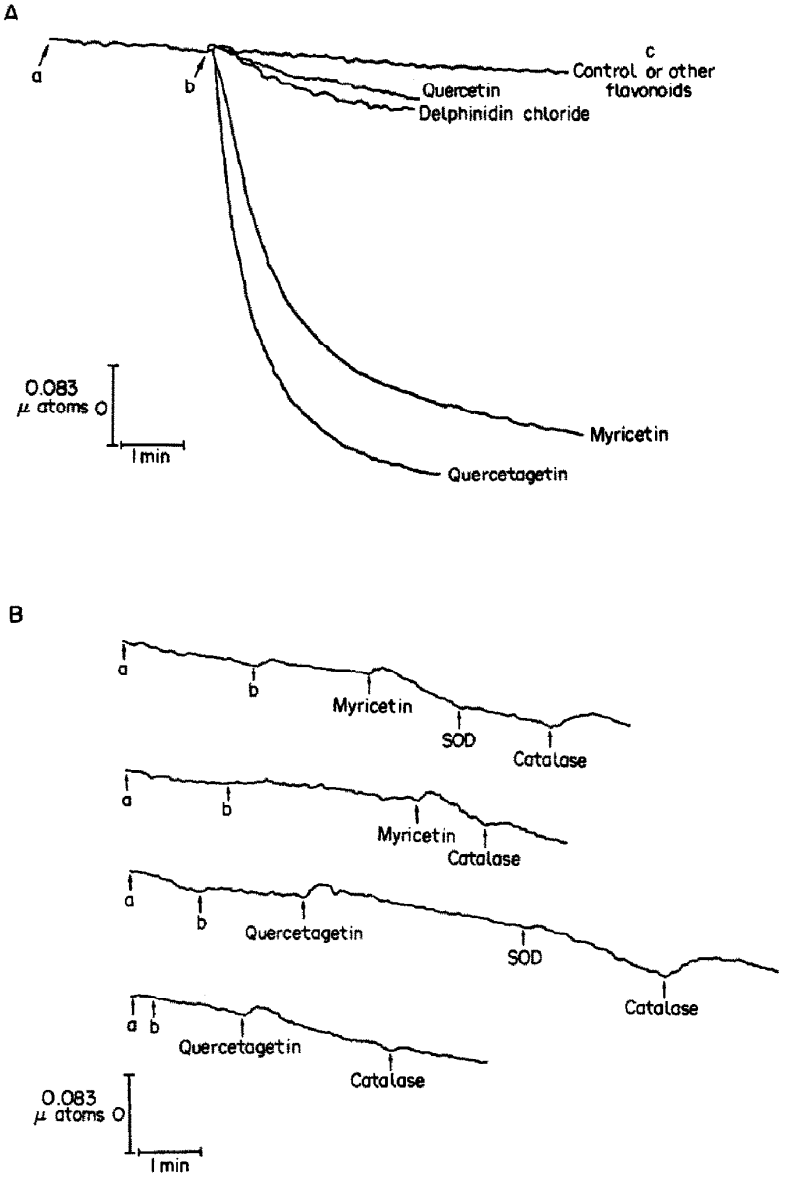


Fig. 2. Flavonoid-induced respiratory bursts in mitochondria. (A) The reaction conditions were as outlined in Methods: (a) MOPS, beef heart mitochondria (BHM) and KCN; (b) flavonoid or (c) substrate. (B) The polarography was done as described in Methods. The respiration medium consisted of: (a) 3 mM MOPS, pH 7.5, BHM (0.2 mg mitochondrial protein/ml; this protein concentration was employed to optimize the slope of the polarographic tracing), and (b) antimycin A (1.8 μM). The final concentration of the flavonoid (myricetin or quercetagetin) was 0.2 mM. SOD and catalase (Sigma Chemical Co.) were added to final concentrations of 25 μg/ml and 0.5 μg/ml respectively.

itoneally grown Sarcoma 180 in mice [29, 30], whereas the non-inhibitors did not possess antineoplastic activity. These findings by analogy suggest that the succinoxidase inhibitory flavonoids may possess antineoplastic activity. Certain flavones have been reported to possess cytotoxicity towards KB cells in culture [13, 15] and antineoplastic activity [13, 14]. However, Edwards *et al.* [13] reviewed the available literature on flavonoids and concluded that flavonoids as a class do not warrant further pursuit as anticancer agents because of the lack of a clear structure–activity relationship. Despite this conclusion, he reported that the two catechol-containing flavonoids tested, 3,5,7,3′,4′-pentahydroxyflavone and 5,7,3′,4′-tetrahydroxy-3-glycosyloxyflavone, possess antineoplastic activity towards Walker carcinoma 256. These findings are consistent with the structural requirements reported herein for succinoxidase inhibition and demonstrate the need to conduct a systematic structure/activity investigation on the antineoplastic activity of the flavonoids. The cytotoxic and antineoplastic properties of this series of flavonoids are currently under investigation as are their effects on mitochondrial NADH-oxidase activity.

Four of the fourteen flavonoids tested produced a substrate-independent increase in oxygen consumption (respiratory burst) which was insensitive to KCN (Fig. 2A). The role of toxic oxygen radicals in the flavonoid-induced respiratory burst in mitochondrial suspensions was then examined. Since quercetagetin and myricetin produced a significantly larger respiratory burst than delphinidin chloride and quercetin at the same concentration (Fig. 2A), they were further investigated for production of oxygen radicals. In the absence of CN^-, the respiratory bursts of both compounds were inhibited by catalase, whereas SOD inhibited the respiratory burst produced by myricetin and exacerbated that of quercetagetin (Fig. 2B). These data demonstrate that both O_2^- and H_2O_2 were produced during the flavonoid-induced respiratory bursts in mitochondria. The production or regeneration of oxygen by catalase was greater when SOD was present, suggesting that H_2O_2 was a secondary product of the flavonoid-induced respiratory burst, being produced during the dismutation of O_2^-.

It is interesting to note that the respiratory bursts were extremely diminished in the absence of CN^- (Fig. 2B); thus, the possibility of a non-enzymatic interaction between flavonoid and CN^- was assessed. Figure 3 demonstrates that (1) the flavonoids autooxidize and (2) the flavonoids myricetin and quercetagetin react non-enzymatically with CN^- to produce a respiratory burst by directly facilitating the auto-oxidation of myricetin and quercetagetin. The auto-oxidation of the flavonoids occurred in various aqueous buffers but not in organic solvents (DMSO and ethanol) (data not shown). Sodium azide supported a markedly reduced auto-oxidation of the flavonoid and thus was employed in place of CN^- to inhibit cytochrome oxidase. Azide-inhibited mitochondria gave a myricetin-induced respiratory burst (data not shown) similar to that observed in the absence of a cytochrome oxidase inhibitor (Fig. 2B). These results confirm that CN^- reacts nonenzymatically with the flavonoids and show that care should be exercised when CN^- is employed for studies with redox-active flavonoids.

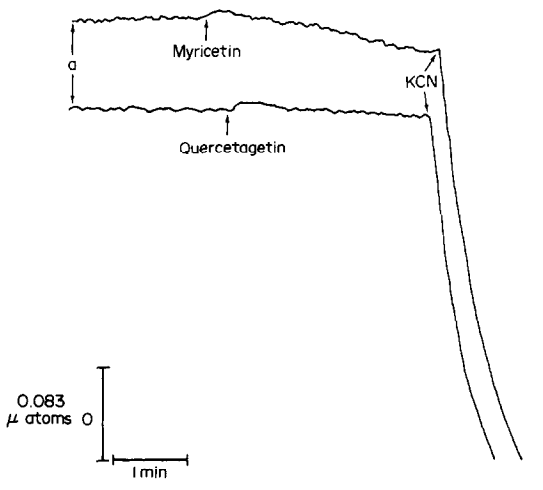


Fig. 3. Effect of cyanide on the auto-oxidation of myricetin and quercetagetin. The reaction medium was 3 mM MOPS, pH 7.5. The flavonoids (0.2 mM) and KCN (5 mM) were added at the indicated points. Polarography was conducted as described in Methods.

Attempts to confirm the presence of O_2^- in the flavonoid-induced respiratory burst by three standard assay methods (SOD-sensitive cytochrome *c* reduction [31], sulfite oxidation [32], and adrenochrome formation [33]) were unsuccessful due to the inability of SOD to inhibit the flavonoid-induced reactions. To further characterize this flavonoid-induced mitochondrial respiratory burst, we investigated the role of O_2^- and H_2O_2 in the auto-oxidation of the two flavonoids at pH 7.5. The non-enzymatic oxygen consumption of myricetin was inhibitable by the addition of SOD and catalase (CAT) (Fig. 4A). These non-enzymatic results are consistent with the findings and conclusions obtained when the flavonoid was added to mitochondrial suspensions (Fig. 2B). They also suggest that O_2^- might be involved in the auto-oxidation of the flavonoids by a chain reaction mechanism as suggested for pyrogallol [34]. This would not be surprising in light of the structural similarity between pyrogallol and the b-ring of myricetin.

To test this hypothesis, the auto-oxidation of myricetin was monitored spectrophotometrically. The absorption spectra at pH 7.5 showed a peak at 380 nm which displayed both hypochromic (decrease in absorbance) and hypsochromic (movement of absorbance maximum towards the blue) shifts with time. SOD inhibited both of these shifts, and an O_2^- generating system (xanthine, xanthine oxidase; X/XO) accelerated them (Fig. 5). The rate of auto-oxidation of myricetin as measured by O_2 consumption was elevated markedly at pH 10.2 (Fig. 4, B and C), and the auto-oxidation was accompanied by a complex spectral pattern characterized by time-dependent auxochromic shifts which paralleled the observed color changes. As at pH 7.5, the auxochromic shifts at pH 10.2 were inhibited by SOD

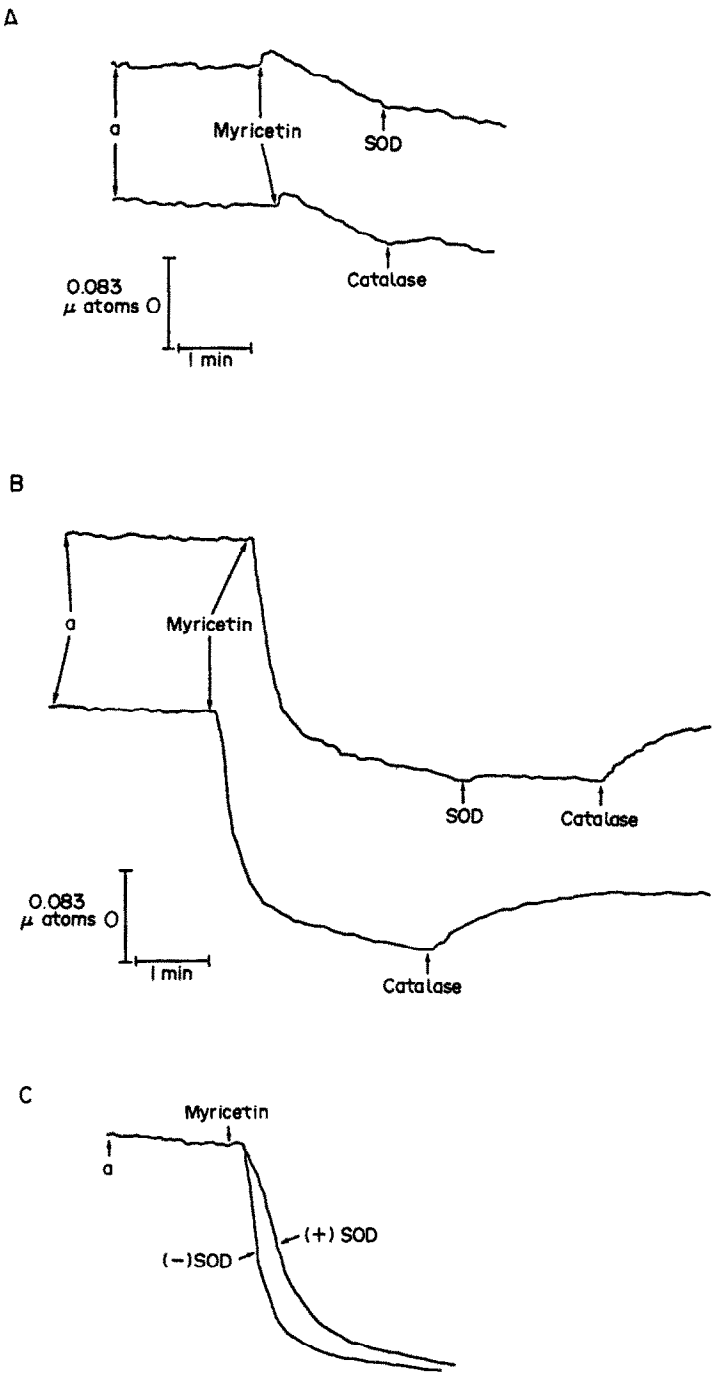


Fig. 4. Polarographic tracings of the auto-oxidation of myricetin. (A) At pH 7.5: (a) 3 mM MOPS, pH 7.5, myricetin (0.2 mM); (B and C) At pH 10.2: (a) 50 mM Na_2CO_3, pH 10.2, myricetin (0.05 mM). SOD (25 μg/ml) and catalase (0.5 μg/ml) were added at indicated points.

and exacerbated by X/XO (Fig. 6). These results demonstrate that O_2^- is involved in the auto-oxidation of myricetin.

The O_2 consumption (auto-oxidation) of quercetagetin at pH 7.5 (Fig. 7A) exhibited responses to SOD and catalase identical to that seen with mitochondria, confirming the generation of O_2^- and H_2O_2 (Fig. 2B). The adsorption spectrum displayed a time-dependent decrease in a 360 nm peak with no other auxochromic shifts. Under these conditions, SOD enhanced the hypochromic shift (Fig. 8B), whereas while X/XO inhibited it and produced a hypsochromic shift (Fig. 8C). At pH 10.2, the rate of auto-oxidation of quercetagetin was increased to that at pH 7.5. Similar to the observations at pH 7.5, the rate of auto-oxidation of quercetagetin at pH

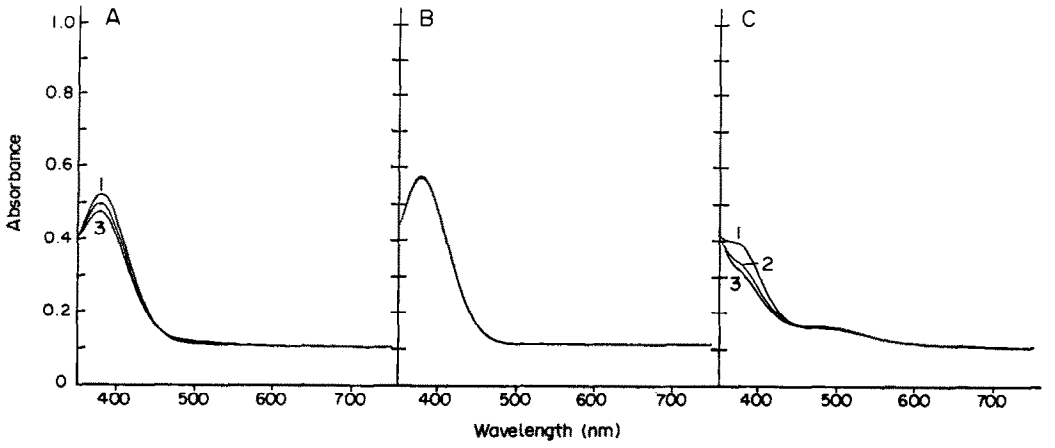


Fig. 5. Absorption spectra of myricetin at pH 7.5. The reaction mixtures contained 3 mM MOPS, pH 7.5. (A) Myricetin (33.3 μM) alone. (B) plus SOD (25 μg/ml). (C) plus X/XO [xanthine (Sigma Chemical Co. (XS), xanthine oxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) (0.12 units/ml)] in a volume of 3.0 ml at 25°. The first scan was recorded 1 min after the start of the reaction; subsequent scans were done at 3-min intervals with a blank cuvette containing all components except the flavonoid.

10.2 was increased by the addition of SOD (Fig. 7B). Also, the addition of CAT resulted in the evolution of oxygen, demonstrating the accumulation of H_2O_2 in the reaction vial. The spectrum of quercetagetin at pH 10.2 exhibited a 390 nm peak which decayed by time-dependent hypochromic and hypsochromic shifts. The addition of SOD inhibited the hypochromic shift by 0.02 absorbance units per minute and enhanced the hypsochromic shift by 10 nM (Fig. 9A and B), whereas the addition of O_2^- from X/XO enhanced both shifts (Fig. 9C).

These data suggest that quercetagetin undergoes auto-oxidation involving O_2^- and H_2O_2. The discrepant observation that the auto-oxidation was inhibited by SOD for myricetin and exacerbated by SOD for quercetagetin remains unexplained. In the case of myricetin, inhibition of auto-oxidation by SOD demonstrates that O_2^- participated in the auto-oxidation, since removal of O_2^- by SOD inhibited the process. Conversely, the auto-oxidation of quercetagetin was increased by the addition of SOD. This suggests that, during auto-oxidation at pH 7.5, a highly reactive intermediate (oxidizing radical) of quercetagetin is generated that is capable of catalyzing the continued oxidiation of querecetagetin and also reacting with O_2^-. By removing the O_2^- from the reaction mixture with SOD, the proposed intermediate is free to react with the native quercetagetin. It is envisioned that, when O_2^- is present, it competes for the reactive intermediate directly and thereby slows down the auto-oxidation of quercetagetin. This proposed mechanism is consistent with the obser-

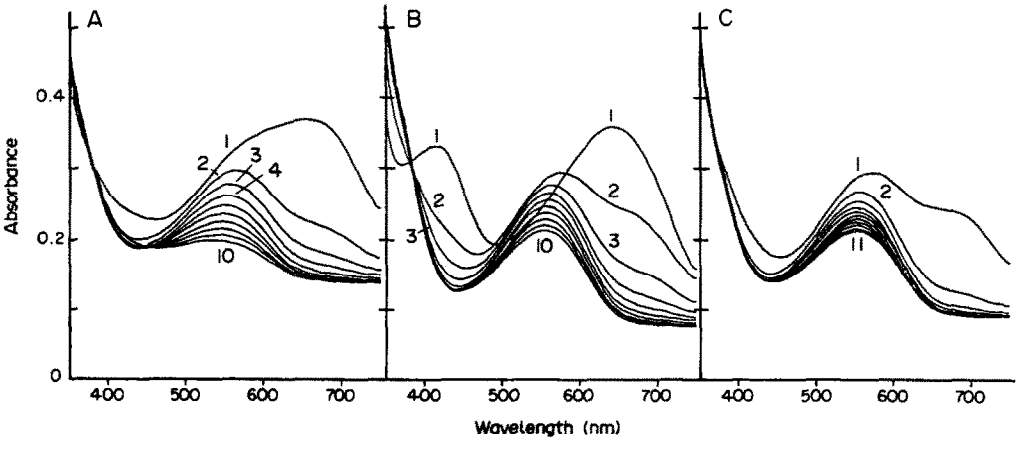


Fig. 6. Absorption spectra of myricetin at pH 10.2. The reaction mixtures contained 50 mM Na_2CO_3, pH 10.2. (A) Myricetin alone. (B) plus SOD. (C) plus X/XO at the concentrations and conditions as described in Fig. 5.

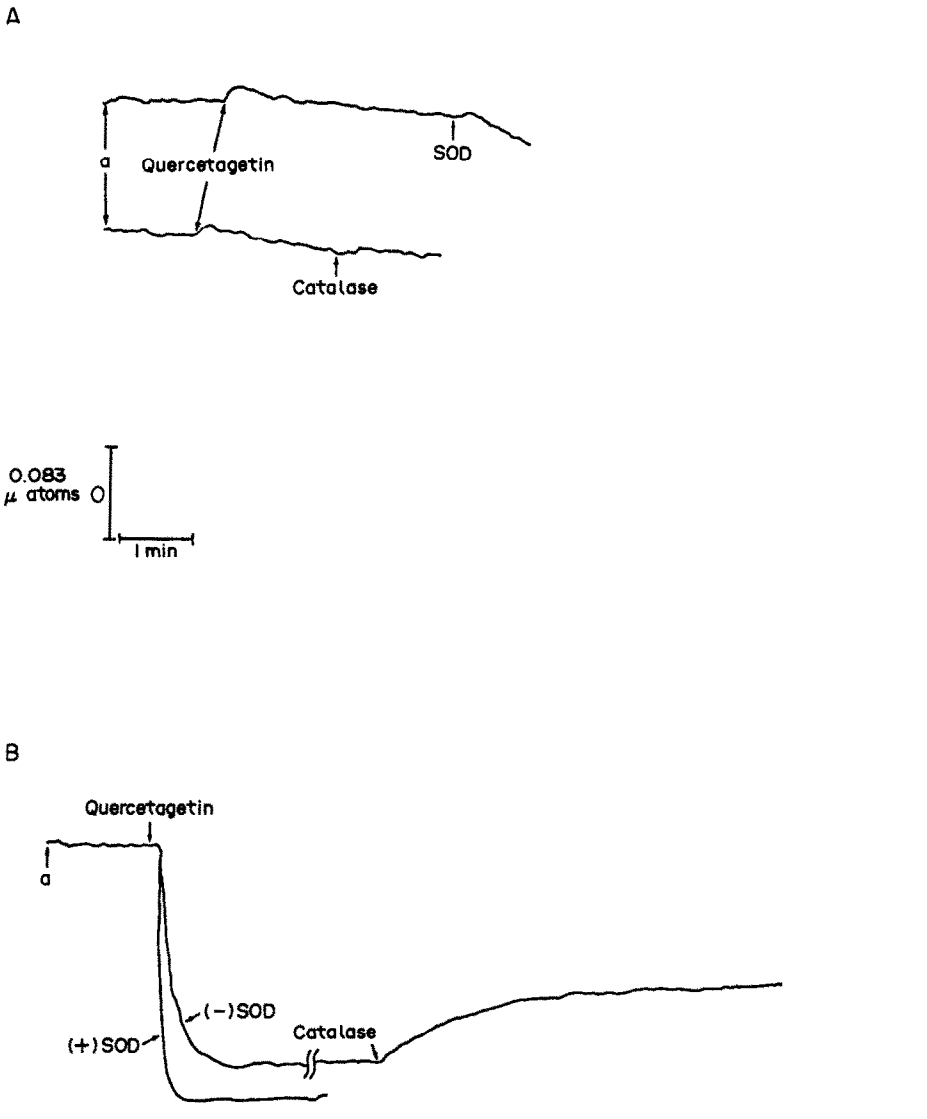


Fig. 7. Polarographic tracings of the auto-oxidation of quercetagetin. (A) At pH 7.5: (a) 3 mM MOPS, pH 7.5, quercetagetin (0.2 mM). (B) At pH 10.2: (a) 50 mM Na_2CO_3, pH 10.2, quercetagetin (0.05 mM). SOD (25 μg/ml) and catalase (0.5 μg/ml) were added at indicated points.

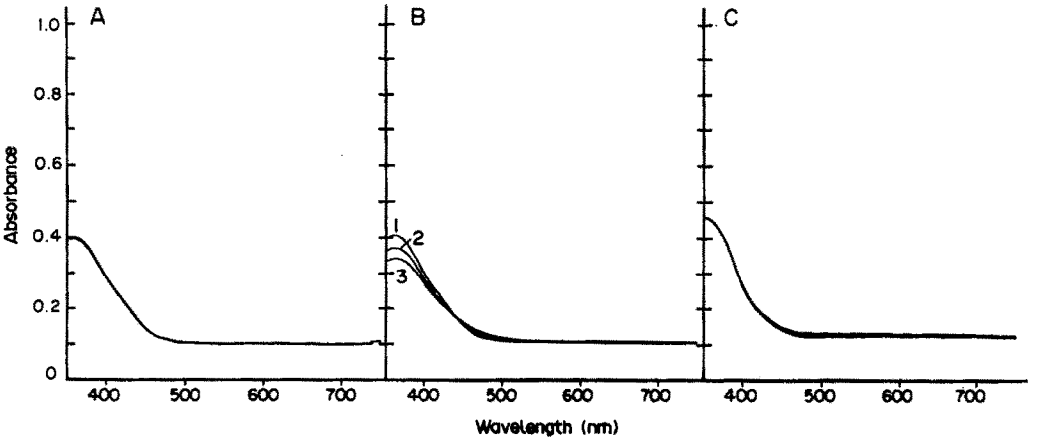


Fig. 8. Absorption spectra of quercetagetin at pH 7.5. The reaction mixtures contained 3 mM MOPS, pH 7.5. (A) Quercetagetin alone. (B) Quercetagetin plus SOD. (C) Quercetagetin plus X/XO at the concentrations and conditions described in Fig. 5.

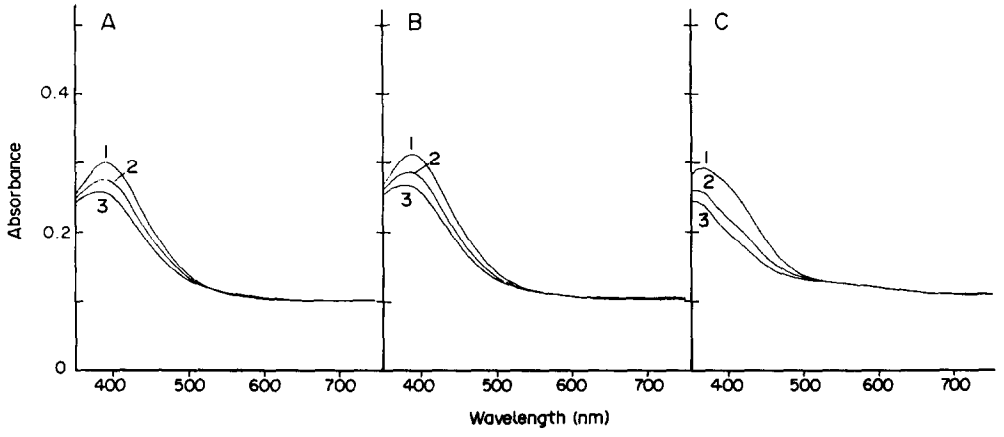


Fig. 9. Absorption spectra of quercetagetin at pH 10.2. The reaction mixtures contained 50 mM Na_2CO_3, pH 10.2. (A) Quercetagetin alone. (B) Quercetagetin plus SOD. (C) Quercetagetin plus X/XO at the concentrations and conditions described in Fig. 5.

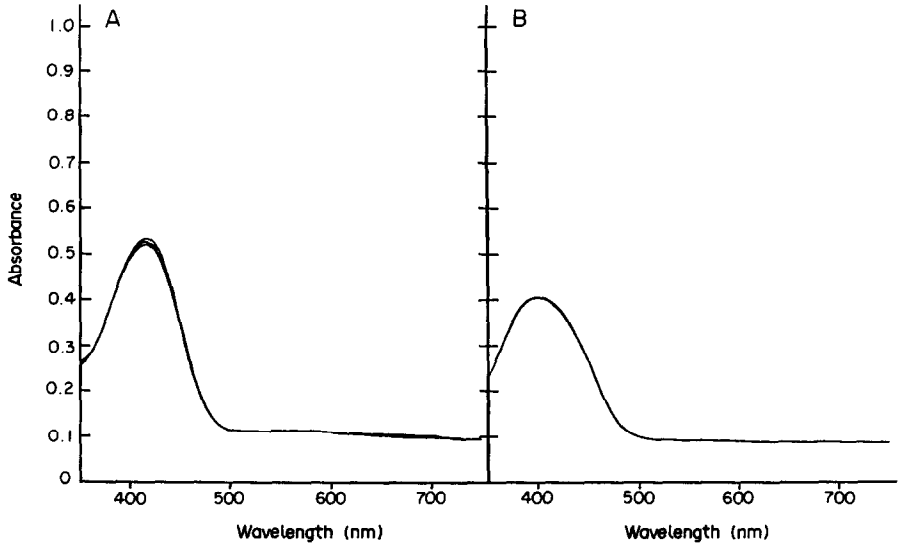


Fig. 10. Absorption spectrum of myricetin and quercetagetin under anaerobic (N_2) conditions at pH 10.2. The reaction mixtures contained 50 mM Na_2CO_3, pH 10.2, and flavonoid at a final concentration of 33.3 μM. The spectra were recorded as described in Fig. 5. (A) Myricetin. (B) Quercetagetin.

vation that elevating O_2^- lowered the rate of auto-oxidation whereas removing the O_2^- increased the rate.

The findings that O_2^- is involved in the auto-oxidation of myricetin is not surprising owing to the similarity in the structure of the myricetin b ring and pyrogallol [34]. Also, O_2^- has been implicated in the auto-oxidation of adrenalin [33] and 6-hydroxydopamine [35], both containing a catechol moiety. Quercetagetin contains a catechol configuration on the b ring; however, in this case O_2^- appears to interrupt the auto-oxidation of quercetagetin. Quercetagetin also contains an adjacent trihydroxy configuration on the a ring, and this complex structure of quercetagetin may contribute to its unique and at present unexplained behavior.

The involvement of active oxygen species depends on the presence of oxygen. Under anaerobic (N_2) conditions (Fig. 10), essentially no changes in the absorption spectrum with time were observed for both compounds which confirms the conclusion that active oxygen species are involved.

The generation of active oxygen species by flavonoids at pH 7.5 implies their potential to induce oxidative stress in cellular organelles analogous to that generated by 2,3-dichloro-1,4-naphthoquinone (CNQ) [36, 37] and adriamycin [38–43].

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