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# INHIBITION OF MITOCHONDRIAL NADH OXIDASE, SUCCINOXIDASE, AND ATPASE BY NATURALLY OCCURRING FLAVONOIDS

CRAIG BOHMONT, LAURA M. AARONSON, KATHY MANN, and RONALD S. PARDINI

*Department of Biochemistry, University of Nevada, Reno, Nevada 89557*

ABSTRACT.—A structure-activity investigation of the inhibition of beef heart mitochondrial NADH oxidase and succinoxidase and rat liver mitochondrial ATPase by flavonoids was conducted. NADH oxidase was the most sensitive to inhibition by flavonoids: 13 of the 18 flavonoids tested inhibited NADH oxidase, whereas only 4 and 5 flavonoids inhibited succinoxidase and ATPase, respectively.

The flavonoids possessing a catechol or pyrogallol moiety, and a 2,3-double bond and a 3-hydroxyl group were the most inhibitory towards the respiratory chain enzymes. The catechol or pyrogallol moiety did not exert preferential activity towards the oligomycin-sensitive ATPase because morin, which contains a *meta*-dihydroxy configuration, was the most potent ATPase inhibitor.

Flavonoids, a class of naturally occurring pigments, have a wide range of effects on biological systems (1-5). They have been shown to have cytotoxic (1-4,6), antimicrobial (1-5), antiviral (6,7), antineoplastic (8), mutagenic (2,5), and carcinogenic (4,5) activity. Biochemical investigations of the mechanisms of the whole-organism toxicity of the flavonoids have shown that they inhibit a wide variety of enzyme systems, including phosphodiesterase (1,5,9), ATPase (1,2,5,10), lens aldol reductase (11,12), and pancreatic ribonuclease (13). We have previously reported that several members of another class of naturally occurring plant derived compounds, the lignans, and several of their structurally related synthetic analogues, inhibit mitochondrial electron transport systems (14-16) and, hence, impair cellular energy production. Because it has been reported that several of the flavonoids inhibit mitochondrial electron transport (17-20) and ATPase activity (10), in this study, we now extend structure-activity considerations by comparing the ability of commercially available naturally occurring flavonoids to inhibit two mitochondrial electron transport systems, NADH oxidase and succinoxidase, with their ability to inhibit the energy transducing system, oligomycin-sensitive ATPase activity.

## MATERIALS AND METHODS

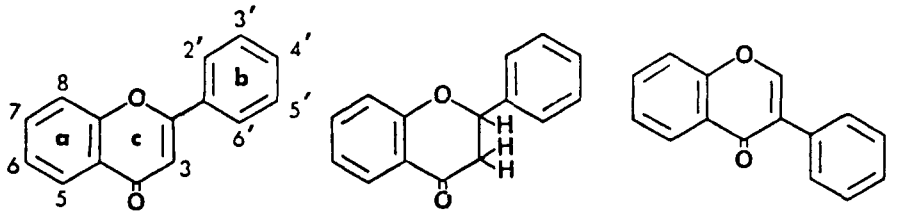
The trivial and proper names and the structures of the flavonoids examined in this study are shown in Table 1, listed approximately in order of increasing hydroxyl substitution, with methyl ether derivatives listed after their corresponding hydroxyl parent compounds. The trivial names of these flavonoids will be used whenever possible throughout this paper.

Flavone, flavanone, morin, myricetin, and rutin were obtained from the Aldrich Chemical Co. Quercetin was obtained from the Sigma Chemical Co. Naringenin was obtained from United States Biochemicals Corp. Acacetin, apigenin, 5-hydroxyflavone, 7-hydroxyflavone, 2,5-dimethoxyflavone, chrysin, fisetin, genistein, kaempferol, and kaempferide were obtained from ICN Pharmaceuticals, Inc., K and K Labs Division, Life Sciences Group. All other compounds and assay biochemicals were on hand and were of reagent grade or the highest purity available.

Mitochondria were isolated from the hearts of freshly slaughtered beef cattle by the differential centrifugation method described by Smith (21). Protein was assayed by the method of Lowry *et al.* (22) using bovine serum albumin as a standard.

Succinoxidase and NADH oxidase enzyme system activities were monitored by a modified manometric procedure (23) at 30°. The total volume of the respiration media was 2.8 ml at pH 7.4 and contained 0.55-0.84 mg mitochondrial protein. For succinoxidase the media contained 33 mM Tris buffer, pH 7.4, 166 mM sucrose, 0.12 mg cytochrome c (Sigma Grade III), and 0.06 ml of soybean phospholipid (Asolectin, Associated Concentrates, Woodside, New York). The flavonoids in EtOH (or EtOH alone for controls) were added to produce a constant EtOH concentration of 3.6%. Succinate was added in 0.2 ml $H_2O$ to a

TABLE 1. Names and Structures of the Flavonoids Examined in this Study




flavones flavanones isoflavones

| Common Name | Chemical Name | Class |
|---|---|---|
| | flavanone | flavanone |
| | flavone | flavone |
| | 5-hydroxyflavone | flavone |
| | 7-hydroxyflavone | flavone |
| Chrysin | 5,7-dihydroxyflavone | flavone |
| Galangin | 3,5,7-trihydroxyflavone | flavonol |
| Naringenin | 5,7,4′-trihydroxyflavanone | flavanone |
| Apigenin | 5,7,4′-trihydroxyflavone | flavone |
| Acacetin | 5,7-dihydroxy-4′-methoxyflavone | flavone |
| Genistein | 5,7,4′-trihydroxyisoflavone | isoflavone |
| Kaempferol | 3,5,7,4′-tetrahydroxyflavone | flavonol |
| Kaempferide | 3,5,7-trihydroxy-4′-methoxyflavone | flavonol |
| Fisetin | 3,7,3′,4′-tetrahydroxyflavone | flavonol |
| Quercetin | 3,5,7,3′,4′-pentahydroxyflavone | flavonol |
| Myricetin | 3,5,7,3′,4′,5′-hexahydroxyflavone | flavonol |
| Morin | 3,5,7,2′,4′-pentahydroxyflavone | flavonol |
| Rutin | 5,7,3′,4′-tetrahydroxyflavone-3-glucorhamnoside | flavonol rhamnoside |
| | 5,2′-dimethoxyflavone | flavone |

final substrate concentration of 50 mM (150 μmol/flask). The reaction was started by adding substrate to the reaction buffer; KOH and a paper wick were present in an adjacent well of the reaction vessel to trap the $CO_2$ produced by the reaction. Thus, the volume compensations needed to maintain constant pressure were equal to the volume of $O_2$ consumed by the reaction.

NADH oxidase incubation conditions were identical to succinoxidase except for the following modifications. Ethylenediamine tetraacetic acid (EDTA) (27 micromolar) in $H_2O$ and 1.2 mg coenzyme $Q_{10}$ (Sigma) in EtOH were added. The flavonoids were also added in EtOH, and the total EtOH concentration was kept at 5% in each assay flask. NADH solutions were freshly prepared for each assay and were added in 0.2 ml in 33 mM Tris buffer to a final NADH concentration of 5 mM (15 μmol/flask). This concentration of NADH was sufficient to ensure that the reaction was enzyme-limited (linear) for at least 20 min.

For both succinoxidase and NADH oxidase activity assays, the reaction buffer was prepared by mixing in the following order: Tris buffer, sucrose, asolection, flavonoid/EtOH, mitochondria, cytochrome C, EDTA (for NADH-oxidase only), and $H_2O$ to make the reaction volume 2.8 ml. Flavonoid and mitochondria were preincubated for 15 min at 30° before the reaction was initiated by adding substrate. After addition of substrate to the reaction buffer, the volume of oxygen consumed was recorded at 5 min intervals. Only assays showing a linear rate of $O_2$ consumption for a minimum of 10 min were considered valid; nonlinear assays were rejected and repeated. The reactions were routinely run for 15-20 min.

ATPase assays were performed according to the method of Gear (24). The reaction mixture contained 80 mM NaCl, 5 mM Tris buffer (pH 7.4), 10 mM succinate, 5 mM $MgCl_2$, 5 mM ATP (Sigma, equine muscle), and 0.35-0.5 mg mitochondrial protein. Controls for background phosphate levels contained boiled mitochondrial protein. The flavonoid inhibitors were added in EtOH; the EtOH concentration was kept constant at 3.3%. Oligomycin controls were used to estimate non-mitochondrial ATPase activity. The reaction was started by addition of ATP and was stopped by precipitating with 200 μl of 0° 2.5 *N* perchloric acid, followed by neutralization with an equal amount of KOH. The final volume was 1.9 ml. Inorganic phosphate was assayed by the turbidimetric assay of Eibel and Lands (25).

For flavonoids that produced concentration-dependent inhibition of NADH oxidase, succinoxidase,

or ATPase activity, the $I_{50}$ (concentration that produced 50% inhibition of activity) was estimated by interpolation of the concentration-response curve.

## RESULTS AND DISCUSSION

The flavonoids were first screened for their ability to inhibit mitochondrial NADH oxidase and succinoxidase at $3.5\times10^{-4}$ M (1 μmol/flask, 1.2 to 1.8 μmol/mg mitochondrial protein) (Table 2). In general, NADH oxidase was more sensitive than succinoxidase to these compounds at this concentration. The methyl ether derivatives kaempferide and acacetin were less effective inhibitors of NADH oxidase than their respective parent compounds, kaempferol and apigenin. Similarly, the glycone rutin was

TABLE 2. Effect of the Flavonoids on NADH Oxidase, Succinoxidase, and ATPase Activities

| Flavonoid[a] | NADH Oxidase | Succinoxidase | ATPase |
|---|---|---|---|
| | Relative Activity (%) | Relative Activity (%) | Relative Activity (%) |
| Flavanone | 6.5±3.0[b] | 84.8± 9.1[e] | 73.4± 9.4[g] |
| Flavone | 14.5±4.5[b] | 82.0± 2.7[e] | 67.4± 6.8[g] |
| 5-Hydroxyflavone | 14.6±4.8[b] | 95.7± 4.3[e] | 98.9± 7.7[g] |
| 7-Hydroxyflavone | 54.2±8.1[b] | 91.3±13.4[e] | 95.1± 7.7[h] |
| Chrysin | 11.2±1.9[b] | 75.8± 0.03[e] | 77.7±11.2[g] |
| Galangin | 48.6[c] | 92.4±11.2[f] | 99.9± 6.3[h] |
| Naringenin | 83.9±4.2[b] | 84.8± 9.1[f] | 43.6± 2.1[h] |
| Apigenin | 12.9±1.6[b] | 93.8± 2.7[e] | 84.6± 9.0[g] |
| Acacetin | 88.6±4.2[b] | 99.3± 7.6[e] | 98.7± 9.5[h] |
| Genistein | 6.6±3.4[d] | 98.3± 7.9[f] | 69.2± 8.8[h] |
| Kaempferol | 59.3±7.9[b] | 84.4± 9.0[f] | 94.2± 3.8[g] |
| Kaempferide | 70.6±3.2[b] | 98.7± 8.6[f] | 96.1±13.9[h] |
| Fisetin | 8.3±3.1[b] | 9.6± 4.7[f] | 17.2± 7.4[g] |
| Quercetin | 6.4±3.1[b] | 21.8±14.1[f] | 7.1± 3.3[g] |
| Myricetin | 12.0±1.0[d] | 14.7± 3.3[e] | 8.4± 0.7[g] |
| Morin | 18.0±3.6[d] | 31.5± 1.4[e] | 13.3± 2.8[g] |
| Rutin | 49.1±4.2[d] | 66.7± 3.4[e] | 66.5± 6.8[h] |
| 2',5-Dimethoxyflavone | 86.9±3.9[d] | 80.8± 2.4[e] | 77.3± 9.6[h] |

Relative activity is the enzyme specific activity in the presence of flavonoid divided by the average specific activity of uninhibited controls × 100%. Control rates for individual batches of mitochondria are given in the footnotes below. All values represent that mean ± standard deviation of 4-12 replicates except where noted.

[a]For NADH oxidase and succinoxidase, the flavonoid concentration was $3.5\times10^{-4}$M, which was 1.2 to 1.8 moles/mg mitochondrial protein, depending on the protein content of the mitochondrial preparation. For ATPase the final concentration in the reaction was $4.2\times10^{-4}$M.

[b]The uninhibited NADH oxidase control was 0.634±0.061 atoms $O_2$ consumed/min/mg protein.

[c]Average of only 2 replicates; limited availability of flavonoid precluded additional point assays; the uninhibited control was as in footnote b.

[d]The average uninhibited NADH oxidase control was 0.841±0.046 atoms $O_2$ consumed/min/mg protein.

[e]The average uninhibited succinoxidase control was 0.600±0.051 atoms $O_2$ consumed/min/mg protein.

[f]The average uninhibited succinoxidase control was 0.348±0.033 atoms $O_2$ consumed/min/mg protein.

[g]The average uninhibited ATPase control was 311.0±37.6 nmoles phosphate liberated/min/mg protein.

[h]The average uninhibited ATPase control was 190.8±31.9 nmoles phosphate liberated/min/mg protein.

less inhibitory than its aglycone, quercetin. These observations indicate that substitution of the hydroxyl groups reduced the activity of the flavones and that the 4′-hydroxyl substitution is important for activity. The flavanone naringenin was less inhibitory against NADH oxidase than its corresponding flavone apigenin, suggesting that 2,3-unsaturation is important for activity. Any compound that did not inhibit NADH oxidase by at least 50% of control at this initial high screening concentration ($3.5\times10^{-4}$ M; close to the limit of solubility for many of these compounds) was considered less active and was not tested further. By this criterion, acacetin, 2′5-dimethoxyflavone, naringenin, kaempferol, and kaempferide were not further tested.

The active flavonoids were tested over a wide range of concentrations to determine their $I_{50}$'s for inhibition of NADH oxidase. The unhydroxylated compounds flavanone and flavone were inhibitors of NADH oxidase producing nearly complete inhibition at high concentrations with estimated $I_{50}$'s of 310 and 240 nmol/mg protein, respectively (Table 3). The higher $I_{50}$ value for flavanone as compared to flavone suggests that the 2,3-double bond is important for flavone inhibition of NADH oxidase. This conclusion is supported by the observations that apigenin has a lower estimated $I_{50}$ value than the corresponding flavanone naringenin with $I_{50}$ values of 920 and >1500, respectively (Table 3). 5-Hydroxyflavone was less potent than flavone ($I_{50}$ of 425 nmol/mg), and it did not completely inhibit NADH oxidase at the highest dose tested. Chrysin (5,7-dihydroxyflavone) was more potent with an $I_{50}$ of 250. This suggests that increasing hydroxylation of the *a* ring increases potency for inhibition of NADH oxidase. The effect of position of the *b* ring on NADH oxidase inhibition is illustrated by comparing apigenin and genistein (Table 3). Genistein (an isoflavone analog of apigenin) with an $I_{50}$ of 365 nmol/mg, was far more potent than its corresponding flavone apigenin ($I_{50}$=920), suggesting that isoflavones may in general be more potent inhibitors than their corresponding flavones. This is not surprising since the potent inhibitor of NADH oxidase, rotenone, is an isoflavone (26).

Flavones more highly substituted on the *b* ring were very potent inhibitors of

TABLE 3. Comparison of the Inhibitory Potency of the Flavonoids ($I_{50}$'s) Against NADH Oxidase, Succinoxidase, and ATPase

| Compound | $I_{50}$[a] | | |
|---|---|---|---|
| | NADH Oxidase (nMoles/mg prot) | Succinoxidase (nMoles/mg prot) | ATPase (nMoles/mg prot) |
| Flavanone | 310± 60 | | |
| Flavone | 240± 75 | | |
| 5-Hydroxyflavone | 425±130 | | |
| 7-Hydroxyflavone | 1400 (54%) | | |
| Chrysin | 250± 50 | | |
| Galangin | 1670[b] | | |
| Naringenin | | | 1800±800 |
| Apigenin | 920± 75 | | |
| Genistein | 365± 50 | | |
| Fisetin | 15± 10[b] | 45± 15[b] | 480±130 |
| Quercetin | 145± 7[b] | 715±100[b] | 205± 85 |
| Myricetin | 35± 30[b] | 45± 25[b] | 370± 90 |
| Morin | 430± 70[b] | 730±410[b] | 120± 30 |
| Rutin | 1886 | | |

[a]Concentration that inhibited enzyme activity by 50%, obtained by interpolation of concentration-inhibition curves.

[b]Previously published (18-20).

NADH oxidase (Table 3). Fisetin and quercetin are dihydroxy substituted with a catechol-like *b* ring. As reported previously (18-22), fisetin had an $I_{50}$ of 15 nmol/mg, and it completely inhibited NADH oxidase at 100 nmol/mg. Quercetin (Table 3) with an additional hydroxyl group at position 5 of the *a* ring was less potent than fisetin [$I_{50}$=145 (18-20)]. Further comparison of the $I_{50}$ values of quercetin (145 nmol/mg) and fisetin (15 mmol/mg) to their corresponding flavanones taxifolin (1036 nmol/mg) and fustin (671 nmol/mg), verifies the importance of the 2,3-double bond in inhibiting NADH oxidase (18-20). Morin (Table 3), with a 2′,4′ substitution pattern on the *b* ring instead of a catechol arrangement, was less potent than either fisetin or quercetin; its $I_{50}$ was 430 nmol/ mg mitochondrial protein (18,20), and it did not completely inhibit NADH oxidase at the highest dose tested (1800 nmol/mg protein). Thus, a catechol structure on the *b* ring contributed to the potency of these flavonoids as inhibitors of NADH oxidase.

Additional hydroxylation of the *b* ring to a pyrogallol structure as in myricetin further increased the potency for inhibition of NADH oxidase. Myricetin's $I_{50}$ was 35 nmol/mg protein (Table 3). Interestingly, myricetin solutions changed from yellow to red in the presence of mitochondrial protein; this was further investigated and was shown to be due to autoxidation, as we recently reported (18-20).

Succinoxidase was less sensitive to inhibition by the preliminary test screening of the flavonoids ($3.5\times10^{-4}$ M) than was NADH oxidase (Table 2). By the criterion of 50% inhibition, only fisetin, quercetin, morin, and myricetin were active inhibitors; chrysin and rutin inhibited only to 65-75% of control at the concentration employed.

The four compounds that actively inhibited succinoxidase at the screening concentration were tested over a range of concentrations to determine their $I_{50}$'s (Table 3) and to compare their inhibition curves against those obtained for NADH oxidase. Fisetin, quercetin, and morin inhibition curves for succinoxidase were not identical to those for NADH oxidase. Fisetin was almost as potent an inhibitor of succinoxidase [$I_{50}$=45 (18-20)] as of NADH oxidase ($I_{50}$=15). However, quercetin and morin were much more potent inhibitors of NADH oxidase with $I_{50}$'s for NADH oxidase of 145 and 430, respectively, and $I_{50}$'s for succinoxidase of 715 and 730 (18), respectively. These findings indicate that their primary site of action in the respiratory chain was in Complex 1 (NADH-coenzyme Q reductase). Myricetin's potency for inhibiting succinoxidase [$I_{50}$=45 (18-20)] was about equal to its potency for inhibiting NADH oxidase ($I_{50}$=35) suggesting that it inhibits the respiratiory chain in a portion common to both succinoxidase and NADH oxidase.

Because quercetin was reported to inhibit mitochondrial ATPase activity, (27) we decided to evaluate other flavonoids for their ATPase inhibition. The ability of the flavonoids to inhibit uncoupled, nonpurified, mitochondrial ATPase was then screened at a single high concentration of 1 μg/flask ($4.2\times10^{-4}$ M) (Table 2). Oligomycin abolished ATPase activity under the same experimental conditions, verifying that the observed activity was the oligomycin-sensitive mitochondrial ATPase. By the criterion of 50% inhibition, fisetin, quercetin, morin, myricetin, and naringenin were active inhibitors of ATPase. These findings are in reasonable agreement with those of Suolinna *et al.* (10).

These active compounds were tested over a wide range of concentrations to estimate their $I_{50}$'s and to compare their inhibition curves with those for NADH oxidase and succinoxidase inhibition (Table 3). In general, compounds with more than one hydroxyl group on the *b* ring, which inhibited both NADH oxidase and succinoxidase, also inhibited ATPase. However, the relative potency of these compounds was different for the three enzyme systems. For example, fisetin and myricetin were much less potent inhibitors of ATPase than of succinoxidase or NADH oxidase. In contrast, morin was a

more potent inhibitor of ATPase than of the other enzymes, and quercetin was nearly as potent an ATPase inhibitor as a NADH oxidase inhibitor. It is apparent from these differences in the rank order of potency that the flavonoids tested in this study show selectivity in their ability to inhibit NADH oxidase, succinoxidase, and ATPase.

Naringenin, which did not meet the criterion for activity in the NADH oxidase or succinoxidase assays, was the least potent of the active compounds in the ATPase assay. Its inhibition curve flattened at higher concentrations, and it did not completely inhibit ATPase at the highest concentration tested.

The three most potent inhibitors of NADH-oxidase were fisetin, myricetin, and quercetin, and the two most potent inhibitors of succinoxidase activity were fisetin and myricetin. All of these inhibitors contained a catechol or pyrogallol structure, a 2,3-double bond, a 3-hydroxyl group, and a 4-keto group. These structural features were reported by MacGregor and Jurd (28) to be required for flavonoid, mutagenic activity towards *Salmonella typhimurium* and by Varma and Kinoshita (29) for inhibition of lens aldol reductase.

These findings corroborate and extend our previous conclusion that a catechol or a pyrogallal moiety in the *b* ring of flavonoids is important for inhibition of mitochondrial electron transport systems (18-20). Furthermore, these findings are consistent with previous reports demonstrating that those model phenolic compounds capable of undergoing oxidation-reduction reactions (*p*-hydroquinone and catechol) were the most potent inhibitors of mitochondrial succinoxidase activity (30,31). Consistent with these observations is the proposal by MacGregor (32) that genotoxic flavonoids may act through quinone intermediates, which are generated in vivo through oxidation-reduction reactions.

Based on these observations, it is tempting to speculate that flavonoids that possess redox active centers like a catechol or pyrogallol inhibit the respiratory chain through a quinone intermediate. Conversely, redox reactivity does not appear to be important for inhibition of the oligomycin-sensitive ATPase system, because morin, which contains a *m*-dihydroxy configuration on the *b* ring, was the most potent inhibitor tested.

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