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INHIBITION OF MITOCHONDRIAL RESPIRATION AND CYANIDE-STIMULATED GENERATION OF REACTIVE OXYGEN SPECIES BY SELECTED FLAVONOIDS*

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Abstract—A continuation of our structure–activity study on flavonoids possessing varied hydroxyl ring configurations was conducted. We tested six additional flavonoids for their ability to inhibit beef heart mitochondrial succinoxidase and NADH-oxidase activities. In every case, the IC_{50} observed for the NADH-oxidase enzyme system was lower than for succinoxidase activity, demonstrating a primary site of inhibition in the complex I (NADH-coenzyme Q reductase) portion of the respiratory chain. The order of potency for inhibition of NADH-oxidase activity was robinetin, rhamnetin, eupatorin, baicalein, 7,8-dihydroxyflavone, and norwogonin with IC_{50} values of 19, 42, 43, 77, 277 and 340 nmol/mg protein, respectively. Flavonoids with adjacent tri-hydroxyl or para-dihydroxyl groups exhibited a substantial rate of auto-oxidation which was accelerated by the addition of cyanide (CN^-). Flavonoids possessing a catechol configuration exhibited a slow rate of auto-oxidation in buffer that was stimulated by the addition of CN^-. The addition of superoxide dismutase (SOD) and catalase in the auto-oxidation experiments each decreased the rate of oxygen consumption, indicating that O_2^- and H_2O_2 are generated during auto-oxidation. In the CN^--stimulated oxidation experiments, the addition of SOD also slowed the rate of oxygen consumption. These findings demonstrate that the CN^-/flavonoid interaction generated O_2^- non-enzymatically, which could have biological implications.

Flavonoids are an integral part of the plant kingdom, present in all photosynthesizing cells. Their widespread occurrence in food plants and their biological effects, including but not limited to antimicrobial, mutagenic, carcinogenic, antioxidant, and cytotoxic properties [1–3], make an understanding of their mechanism(s) of action important. Inhibition of mitochondrial enzymes by flavonoid constituents may contribute to some of their biological activities [4]. The hydroxyl group configurations of various flavonoids have been linked to the potency of inhibition in the succinoxidase system and inhibition of succinoxidase has also been linked to their ability to participate in oxidation–reduction reactions [4]. Some biologically active flavonoids have hydroxyl group configurations similar to those flavonoids capable of inhibiting succinoxidase systems [2, 4]. In a study comparing the structures of flavonoids that inhibit NADH-oxidase and succinoxidase enzyme systems, it was determined that the $C_{2,3}$-double bond, the C_4-keto group, and 3′,4′,5′-hydroxyl are important for inhibiting NADH-oxidase [4–6]. We have extended this study to include a series of flavonoids of varying hydroxyl group configurations for which no data are currently available (Fig. 1).

Previous experimentation has shown that some flavonoids cause a substrate-independent cyanide (CN^-) insensitive respiratory burst in isolated mitochondria and undergo auto-oxidation, which is associated with the production of superoxide (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical; this redox activity may also contribute to the overall cytotoxicity of the flavonoids [4, 7, 8]. Accordingly, these flavonoids were also screened for their ability to produce a respiratory burst and toxic oxygen radicals. The rate of flavonoid auto-oxidation has been shown to be stimulated by the presence of CN^- [4]; thus, the ability of CN^- to enhance the auto-oxidation of these flavonoids was assessed.

MATERIALS AND METHODS

Beef heart mitochondria (BHM)‡ were isolated by differential centrifugation and stored as described previously [9]. Aging of the mitochondria to uncouple respiration from phosphorylation was achieved by repeated freezing and thawing. Mitochondrial protein was determined by the method of Lowry *et al.* [10].

The activities of BHM succinoxidase and NADH-oxidase were assayed by measuring oxygen consumption, in the presence and absence of test flavonoids, polarographically with a YSI model 53 oxygen monitor equipped with a Clark electrode

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‡ Abbreviations: BHM, beef heart mitochondria; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; SOD, superoxide dismutase; and CAT, catalase.

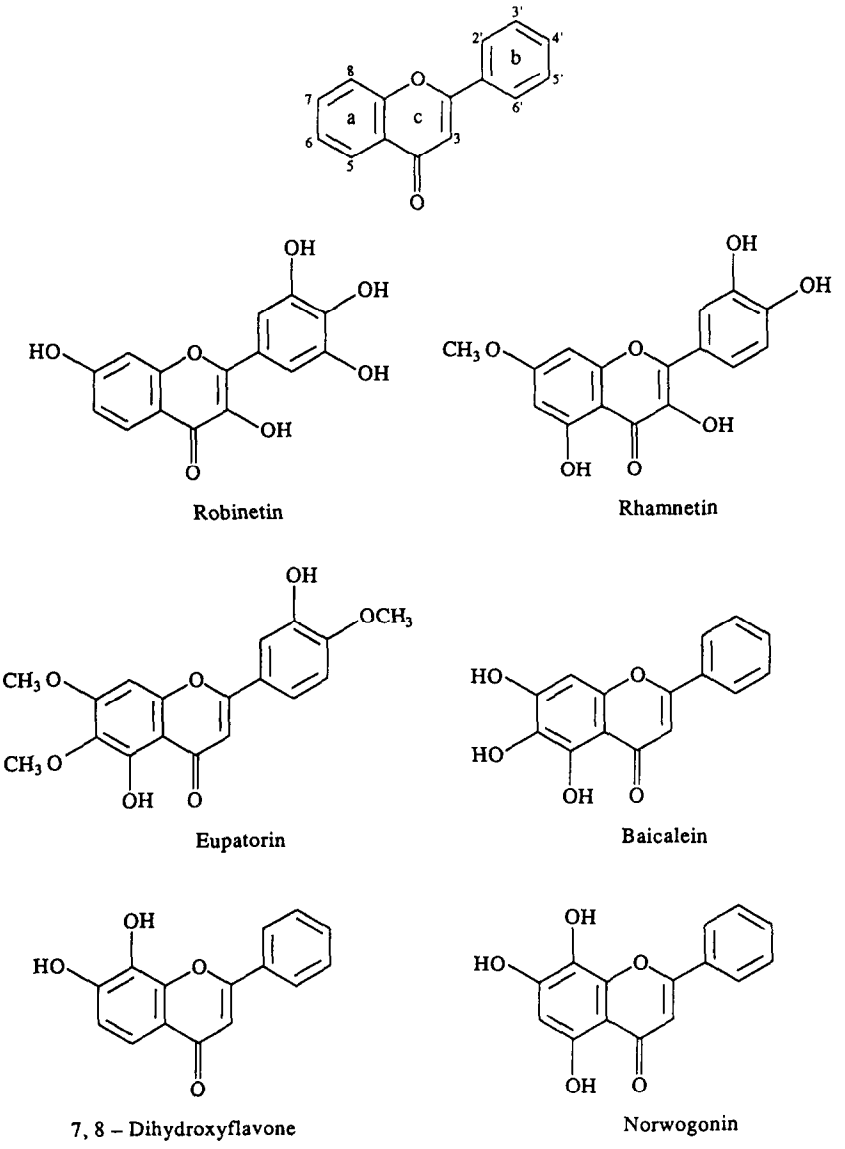

Fig. 1. Structures and numbering system of flavonoids tested.

(Yellow Springs Instrument Co., Yellow Springs, OH) in a water jacketed chamber (Gilson Medical Electronics, Inc., Middleton, WI), a magnetic flea stirbar, a strip chart recorder (Linear 1800, Linear Instruments Corp., Reno, NV), and a Bascom-Turner model 4120T analog to digital electronic recorder (Bascom-Turner Instruments, Newton, MA). The reaction mixture consisted of 33 mM Tris-HCl buffer (pH 7.5), 0.33 mg/mL asolectin (Associated Concentrates, Woodside, NY), 27 μM EDTA (NADH-oxidase only), 0.5 mg/mL BHM and substrate, either 50 mM disodium succinate (for succinoxidase) or 75 mM NADH (for NADH-oxidase), in a final volume of 2 mL. The oxygen electrode was calibrated over a 0–100% saturation scale with the reaction mixture minus the substrate, which was air saturated at the experimental temperature of 30°. The test compounds were prepared in dimethyl sulfoxide (DMSO), which was added at a maximum concentration of 0.05 mL DMSO/mL of reaction mixture. The test compounds were allowed to preincubate for 30 min before the substrate was added. Controls were conducted in the absence of flavonoid and in the presence of substrate. The IC_{50} values were estimated by extrapolation from titration curves. The number of replicates for each concentration of flavonoid was from 3 to 6 and the specific activities of the controls ranged between 0.20 and 0.32 nanoatoms of oxygen consumed per min per mg mitochondrial protein. Flavonoid auto-oxidation and its stimulation by CN^- were also monitored polarographically in 3 mM 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer (pH 7.5) at 30°.

Table 1. Inhibition of mitochondrial respiration by flavonoids

Common name	Name	IC_{50} (nmol/mg protein)	
		Succinoxidase	NADH-oxidase
Robinetin	3,3′,4′,5′,7-Pentahydroxyflavone	738	19
Rhamnetin	3,3′,4′,5-Tetrahydroxy-7-methoxyflavone	367	42
Eupatorin	3′,5-Dihydroxy-4′,6,7-trimethoxyflavone	>1000*	43
Baicalein	5,6,7-Trihydroxyflavone	383	77
	7,8-Dihydroxyflavone	>1000*	277
Norwogonin	5,7,8-Trihydroxyflavone	432	340

* Compounds failed to inhibit succinoxidase by 50% at a concentration of 1000 nmol/mg of mitochondrial protein.

Rhamnetin and eupatorin were purchased from the Spectrum Chemical Mfg. Co. (Gardena, CA). Robinetin was obtained from Roth (Atomergic Chemetals Corp., Plainview, NY) and baicalein from Sarsynthese (Merignac, France). Norwogonin and 7,8-dihydroxyflavone were the gifts of Dr. James T. MacGregor (USDA Western Regional Research Center, Berkeley, CA). Tris–HCl, EDTA, MOPS, disodium succinate, β-NADH and catalase (CAT) were all obtained from the Sigma Chemical Co. (St. Louis, MO). KCN was purchased from J. T. Baker (Phillipsburg, NJ), superoxide dismutase (SOD) was from DDI (Mountain View, CA), and DMSO from Burdick & Jackson Laboratories Inc. (Muskegon, MI).

RESULTS AND DISCUSSION

The potencies of six flavonoid compounds for inhibiting the succinoxidase and NADH-oxidase enzyme systems are listed in Table 1. Comparisons of the IC_{50} values show that all of the flavonoids tested were more potent inhibitors of the NADH-oxidase system than of succinoxidase, indicating that the primary site of inhibition is in complex I (NADH-coenzyme Q reductase). The order of potency for inhibition of NADH-oxidase activity was robinetin > rhamnetin = eupatorin > baicalein > 7,8-dihydroxyflavone = norwogonin with IC_{50} values of 19, 42, 43, 77, 277 and 340 nmol/mg protein, respectively. These data are consistent with previous reports that flavonols, in general, are more potent inhibitors of the NADH-oxidase system than the succinoxidase system [5].

The compounds tested for NADH-oxidase inhibition follow the pattern previously reported for 3,5,7-trihydroxyflavonoids with varying hydroxyl ring substitution patterns on the B ring. The most potent inhibitor had a B-ring configuration of 1,2,3-trihydroxyl (robinetin), with the catechol on the B ring next in potency (rhamnetin). Eupatorin was next, with a mono-hydroxyl group on the B-ring; baicalein, norwogonin and 7,8-dihydroxyflavone were the weakest inhibitors tested with no B-ring hydroxyl groups.

The most potent flavonoid tested was robinetin which had an IC_{50} value of 19 nmol/mg protein for NADH-oxidase, a potency which is comparable to the potent respiratory poisons butein (18 nmol/mg protein) and fisetin (15 nmol/mg protein) [5]. Out of all of the flavonoids tested, these are the most potent inhibitors of NADH-oxidase reported to date [5, 6]. Previous studies predicted that flavonoids devoid of a 5-hydroxyl group would be more potent inhibitors since 5-hydroxyflavone is less potent toward NADH-oxidase than flavone [6], and quercetin (3,5,7,3′,4′-pentahydroxyflavonol) is less potent than fisetin (3,7,3′,4′-tetrahydroxyflavonol), the corresponding flavonol minus the 5-hydroxyl group. These findings predicted that robinetin would be a more potent inhibitor of NADH-oxidase than myricetin. The present study supports this prediction in that robinetin (3,7,3′,4′,5′-pentahydroxyflavonol) was a more potent inhibitor of NADH-oxidase than myricetin (3,5,7,3′,4′,5′-hexahydroxyflavonol) with IC_{50} values of 19 and 35, respectively [5]. Thus, the absence of a hydroxyl substituent in the 5 position increases the potency of flavonoid inhibition of mitochondrial respiration. Interestingly, rhamnetin with a 3,5,3′,4′-tetrahydroxy-7-methoxyflavonol configuration is a more potent inhibitor than quercetin with a 3,5,7,3′,4′-pentahydroxy configuration, with IC_{50} values of 42 and 145, respectively. This finding indicates that a methoxyl configuration on the A-ring increases the inhibitory activity when compared to a hydroxyl group at the same position. In contrast, we know from a previous study [6] that converting a hydroxyl to a methoxyl on the B-ring decreases activity. Acacetin (5,7-dihydroxy-4′-methoxy) and kaempferide (3,5,7-dihydroxy 4′-methoxy) were poorer inhibitors than their unmethylated counterparts apigenin and kaempferol, respectively, with percent inhibition at 3.5×10^{-4} M being 88.6, 70, 12.9, and 59, respectively. It is interesting to note that the most potent inhibiting flavonoid that undergoes oxidation-reduction is robinetin. The flavonoids undergoing auto-oxidation include robinetin, myricetin, baicalein, quercetin, quercetagetin, 7,8-dihydroxyflavone, norwogonin and delphinidin with IC_{50} values toward NADH-oxidase being 19, 35, 77, 145, 177, 277, 340 and 1000, respectively. Thus, robinetin may be an important redox active flavonoid for inducing oxidative stress and a model redox cycling compound.

In previous work, we have demonstrated that those flavonoids capable of redox cycling and

producing toxic oxygen species possessed lower oxidation potentials than the flavonoids that did not auto-oxidize [11]. In addition, a semiquinone species was observed when myricetin was auto-oxidized in the presence of Mg^{2+}, a spin stabilizer for the *o*-semiquinones [8]. Semiquinones could undergo disproportionation to form a fully oxidized *o*-quinone and a fully reduced catechol. Thus, it is likely that semiquinones, fully reduced catechols, and fully oxidized quinones are all present in our reaction mixture. *o*-Quinones can react through a Michael addition with thiols. Carpenedo *et al.* [12] reported that quercetin inhibits mitochondrial electron transport and that cysteine and DTT reverse the inhibition. Furthermore, Roberts [13] reported that flavonol *o*-quinones react with cysteine and glutathione forming thiol conjugates. Collectively, these observations are consistent with a flavonoid quinoid species reacting with a key thiol and inhibiting the respiration. Alternatively, the quinoid by-product could "short circuit" the respiratory chain and thereby inhibit electron transport in a reversible fashion [14, 15]. It has been speculated previously that several biological activities observed for the flavonoids are dependent on their redox activity [11]. In fact, the structural requisites for inhibition of mitochondrial NADH-oxidase [5] and succinoxidase [4], mutagenicity [2], antioxidant activity, inhibition of lens aldose reductase and neutrophil NADPH-oxidase [1–3] are all similar and consistent with the ability to undergo oxidation [4–7, 11].

Eupatorin has been reported to be cytotoxic toward tumor cells in culture [16], and the findings reported herein demonstrate a preferential inhibition of NADH-oxidase activity. The antineoplastic agent 4′-dimethyl-epipodophyllotoxin thenylidine glucoside (VM-26) is a lignan that is biosynthetically related to flavonoids [17]. VM-26 has been reported to inhibit NADH-linked respiration in isolated mitochondria, sub-mitochondrial particles, and Ehrlich ascites tumor (EAT) cells in suspension [18]. The mitochondrial electron transport inhibitors antimycin A [19] and rotenone (a flavonoid derivative) [20, 21] have been shown to inhibit the growth of EAT cells *in vivo* and *in vitro*. These findings suggest that inhibition of mitochondrial respiration may be an underlying mechanism of the cytotoxicity of the flavonoid class of xenobiotics. Furthermore, these findings support the postulate that mitochondrial respiration may be a target for the chemotherapy of cancer [21, 22].

The abilities of each of the flavonoids to auto-oxidize are shown in the oxygen electrode tracings in Fig. 2. Flavonoids with adjacent trihydroxyl (pyrogallol) or para-dihydroxyl (hydroquinone) configurations underwent a high rate of auto-oxidation. By analogy with other flavonoids [8], this activity may be attributed to the flavonoid being oxidized by molecular oxygen, resulting in the formation of semiquinones and quinones. This is illustrated by norwogonin which has a para-dihydroxyl substitution on the A-ring and shows the most rapid rate of oxygen consumption among the flavonoids tested. Robinetin and baicalein, which have pyrogallol-like hydroxyl configurations, exhibited a slower but significant rate of auto-oxidation. Robinetin, which has the pyrogallol configuration on the B-ring, auto-oxidized much faster than baicalein, which has the same hydroxyl group configuration located on the A-ring. This is not surprising, considering the fact that with the B-ring there exists the possibility of extended conjugation through the $C_{2,3}$-double bond that is not possible with the A-ring. In addition, in the case of robinetin, there is an additional hydroxyl group at the C_3-position. An electrochemical study of other flavonoids in aqueous medium has shown that extended conjugation and/or the C_3-hydroxyl group decrease the midpoint potential ($E_{1/2}$) of these compounds, as would be expected [11]. Based on this previous study, robinetin probably has a lower $E_{1/2}$ than baicalein, hence it would be easier for it to auto-oxidize. Flavonoids possessing a catechol configuration auto-oxidized very slowly (7,8-dihydroxyflavone) or not at all (rhamnetin). The flavonoid eupatorin, which did not possess oxidizable hydroxyl configurations, did not auto-oxidize. These findings serve to further confirm the conclusion that there is a requirement for hydroxyl configurations capable of supporting such redox reactions (i.e. hydroquinone and/or catechol). These findings are consistent with what is known about quinone chemistry in that the relative rates for auto-oxidation in decreasing order are hydroquinone > pyrogallol > catechol. As a consequence, when comparing para-oxygenated versus ortho-oxygenated compounds, their relative stabilities are such that the para-oxygenated compounds are typically most stable as the quinone (e.g. 1,4-benzoquinone) and ortho-oxygenated compounds are most stable as the hydroquinone (e.g. catechol). The addition of SOD and CAT both decreased the rate of O_2 consumption induced by flavonoids. Since both SOD and CAT catalytically regenerate oxygen, this demonstrates that $O_2^{\cdot-}$ and H_2O_2 are produced during the flavonoid auto-oxidation process (Fig. 3). These results also suggest $O_2^{\cdot-}$ might be involved in the propagation of the auto-oxidation of these flavonoids by a chain reaction mechanism as we previously proposed for another series of flavonoids [4]. This possibility is supported by the greater than 50% inhibition of the flavonoid-induced oxygen consumption by SOD (Fig. 3). Confirmation of $O_2^{\cdot-}$ formation by direct measurement (e.g. SOD-sensitive cytochrome *c* reduction, sulfite oxidation and adrenochrome formation) is not possible due to the inability of SOD to inhibit the reduction of these electron acceptors [4].

Flavonoids have been shown to react non-enzymatically with CN^- to facilitate their auto-oxidation [4]. Accordingly, we tested this series of flavonoids for their ability to react with CN^- (Fig. 2). The addition of CN^- exacerbated the rate of auto-oxidation and, in the case of rhamnetin, which did not auto-oxidize in buffer alone, CN^- induced oxygen consumption. The addition of SOD also slowed the rate of CN^--stimulated oxygen consumption (Fig. 3). Since CN^- was added at a concentration that was not inhibitory to SOD, these findings demonstrate that the non-enzymatic reaction with CN^- also stimulates the generation of $O_2^{\cdot-}$. Surprisingly, the CN^--stimulated oxygen con-

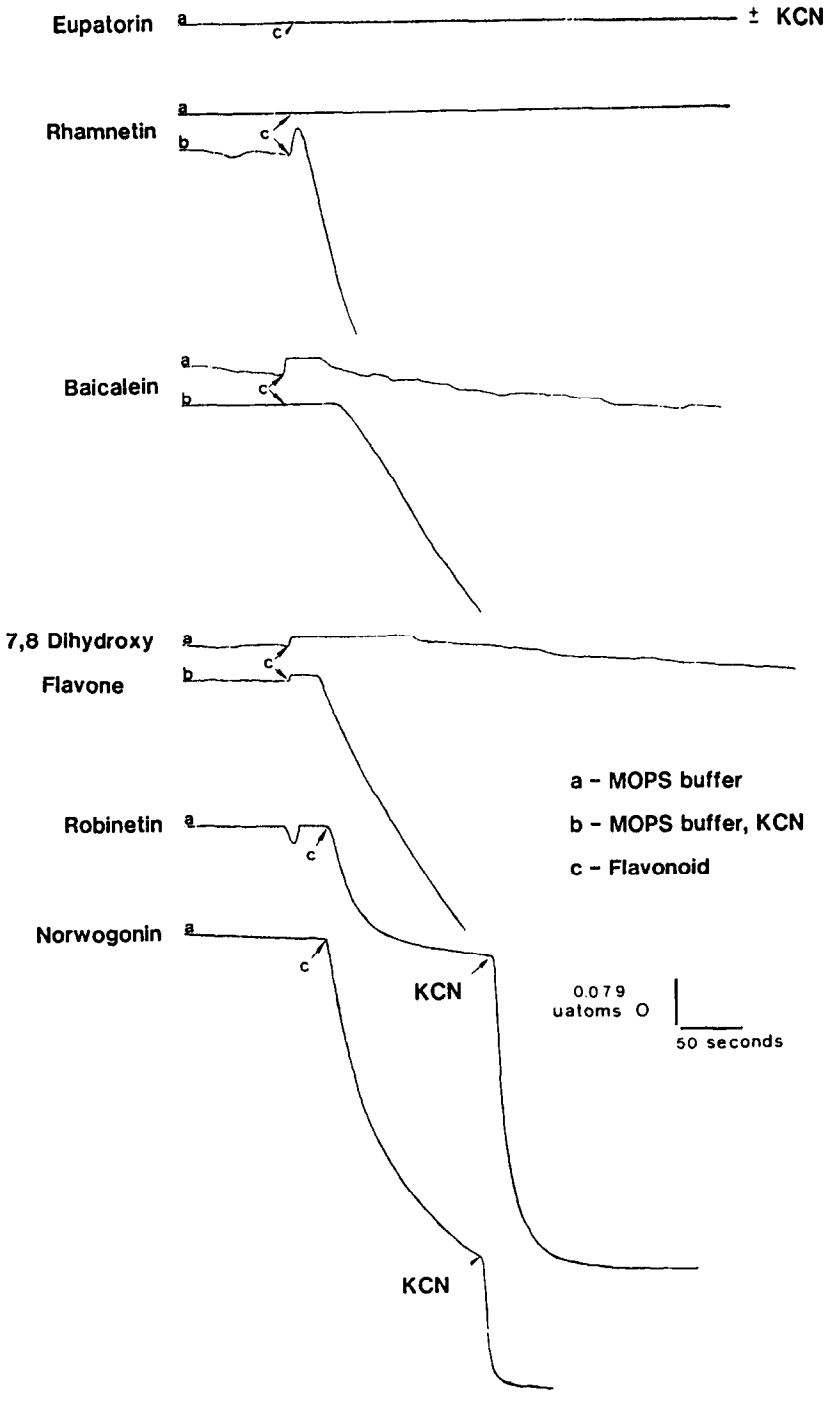

Fig. 2. Cyanide-enhanced auto-oxidation of flavonoids. Polarography was conducted as outlined in Materials and Methods. The flavonoids and KCN were added, as indicated, to final concentrations of 1.25 and 5 mM, respectively.

sumption by 7,8-dihydroxyflavone was inhibited by the presence of mitochondria (Fig. 4); this flavonoid was the only one for which this phenomenon was observed. At the present time, this behavior remains unexplained. This inhibition of CN^--stimulated auto-oxidation of 7,8-dihydroxyflavone by mitochondria raised the question of whether the inhibition seen with SOD was purely a non-specific protein effect instead of SOD-catalyzed dismutation of $O_2^{\cdot-}$. The addition of 25 μg/mL bovine serum albumin had no measurable effect on the rate of auto-oxidation of 7,8-dihydroxyflavone. However, the addition of

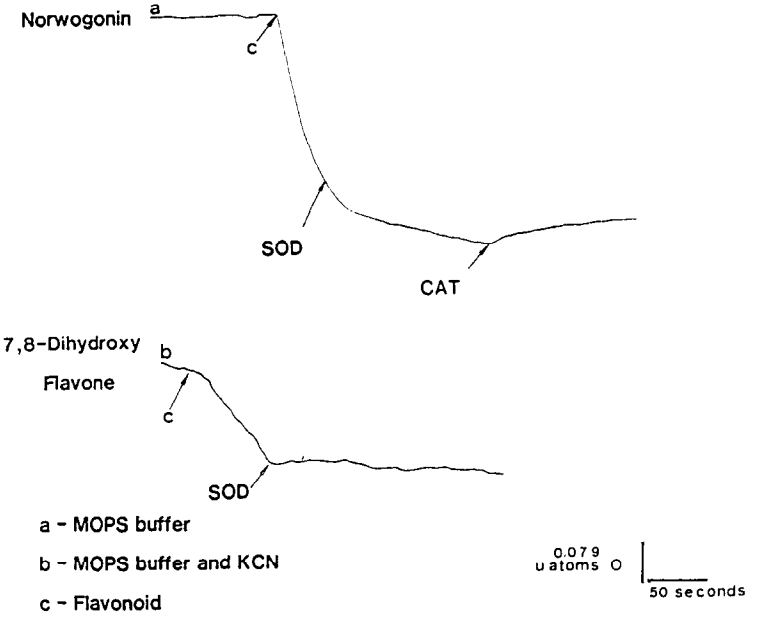

Fig. 3. Effects of SOD and CAT on flavonoid auto-oxidation and its enhancement by CN^-. Oxygen consumption was again measured polarographically with the flavonoids being added to a final concentration of 1.25 mM. (Top trace) without CN^-: SOD (25 μg/mL) and CA (0.5 μg/mL) were added at indicated points; (Bottom trace) with CN^- (2.5 mM): SOD (250 μg/mL) was added as indicated.

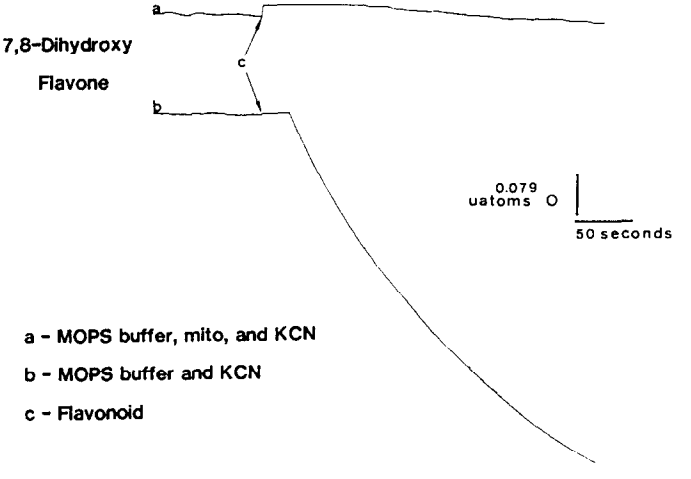

Fig. 4. Inhibition of CN^--mediated oxygen consumption by mitochondria. KCN (5 mM), flavonoid (1.25 mM) and BHM (0.5 mg/mL) were present or added as indicated.

soybean phospholipid (asolectin) as a micellar preparation to a final concentration of 1.65 mg/mL inhibited the rate of 7,8-dihydroxyflavone auto-oxidation by about 72% and the rate of CN^--stimulated auto-oxidation of 7,8-dihydroxyflavone by approximately 37%. Subsequent addition of SOD further inhibited oxygen consumption (data not shown), indicating that the inhibition of 7,8-dihydroxyflavone auto-oxidation by SOD is not a non-specific protein effect. This also suggested that it may be the mitochondrial phospholipids and not the protein that are, in part, responsible for the inhibition of the CN^--stimulated auto-oxidation of 7,8-dihydroxyflavone by mitochondria. Since the redox potential is influenced by both solvent and pH, the phospholipid environment could be a major factor. For example, it is known that micelles produce microenvironments that markedly affect the redox behavior of dissolved solutes [23]. These facts form the basis for the determination of redox

potentials in octanol to mimic a membrane environment. Further studies are needed to confirm if indeed this is the case.

The CN^--catalyzed oxidation of compounds is not new. Cyanide has been reported to catalyze the oxidation of α-hydroxyaldehydes and related compounds with the concomitant production of O_2^- [24, 25]. The proposed mechanism involves tautomerization to an enediol to which CN^- can combine, and the complex then auto-oxidizes to yield O_2^- and ultimately regenerates the CN^-. Many of the flavonoids that interacted with CN^- possessed a $C_{2,3}$-double bond, C_3-hydroxyl and C_4-keto group on the C-ring. This configuration resembles an α-hydroxyl unsaturated ketone which one could envision to tautomerize to form an enediol and undergo auto-oxidation by a similar mechanism. In general, it was found that among the compounds tested non-terminal α-hydroxyketones and diketones were inactive [24]. However, among the flavonoids we tested, most of those that reacted with CN^- auto-oxidized in the absence of CN^-, consistent with their electrochemical properties [11], and this auto-oxidation was enhanced by the presence of CN^-. In addition, dialuric acid, the reduced form of alloxan (a cyclic compound), auto-oxidizes, produces O_2^- [26], and has been found to be highly reactive with CN^- [24]. Part of the supporting evidence given for the mechanism involving an enediol was the inhibition of the CN^--catalyzed oxidation of α-hydroxyaldehydes by borate [24]. The addition of borate (2 mM) almost completely inhibited the CN^--stimulated auto-oxidation of rhamnetin and 7,8-dihydroxyflavone (data not shown), which by analogy suggests that an enediol intermediate was involved. However, since both of these flavonoids possess a catecholic hydroxyl configuration somewhere on the molecule and (catecholato) borate complexes are well known, it is possible that borate simply complexed this redox active center [27, 28]. In addition, Zn^{2+} (2 mM) also inhibited the auto-oxidation of these flavonoids to an extent equal to or greater than borate (data not shown). Metal ions such as Zn^{2+} and Mg^{2+} are employed for spin stabilization to enhance the steady-state concentration of *o*-semiquinone radicals under acidic and neutral pH for electron spin resonance investigation [29]. Using Mg^{2+} as the stabilizing ion, we have detected semiquinone radicals of flavonoids produced during auto-oxidation [8]. Therefore, at present it is unclear what part of the molecule is being affected by these ions and whether borate and zinc react at the same center on the molecule. Finally, addition of Mg^{2+} (2 mM) stimulated the rate of CN^- enhanced auto-oxidation of these two flavonoids, further confounding the issue (data not shown). We are currently investigating the mechanism of this CN^- reaction. Since CN^- reacts with flavonoids and many other compounds, some of which are metabolic intermediates, great care must be exercised when CN^- is employed in biochemical studies to inhibit respiration or any other enzyme(s) [4, 24, 25].

The interaction with CN^- may be biologically significant since flavonoids have been reported to serve as allelochemicals affecting plant–insect interaction [30]. The generation of oxygen radicals by flavonoids has been implicated as a mechanism for their allelopathic properties [31–33]. Since flavonoids are ubiquitous in the plant kingdom and many plants also contain cyanogenic glycosides that can release CN^-, the CN^- could then react with the flavonoids and exacerbate the production of toxic oxygen species from flavonoids. This presents a new role for cyanogenic glycosides in generating oxidative stress, and it may be particularly important in that it has been reported that many insects feed with impunity on cyanogenic compounds [34]. Also the mitochondria of some of these insects exhibit target-site insensitivity to CN^- [35, 36].

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