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INTERFERENCE BY FLAVONE AND FLAVONOLS WITH CHLOROPLAST-MEDIATED ELECTRON TRANSPORT AND PHOSPHORYLATION

DONALD E. MORELAND and WILLIAM P. NOVITZKY

U. S. Department of Agriculture, Agricultural Research Service, Departments of Crop Science and Botany, North Carolina State University, Raleigh, NC 27695-7620, U S A

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Key Word Index—*Spinacia oleracea*, Chenopodiaceae, spinach, chloroplasts, thylakoids, electron transport, phosphorylation, uncoupler, energy transfer inhibition, Q_B-protein, flavone, flavonols

Abstract—The effects of flavone and seven flavonols on the light-induced electron transport and phosphorylation of isolated spinach (*Spinacia oleracea* L) chloroplasts were investigated With the exception of flavonol (3-hydroxyflavone), all of the compounds interacted with components of both the ATP-generating and electron transport pathways. Flavonol only interacted with the phosphorylation pathway. Interference with the phosphorylation pathway was evidenced by the greater sensitivity of the phosphorylation reaction than coupled whole-chain electron transport, inhibition of cyclic phosphorylation, inhibition of the light-activated Mg^{2+}-ATPase, and inhibition of the heat-activated Ca^{2+}-ATPase associated with CF_1 The overall decreasing order of effectiveness for inhibition of cyclic phosphorylation was galangin > quercetin = kaempferol = myricetin = flavonol > fisetin > flavone > morin. On the electron transport pathway, all of the compounds, except flavonol, interacted with the Q_B-protein complex as evidenced by inhibition of uncoupled electron transport, alteration of chlorophyll fluorescence transients, and competitive displacement of previously bound radiolabeled atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. The decreasing order of effectiveness for inhibition of uncoupled electron transport was: fisetin > quercetin = galangin > kaempferol > flavone > morin.

INTRODUCTION

Flavonoids are commonly found in, and widely distributed among, families of higher plants The physiological and biological activity of flavonoids and their synthetic analogues in general, and of quercetin in particular, have been studied both extensively and intensively in mammalian systems. The wide variety of responses elicited either directly or indirectly include: inhibition of tumour growth and carcinogenesis; anti-inflammatory and anti-allergenic activity, induction of mutagenicity; induction of cytochrome P-450 monooxygenases, inhibition of specific cytochrome P-450 isozymes; activity as an antioxidant, chelator, and free-radical scavenger; inhibition of glycolysis, inhibition of fatty acid mobilization and metabolism; inhibition of membrane transport systems including Ca^{2+}/Mg^{2+}-ATPase and Na^+/K^+-ATPase; inhibition of histamine release; inhibition of protein kinases and aldose reductase; and inhibition of mitochondrial electron transport and ATP generation [1, 2]

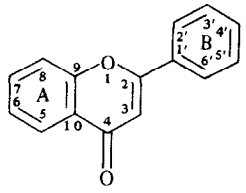
The action of flavonoids on plant metabolism has not been studied as extensively as on animal metabolism [3]. However, the compounds do constitute an important class of allelopathic agents. Inhibition of photosynthesis by flavonoids in intact plants and microorganisms has been observed as well as inhibition of CO_2-dependent oxygen evolution and photophosphorylation of intact isolated chloroplasts by kaempferol and fisetin [4]. There is, however, limited information at the molecular level relative to the mechanism(s) through which flavonoids interfere with photosynthesis Quercetin and kaempferol have been shown to inhibit coupled electron transport and phosphorylation of isolated thylakoids, but to have only a marginal effect on uncoupled or basal electron flow [5, 6]. Consequently, the compounds were postulated to act as energy transfer inhibitors, but the action was considered to be different from that of reference standards [5, 6]. Luteolin (a flavone), quercetin (a flavonol), and taxifolin (a flavanonol) acted as energy transfer inhibitors against isolated thylakoids at sub-molar concentrations. At higher concentrations, luteolin and quercetin, but not taxifolin, also interfered with the electron transport chain at or near the Q_B-protein complex (the secondary quinone electron acceptor of photosystem II) [7].

The objectives of the study reported herein were to: (i) compare the effects of flavone and a series of flavonols on photoinduced electron transport and phosphorylation in isolated thylakoids and (ii) identify sites of action of the compounds The chemistry and general structure of the flavonoids examined are presented in Table 1. The compounds are characterized by having two aromatic rings (A and B) linked by a pyrone ring Flavone and the flavonols are considered to assume a planar conformation because of the double bond between C-2 and C-3 [8] The flavonols, in contrast to flavones, are hydroxylated at the C-3 position. The effects of quercetin on

*Address for correspondence USDA/ARS, N C State University, Crop Science Department, Box 7620, Raleigh, NC 27695-7620, U S A

Table 1 Structure of flavonoids studied

General formula



Compound	Hydroxylation at ring position(s)						
	3	5	7	2	3′	4′	5′
Flavone							
Flavonol	-						
Fisetin	-					-	
Galangin		-	-				
Kaempferol	-	-					
Morin	-	-		-			
Quercetin	-	-					
Myricetin			-				-

some of the reactions examined in the present study were reported previously [7], but they were re-evaluated here for comparative purposes

RESULTS

Whole chain electron transport and phosphorylation

As shown by the dose-response curves (Fig 1), flavone inhibited non-cyclic coupled electron transport and photophosphorylation, cyclic photophosphorylation, and uncoupled electron transport mediated by spinach thylakoids. I_{50} values obtained from dose-response curves for flavone and all of the flavonoids are presented in Table 2 In general, non-cyclic coupled phosphorylation was the most sensitive reaction, i.e had the lowest I_{50} value, whereas uncoupled electron transport was the least sensitive, i e had the highest I_{50} value Inhibition of coupled electron transport can result from an effect on the phosphorylation pathway (energy transfer inhibition), which acts to impede electron flow, or can be a direct result of an action on a component of the electron transport chain (electron transport inhibition)

The two effects can be differentiated by the addition of an uncoupler of phosphorylation to dissipate the energized state of the membrane [9] If the inhibition of electron transport coupled to ATP synthesis is caused by interference with a component of the energy transfer pathway, the inhibition will be relieved by the addition of an uncoupler However, if electron transport inhibition is the cause, the presence of an uncoupler will not relieve the inhibition. As shown in Fig 1, uncoupled electron transport is inhibited, but the reaction is somewhat less sensitive than the coupled reaction. Effects imposed on an uncoupled system provide a direct measure of interference with electron transport that is free from interactions with the energy transport system Similarly, effects imposed on cyclic photophosphorylation provide a direct measure of interference with the energy transfer pathway free from interactions imposed on at least the PS (photosystem) II segment of the electron transport pathway, if the compounds do not affect electron transport associated with PS I Flavone inhibited cyclic phosphorylation at a slightly higher I_{50} concentration than was obtained for whole chain phosphorylation (Fig 1 and Table 2) Hence, flavone had two effects (i) the stronger effect involved an interaction with a component of the ATP synthesizing system (energy transfer inhibition) and (ii) the weaker effect was expressed on the electron transport chain associated with PS II (electron transport inhibition)

Flavonol which is formed by the addition on the flavone nucleus of a hydroxyl group at C-3, inhibited only the phosphorylation reactions and did not inhibit electron transport (Table 2) Only flavonol affected electron transport associated with PS I as measured by the reduced DPIP (2,6-dichlorophenolindophenol)/MV (methyl viologen) assay (data not shown) Flavonol stimulated the basal rate of electron transport by this system The rate was doubled at 115 μM, which confirmed its uncoupling action Hydroxylation of the A and B rings restored inhibition of electron transport and decreased or eliminated uncoupling activity (Table 2) Effects of myricetin on electron transport could not be measured because of a chemical interaction with constituents in the reaction mixture that caused rapid consumption of oxygen in the absence of thylakoids (possibly spontaneous oxidation)

Interference with energy transfer

Because the flavonoids did not inhibit electron transport in the PS I region, inhibition of cyclic phosphorylation reflects their interaction with the ATP synthesizing machinery All of the test compounds inhibited cyclic phosphorylation at molar concentrations equal to, or slightly higher than, those that caused inhibition of non-cyclic phosphorylation (Table 2, cf Columns 4 and 2) Consequently, effects on reactions directly associated with the coupling factor (CF_0 CF_1) complex were expressed The thylakoid coupling factor is a latent ATPase ATP hydrolysis can be induced by light-activation in the presence of sulphydryl reagents such as DTT (dithiothreitol) [10] The light-activated Mg^{2+}-ATPase is dependent on an intact CF_0–CF_1 complex Consequently, it is inhibited by both CF_0 and CF_1 inhibitors [7] All of the flavonoids inhibited the light-activated Mg^{2+}-ATPase of spinach thylakoids (Table 3)

CF_0 inhibitors interact with the hydrophobic part of the complex and block the proton channel through the membrane, and include DCCD (dicyclohexylcarbodiimide) and chlorotriorganotins. CF_1 inhibitors are considered to interact directly with the catalytic, hydrophilic segment of the complex and include Nbf-Cl (4-chloro-7-nitrobenzofuran) and phlorizin

The flavonoids inhibited the activity of the isolated CF_1, measured as Ca^{2+}-ATPase, although flavonol was only slightly inhibitory (Table 3) The Ca^{2+}-ATPase was affected by CF_1, but not by CF_0 inhibitors [7] Hence, the flavonoids interact with and inhibit the activity of the CF_1 portion of the coupling factor complex of thylakoid membranes much like Nbf-Cl and phlorizin as was shown previously for luteolin and taxifolin [7]

The flavonoids differed somewhat in their relative order of inhibitory effectiveness in the three assays with intact CF_0–CF_1 complexes that provided a measure of their activity against photophosphorylation, i e coupled

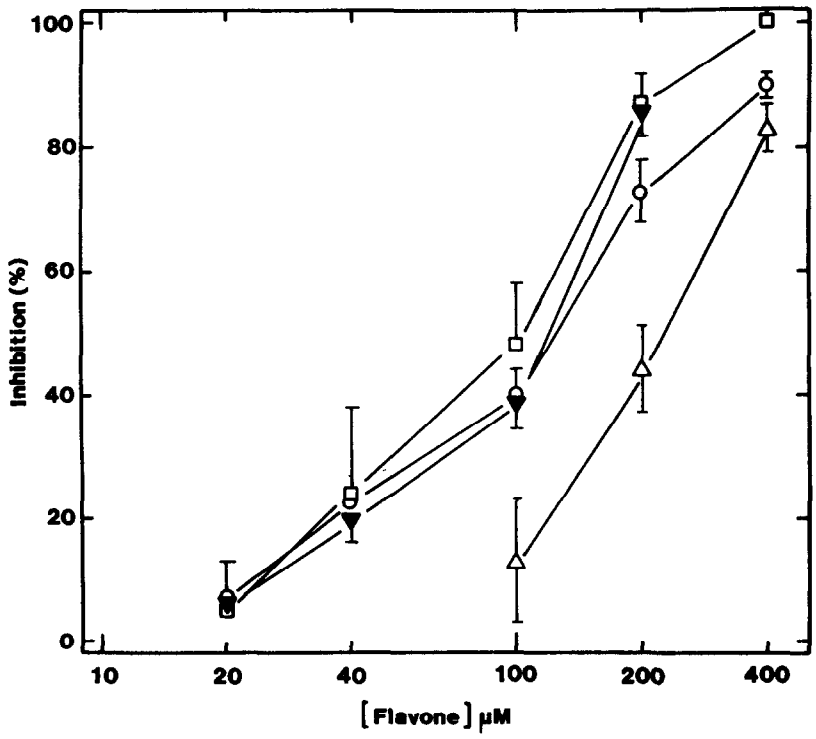


Fig 1. Dose-response curves plotted semilogarithmically obtained with flavone for inhibition of photoinduced electron transport and phosphorylation in isolated spinach thylakoids □——□, coupled phosphorylation and ○——○, electron transport, ▼——▼, cyclic phosphorylation; and △——△, uncoupled electron transport. Data are presented as means ±s.d. (shown by error bars) for three isolations of thylakoids. Averaged specific activities of the control reactions are listed in Table 2 Assay conditions are detailed in the Experimental section

Table 2 Inhibition by flavone and flavonols of non-cyclic coupled electron transport and phosphorylation, cyclic phosphorylation, and uncoupled electron transport mediated by spinach thylakoids

	Assay*			
	Non-cyclic coupled			
Compound	Phosphorylation	Oxygen uptake	Cyclic phosphorylation	Uncoupled oxygen uptake
	I_{50} (μM)			
Flavone	99±28	122±12	118±8	220±20
Flavonol	27±2	NE	64±5	NE
Fisetin	79±8	86±10	92±14	70±11
Galangin	7±2	11±3	14±1	123±12
Kaempferol	34±10	58±12	57±6	203±15
Morin	178±34	242±18	227±21	613±81
Quercetin	63±3	79±4	62±12	121±29
Myricetin	81±19	ND†	67±6	ND†

*H_2O served as the electron donor and MV as the electron acceptor. O_2 consumption was measured polarographically and phosphorylation was measured potentiometrically Data are presented as I_{50} values ±s.d. obtained with three isolations of thylakoids. Average specific activities of the controls were: 136±9 μmol O_2 consumed and 389±22 μmol Pi esterified/mg Chl/hr for the coupled reactions, 522±3 μmol Pi esterified/mg Chl/hr for cyclic phosphorylation, and 173±19 μmol O_2 consumed/mg Chl/hr for the uncoupled reaction Assay conditions are detailed in the Experimental section.

NE = no effect. ND = not determined.

†Myricetin reacted chemically with constituents of the reaction medium.

Table 3 Effects of flavone and flavonols on ATPase activities associated with spinach thylakoids

Flavonoid	Assay*	
	Light-induced Mg^{2+}-ATPase	Ca^{2+}-ATPase
	I_{50} (μM)	
Flavone	173±8	450±30
Flavonol	35±4	>1000
Fisetin	120±33	58±2
Galangin	10±1	144±14
Kaempferol	83±14	65±5
Morin	605±60	323±21
Quercetin	18±1	35±2
Myricetin	38±4	30±2

*Phosphate liberated by the hydrolysis of ATP was measured colorimetrically Data are presented as averaged I_{50} values ± s d obtained from three replications The average specific activities were 38±8 μmol Pi liberated/mg Chl/hr and 369±19 μmol Pi liberated/mg protein/hr for the Mg^{2+}- and Ca^{2+}-ATPase, respectively

non-cyclic phosphorylation, cyclic phosphorylation, and the light-induced ATPase (Tables 2 and 3) In all three assays, galangin was most active and morin was least active, with flavone being the second least active compound. The order of inhibitory effectiveness for the remainder of the flavonoids varied among the three assays between the two extremes The order also differed for activity against isolated CF_1 as reflected by inhibition of the Ca^{2+}-ATPase (Table 3) Myricetin was the most active compound followed by quercetin Morin and flavone were the least active of the inhibitory compounds and flavonol was essentially inactive

Electron-transport interference

When the influence of the ATP-generating pathway was removed by an uncoupler, all the flavonoids, except for flavonol, which had no effect, and myricetin, which interfered with the assay, inhibited electron transport from water to MV (Table 2, last column) The I_{50} concentrations for the inhibition of uncoupled electron transport were two to three times greater than for the coupled assay, except for fisetin

As stated previously, the flavonoids, with the exception of flavonol, had no measureable effect on electron flow mediated by reduced DPIP and MV (data not shown) Hence, the flavonoids that were tested apparently do not inhibit electron transport beyond the site of PQ (plastoquinone) oxidation and cytochrome *f*

Chlorophyll fluorescence

A small percentage of the excitation energy absorbed by the chlorophylls associated with PS II is emitted as fluorescence which has characteristic transients [11] Alterations induced in the transients have been used to characterize effects imposed by various treatments on PS II-associated reactions. A typical chlorophyll fluorescence induction transient for isolated spinach chloroplasts is shown in Fig 2 (control curve) Dark-adapted chloroplasts possess a very low level of fluorescence which was set as 0 on the ordinate. Upon illumination, there is an immediate increase to a low initial level of fluorescence yield (F_o) that is followed by a rapid increase to an intermediate level (F_I) The fluorescence then increases curvilinearly over a short interval (3 5 sec) with the achievement of maximum fluorescence intensity (F_M) The (F_O to F_I rise is considered to be caused by the rapid reduction of some of the primary electron acceptors (Q_A) associated with the PS II reaction centres, whereas the slower F_I to F_M rise corresponds to the reduction of secondary PS II electron acceptors by the secondary electron acceptors (Q_B)

Inhibitors of PS II, such as atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine] (Fig 2, top curve), block the reduction of Q_B by Q_A which results in an increase in the intensity of F_I Complete inhibition of electron flow through Q_B results in F_I being equal to F_M. As shown, fisetin caused the level of F_I to increase in a concentration-dependent manner, much like atrazine The curves presented in Fig 2 have been normalized with respect to F_M, to correct for fluorescence quenching caused by fisetin Absorbance of the excitation light by fisetin not only decreased the fluorescence intensity, but also slowed the rise time to F_M by reducing the photon flux available for photochemistry A representative dose-response curve for inhibition of fluorescence by fisetin is shown in the inset of Fig 2 (I_{50}=76 μM). All of the flavonoids except flavonol altered the chlorophyll fluorescence transients much like fisetin. However, some of the compounds caused drastic quenching of fluorescence Correcting for drastic quenching did not always provide meaningful numbers Consequently, no attempt was made to determine I_{50} values for all of the flavonoids. The results suggest that except for flavonol, the compounds inhibit electron transport by interfering with the function of the Q_B complex.

Competitive binding

Binding studies have been used to characterize interactions of inhibitors with the PS II complex [12–15] Atrazine, and many other herbicides, have been shown to reversibly bind, with high affinity, to the Q_B binding site associated with the PS II reaction centre complex. Binding of atrazine to the site results in inhibition of electron transport by preventing the reduction of PQ. If the flavonoids inhibit at the Q_B site of PS II, then they should competitively displace a radiolabelled herbicide such as [^{14}C]-atrazine from the thylakoid membrane In this study, atrazine was added at a concentration of 0 1 μM and the chlorophyll concentration was increased 2.5 times above that used in the electron transport assay, in order to obtain reasonably high levels of radiolabelling Under these conditions, 0 1 μM atrazine inhibited the reaction by *ca* 45% All of the flavonoids, with the exception of flavonol, displaced radiolabelled atrazine from the thylakoid membranes in a concentration-dependent manner (Fig 3) The relative order of effectiveness was galangin > quercetin > fisetin = kaempferol = morin = flavone > myricetin, with only marginal displacement by flavonol

The order of displacement effectiveness was somewhat different from the order reflected in inhibition of uncoupled oxygen uptake (Table 2, last column). In the latter assay, galangin was the most inhibitory compound,

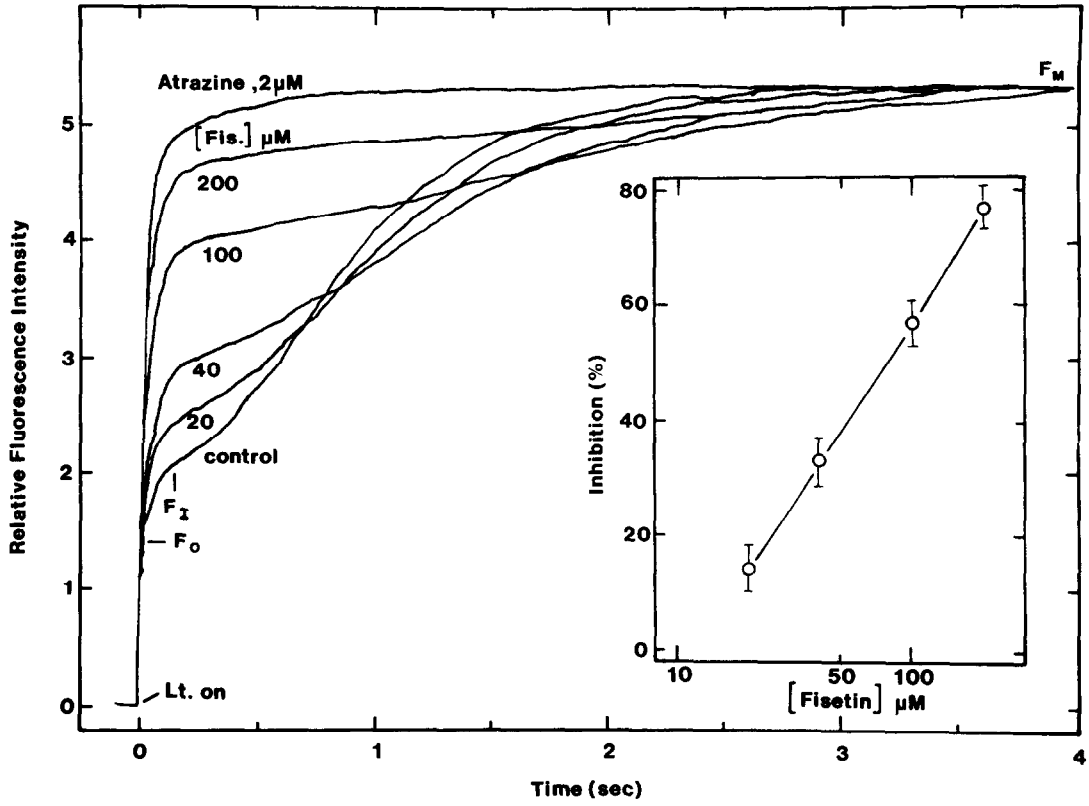


Fig 2. Effects of fisetin on the chlorophyll fluorescence induction transients of isolated spinach thylakoids. The representative traces depict the concentration-dependent alterations induced by fisetin (Fis.) compared to the photosystem II inhibitor atrazine (2.0 μM) F_O, F_I, and F_M = initial, intermediate, and maximum levels of fluorescence intensity, respectively Conditions under which the experiments were conducted are detailed in the Experimental section. Inset. Dose-response curve plotted semilogarithmically for inhibition of the variable fluorescence component ($F_M - F_I$) by fisetin. Data are presented as means ± s d (shown by error bars) for three isolations of thylakoids

whereas fisetin was most effective in the displacement study. Quercetin was the second most active compound in both studies. The reasons for the differential responses are not obvious at this time.

Considerable differences in order of effectiveness among the flavonoids was evident in the three assays that reflect interference with electron transport, i.e. coupled whole chain electron transport, uncoupled electron transport, and the displacement of atrazine (Table 2, Fig. 3). Similarly to inhibition of CF_0–CF_1 dependent phosphorylation, galangin was the most inhibitory compound of non-cyclic electron transport and in displacing bound atrazine. Galangin together with quercetin followed fisetin in order of inhibitory activity against uncoupled electron transport (Table 2, last column). Morin, again showed a low order of inhibitory effectiveness. Flavonol essentially had no effect. The reasons for the differential responses remain to be identified.

DISCUSSION

The data obtained in this study suggest that with the exception of flavonol (3-hydroxyflavone), flavone and the flavonols interfered with light-induced reactions of isolated spinach thylakoid membranes on both the phosphorylation and the electron transport pathways. Flavonol, however, primarily acted on the phosphorylation pathway and was the only compound that acted as an uncoupler. Interference with the phosphorylation pathway by the flavonoids was evidenced by the greater sensitivity of the phosphorylation reaction than the coupled electron transport reaction in studies that involved whole-chain electron transport (Fig 1, Table 2); inhibition of cyclic phosphorylation (Table 2); inhibition of the light-activated Mg^{2+}-ATPase (Table 3); and inhibition of the heat-activated Ca^{2+}-ATPase (Table 3).

Interference with a component of the electron transport system of thylakoids, with the exception of flavonol and possibly myricetin, was initially measured as inhibition of ammonium chloride-uncoupled electron transport (Fig. 1, Table 2). No suggestion for an interaction of the flavonoids with the PS I portion of the electron transport chain was found. The displacement of [^{14}C]-atrazine from the Q_B-binding site of PS II by all of the flavonoids except flavonol indicated that one possible site of inhibition on the electron transport chain involved the Q_B-complex (Fig 3). However, the somewhat different I_{50} values for displacement of radiolabelled atrazine and uncoupled electron transport may be a reflection of non-specific effects that resulted from partitioning of the flavonoid molecules into the lipid bilayer of the thylakoid membranes. Interference with electron transport occur-

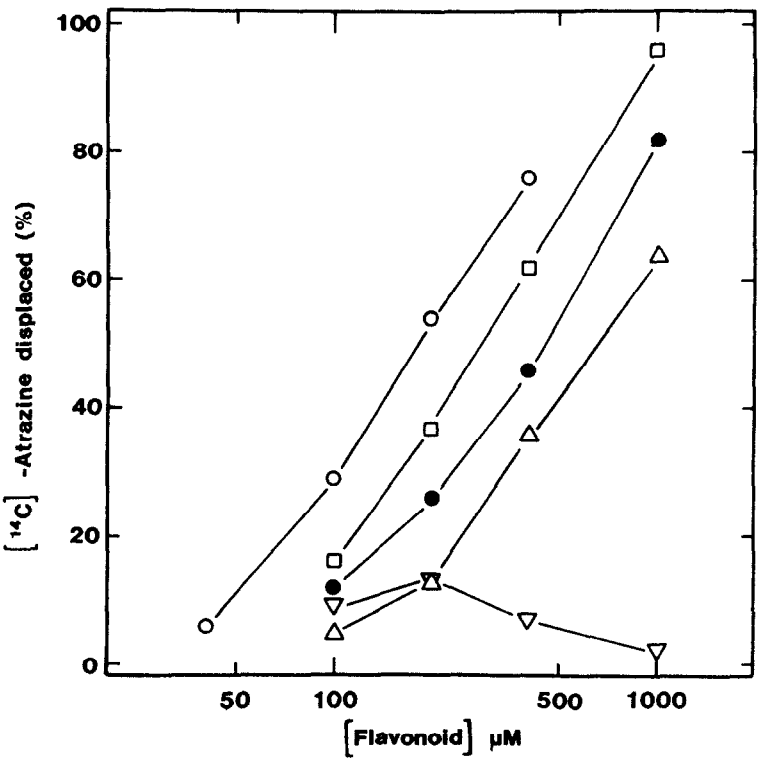


Fig 3. Dose-response curves plotted semilogarithmically that show displacement of previously applied [^{14}C]-atrazine by flavonoids from isolated spinach thylakoids o——o, galangin, □——□, quercetin, ●——●, overlapping data points for flavone, morin, fisetin, and kaempferol, Δ——Δ, myricetin, and ▽ — ▽, flavonol

red at higher flavonoid concentrations than did the effects on phosphorylation and, thus, may be viewed as a secondary effect.

The pattern of hydroxylation of the compounds affected the way the flavonoids interacted with the thylakoids The non-hydroxylated parent compound, flavone, was moderately active in all of the assays. Hydroxylation at C-3 (flavonol) caused loss of electron transport inhibition and the appearance of uncoupling activity. Trihydroxylation at the 3,5,7 positions (galangin) restored electron transport inhibition, maximized inhibition of photophosphorylation and electron transport, and eliminated uncoupling activity. The further hydroxylation of the B-ring tended to reduce inhibitory effectiveness with respect to effects on phosphorylation. Kaempferol, with a 4'-OH was less inhibitory than galangin, quercetin (3',4') even less, and myricitin (3',4',5') even less so. Hydroxylation in the 2' position (morin) caused a large decrease in inhibitory effectiveness as shown by the marked difference between morin and quercetin in all of the assays Fisetin, which is 5-dehydroxyquercetin, was less active against phosphorylation, but was more active against electron transport, than was quercetin

Of the flavonoids included in this study, only the action of quercetin on ATP synthesis in chloroplasts and mitochondria has been investigated in detail [16]. Quercetin has been shown to inhibit different types of ATPases [16]. In studies with highly purified and trypsin-treated CF_1, quercetin was reported to inhibit the activity of Ca^{2+}-ATPase [17] In subsequent studies, the Ca^{2+}-ATPase activity was shown to be associated with the α- and β-subunits of CF_1, and quercetin was found to bind to two sites associated with the subunits [18]. The binding sites of quercetin were considered to be distinct from the 'tight' nucleotide-binding sites or the Nbf-Cl binding site

X-ray diffraction studies of crystallized flavonoids indicate that the compounds form both intramolecular and extramolecular hydrogen bonds Extramolecular hydrogen bonding can result in the formation of cyclic dimers, at least for flavonol [19] and quercetin [2] as well as polymeric chains Specifically, intramolecular bonds are formed between the hydroxyl groups on C-3 or C-5 and the neighbouring exocyclic carbonyl (C-4) oxygen The result is a shortening of the C-4, 10 bond with a strengthening of the double bond character as the C-O bond is lengthened [2]. Intermolecular hydrogen bonds can be formed with water or other acceptors by the hydroxyl group on C-7 and adjacent hydroxyls at C-3' and C-4'. Quercetin exists in a planar conformation with the exocyclic phenyl ring (ring B) and the hydroxyl groups all lying within the molecular plane [2, 19, 20]. Assumption of a planar conformation has been correlated with the ability of flavonoids to penetrate rapidly into the hydrophobic interior of unilamellar vesicles [21].

Many investigators have compared the action of quercetin and several of the flavonoids studied herein in various reactions Included are the extensive structure/activity studies with bioflavonoids as inhibitors of glycolysis that were conducted by Racker [22]. Racker concluded that tetra- and penta-hydroxyflavones with hydroxyl groups at 7, 3', 4' and at either 3 or 5, or both, were most effective inhibitors, whereas a hydroxyl at 5' (myricetin) resulted in weakened activity Trihydroxy compounds were poor inhibitors and dihydroxy or

monohydroxy compounds were essentially inactive. The double bond at C-2, 3 was essential for inhibitory activity, as was the free hydroxyl on C-3 [22]

Other than the observations of Racker, it is difficult to develop general structure/activity correlations from the wide range of responses elicited by the flavonols In some assays, for example inhibition of benzo[α]pyrene hydroxylase activity [23] and inhibition of Δ^{12}-lipoxygenase activity [24], quercetin was the most active and morin was the least active of the flavonols tested. However, in other responses, such as inhibition of deiodinase activity [25] and as antioxidants [26], morin was most active and quercetin was least active

If hydrogen bond formation is important in the expression of inhibition, all of the flavonoids studied herein except flavone could form intramolecular hydrogen bonds through either the C-3 or C-5 hydroxyls, or both. All of the compounds except flavone and flavonol could also form intermolecular hydrogen bonds through the C-7 hydroxyl and the B-ring hydroxyls. Galangin can only form intermolecular hydrogen bonds through the C-7 hydroxyl. For both inhibition of photophosphorylation and electron transport, galangin ranked either first or second as being the most active compound tested The only exception was for inhibition of Ca^{2+}-ATPase where it ranked fifth Apparently, intermolecular bonding that involves both rings was not a requirement for inhibition in the studies reported herein.

Quercetin with the potential to form hydrogen bonds through the hydroxyls at C-3′ and C-4′ was the second or third most active compound in all assays except inhibition of non-cyclic phosphorylation where it ranked fourth. Myricetin, the most highly hydroxylated flavonol, had a relatively low order of activity in some of the assays except for inhibition of Ca^{2+}-ATPase, where it was the most inhibitory compound. However, it was as active as quercetin in inhibiting cyclic phosphorylation and the light-induced ATPase Morin with a C-2′-hydroxyl group was the least active of the flavonoids except for displacement of atrazine Flavone which lacks hydroxyl substituents had a relatively low order of activity in most assays, but was, in general, more inhibitory than morin The responses suggest that the capacity for both intra- and intermolecular hydrogen bonding is not an absolute requirement for inhibition of either photophosphorylation or electron transport

Flavonol only interfered with photophosphorylation and ranked second or third in order of activity against the phosphorylation reactions except for inhibition of the Ca^{2+}-ATPase where it was essentially inactive. The high activity against the light-induced ATPase, which requires an intact coupling factor complex (CF_0–CF_1), and limited activity against isolated CF_1 (Ca^{2+}-ATPase) suggests that flavonol could be a CF_0 inhibitor.

The flavonoids examined in this study, with the possible exception of flavonol, have been isolated from natural sources as aglycones as well as in conjugated forms [27]. Quercetin, kaempferol, and myricetin are widely distributed in higher plants Examples of genera in which the other flavonoids occur include: *Alpinia*, *Pinus* and *Populus*, for galangin; *Acacia*, *Rhus* and *Robinia*, for fisetin; *Morus*, *Artocarpus* and *Toxylon*, for morin, and *Primulus* and *Dionysia*, for flavone [27]

The flavonoids can be expected to interfere with photosynthesis of intact plants and microorganisms if they should partition into chloroplasts in the unconjugated form and achieve concentrations that approach 100 μM. Flavonoids have been reported to be present in leaf epidermal cells and pea plumules at concentrations of 3 to 10 mM [28, 29] Localized concentrations within cells and organelles could, conceivably, be much higher. Flavonoids, including glycosides of quercetin, have been isolated from chloroplast preparations of plants at concentrations considerably higher than the whole leaf extracts [30]. Evidence also has been provided that quercetin as well as its glycosides (quercetin and rutin) can move across the chloroplast envelope and into the stroma [31].

Flavonoids can scavenge superoxide radicals and hydrogen peroxide, and in doing so, protect carotenoids from photooxidation [31, 32] Thus, under conditions where concentrations of ADP and NADP might limit the rate of electron transport, flavonoids could reduce the rates of ATP hydrolysis and electron transport. The flavonoids could also minimize the deleterious effects of photooxidations caused by the reduced state of the thylakoid membrane when electron transport is limiting

The interactions of flavonoids with chloroplasts could, conceivably, result in two different phenomena. As allelochemicals, flavonoids that are released into the environment may inhibit photosynthesis in target organisms. Additionally, via homeostatic control mechanisms, they may be involved in the regulation of energy metabolism within the plant, where they are synthesized, by modulating photosynthetic electron transport and phosphorylation.

EXPERIMENTAL

Thylakoids were isolated from freshly harvested, growth chamber-grown spinach (*Spinacia oleracea* L.) by the method of Armond *et al* [33] Chlorophyll (Chl) concns were estimated according to Arnon [34] Photochemical reactions were conducted at 25° at a photon fluence rate of 750 μmol/m²/sec (PAR). Effects on whole chain electron transport and phosphorylation were measured in a medium (2 0 ml vol.) that contained 0.1 M sorbitol, 10 mM Na-Pi buffer (pH 8.0), 5 mM $MgCl_2$, 10 mM NaCl, 0.05 mM MV, 0 15 mM ADP, and thylakoids (40 μg Chl) Electron flow was monitored with a Clark-type platinum electrode as O_2 consumed during the autooxidation of reduced MV Esterification of ADP was monitored with a pH electrode [35] Uncoupled electron flow was measured in the above medium with 5 mM NH_4Cl added and ADP omitted. Electron transport associated with PS I was measured in the basic medium with 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, diuron) to block PS II and reduced DPIP (50 μM DPIP + 5 mM Na-ascorbate) as electron donor added, and ADP omitted. Cyclic phosphorylation was measured in a medium that consisted of 20 mM KCl, 10 mM K-Pi buffer (pH 7 8), 5 mM $MgCl_2$, 0 05 mM phenazine methosulfate (PMS), 0 67 mM DTT, 1 mM ADP, and thylakoids (40 μg Chl) in a 2.0 ml vol

Effects on the Mg^{2+}-ATPase associated with thylakoids was assayed in a medium that contained 50 mM tricine–NaOH (pH 8 0), 10 mM NaCl, 5 mM $MgCl_2$, 6 mM DTT, 0 02 mM PMS and 20 μg Chl in a 1 0 ml vol. The ATPase was activated by illumination of the thylakoid suspensions with intense white light for 5 min Activity was initiated by the addition of 5 mM ATP and was allowed to proceed in the dark for 15 min at 25°. The reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The Pi liberated was

measured colorimetrically [36] after centrifugation to remove pptd protein. CF_1 was isolated by $CHCl_3$ extraction in the presence of EDTA [37] The latent ATPase was heat activated (60° for 4 min) and assayed for Ca^{2+}-ATPase activity [38]

Chlorophyll fluorescence induction transients were measured as described previously [39] The fluorescence curves were corrected for quenching caused by absorption of the blue excitation beam by the flavonoids Effect of the flavonoids on fluorescence was quantified as inhibition of the variable component of the treatments $(F_M-F_I)_{treatment}$ relative to the variable component of controls $(F_M-F_I)_{control}$

Competitive binding studies with [^{14}C]-atrazine were performed as described previously [39] The Chl concentration in the assay was increased to 50 μg/ml in order to obtain reasonably high radiolabel levels for scintillation counting

Flavonol, fisetin, galangin, and morin were obtained from Aldrich The remaining compounds were supplied by Sigma Stock solutions of the desired concentrations of the flavonoids were prepared in DMSO The final concn of solvent was held constant at 1% (v/v) in all assays including the controls The data presented for the several studies were averaged from determinations made with a minimum of three separate replications and isolations

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