



The Uncoupling Efficiency and Affinity of Flavonoids for Vesicles

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ABSTRACT. The relative hydrophobicity and interaction of flavonoids with artificial membranes using vesicles was studied. At the same degree of hydroxylation, flavones were slightly more hydrophobic than flavanones. Flavonoids possess a hydrophobic character and are weak acids. For this reason, their uncoupling efficiency of the membrane potential was studied using cytochrome *c* oxidase vesicles. With emphasis on naringenin, it was shown that flavonoids affect both the transmembrane potential difference (*V*) and the transmembrane pH difference (*V*). Flavones were slightly more effective in uncoupling the membrane potential than flavanones; the 7OH group seems to play an important role. Hydroxylation of the exocyclic phenyl group decreased the uncoupling efficiency for all flavonoids studied. The flavonol quercetin exhibited hardly any uncoupling activity. Glycosylation abolished all uncoupling activity. The affinity of flavonoids for vesicle membranes was also studied using the fluorescence quenching of the membrane probe diphenylhexatriene. Flavonols exhibited a substantially higher affinity for liposomes than flavanones. This difference in affinity is assumed to be caused by the far more planar configuration of the flavonols in comparison with the tilted configuration of flavanones. Due to this planar configuration, it seems reasonable to assume that flavonols could more easily intercalate into the organised structures of the phospholipids within the vesicle membranes than flavanones. It is concluded that, *in vivo*, hardly any uncoupling activity of flavonoids can be anticipated. However, the quercetin plasma concentration *in vivo* can be such that, based on the affinity study, part of this flavonol could be associated with biological membranes to function there as, for example, an antioxidant. *BIOCHEM PHARMACOL* 60;11:1593–1600, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. flavonoids; relative hydrophobicity; membrane potential; uncoupling efficiency; membrane affinity

Flavonoids comprise a major group of plant secondary metabolites containing over 4000 different chemical structures [1]. In recent decades, this class of compounds has attracted substantial attention due to the assumed link between its antioxidant properties [2] and its beneficial effects on health, with reference to coronary heart diseases and cancer [3]. Due to their chemical composition, the flavonoid aglycons span the whole range from fully water-to fully lipid-soluble, allowing them to perform their anti-oxidative mode of action both in water solutions and in biological membranes. However, the majority of the flavonoids seems to be present in the glycosylated form, both in fresh as well as in processed vegetables [4, 5]. As suggested by Hollman *et al.* [6] and shown by Gee *et al.* [7], the type and degree of glycosylation may be of relevance in

permitting their transport over the intestinal border, using the sodium-dependent glucose co-transporter system.

Given the recent focus on their antioxidant properties, the non-antioxidative properties of flavonoids, especially their interactions with biological membranes and the potential physiological consequences, have been under-exposed. For example, Cojocaru *et al.* [8, 9] showed, using artificial planar membranes, that glycosylated flavonols and their aglycons, in the presence of an applied potential difference across the planar membranes, increase the specific membrane conductance linearly with their concentration. They concluded that flavones are protonophores. Saija *et al.* [10, 11] researched the interaction of flavonoids with vesicles using differential scanning calorimetry. They demonstrated that flavonoid incorporation into liposomal membranes causes stress in the packaging of the bilayer, thereby altering its barrier functions. In his work, Ravel addressed the structure–function relationship between the efficiency of the uncoupling of the membrane potential of plant mitochondria by flavones and flavonols [12] as well as chalcones [13]. It is interesting to note that the chalcone phlorentin increases the cation flux through lipid bilayers [14]. Furthermore, the flavonol platanetin is one of the most potent natural uncouplers of the mitochondrial mem-

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brane [15]. Mirzoeva *et al.* [16] showed that the flavonol quercetin, a component of propolis, affects the membrane potential of the phototrophic bacterium *Rhodobacter spheroides*. Versantvoort *et al.* [17], in their work on multi-drug resistance, showed that (iso)flavonoids affect the membrane potential of multidrug-resistant cells, and as a consequence the efflux of daunorubicin and the dye rhodamine 123. Ong and Khoo [18] showed that the flavonol myricetin mimics insulin in stimulating lipogenesis and glucose transport in rat adipocytes *in vitro*. Myricetin was found to stimulate lipogenesis in rat adipocytes and enhance the stimulatory effect of insulin, but did not have any effect on insulin receptor autophosphorylation or on the tyrosine kinase activity of the receptor.

The present study was undertaken to obtain information about both the binding of flavonoids to vesicle membranes and the uncoupling efficiency of related flavones and flavanones. To quantify the uncoupling efficiency of related flavones and flavanones, use was made of one of the most adequate and simplest systems, vesicles containing cytochrome *c* and cytochrome *c* oxidase.

MATERIALS AND METHODS

Water–Oil Partitioning

The relative hydrophobicity of the flavonoids was determined by using olive oil as hydrophobic phase [19]. The corresponding flavonoid was added to the aqueous phase of a biphasic solution consisting of olive oil and distilled water, buffered with 20 mM *N*-morpholinoethane sulphonic acid (MES) (pH 5.0). Upon vigorous mixing, the phases were separated by centrifugation, and aliquots of 1 mL were collected from the aqueous phase. Spectra were recorded with a Pye Unicam spectrometer (type sp-100) before and after extraction, after which the affinity for the hydrophobic phase was calculated.

Preparation of Phospholipid Vesicles

L- α -Dioleoyl phosphatidylcholine was dried under a stream of N₂ and suspended into 50 mM MES, pH 6.0, at a concentration of 25 mg of phosphatidylcholine.mL⁻¹. The suspension was dispersed using a bath sonicator (Sonicor Instruments). Phospholipid vesicles were prepared by sonication (MSE Scientific Instruments) for 300 sec in total at an amplitude of 3 μ m (peak-to-peak) at 4° under a constant stream of N₂. Alternating periods of 15-sec sonication and 45-sec cooling were used.

Equilibrium Binding Measurements

Equilibrium binding of flavonoids to phospholipid vesicles was measured using a fluorescence quenching assay [20, 21]. Liposomes were suspended in 50 mM potassium phosphate, pH 6.0, and labelled with the intrinsic membrane probe

DPH. A phospholipid-to-DPH* ratio of 200 was used. DPH fluorescence was measured with a Perkin Elmer LS-50 Fluorometer equipped with a thermostatted, magnetically stirred cell holder. Excitation and emission of DPH was at 357 and 430 nm, respectively. At their effective concentrations, flavonoids caused no interference of the fluorescence measurements by inner filter effects.

Preparation of Cytochrome *c* Oxidase Vesicles

Cytochrome *c* oxidase was isolated from beef heart mitochondria [22]. Reconstitution into liposomes composed of acetone/ether-washed *Escherichia coli* phospholipids was performed by detergent dialysis [23, 24]. COVs were stored in liquid nitrogen, as described previously [25, 26]. Before use, COVs were slowly thawed at room temperature and briefly sonicated.

Measurement of $\Delta\psi$ and ΔpH in Cytochrome *c* Oxidase Vesicles

$\Delta\psi$, inside negative, was measured continuously in a temperature-controlled vessel equipped with a TPP⁺-selective electrode [27] that monitors the external concentration of the lipophilic cation TPP⁺. The incubation mixture contained: 50 mM potassium phosphate, pH 6.0 (or as indicated), 2.5 mM MgCl₂, 2 μ M TPP⁺, 10 μ M cytochrome *c*, 20 nM nigericin, and COVs (38 pmol oxidase) in a final volume of 1 mL at 25°. The suspension was aerated by a constant flow of water-saturated oxygen over the surface. Cytochrome *c* was reduced by 10 mM ascorbate-KOH (pH 6.0, or as indicated) in the presence of 200 μ M TMPD. Flavonoids were added from an ethanolic stock solution before energisation. At the end of each experiment, $\Delta\psi$ was completely dissipated by the addition of 100 nM valinomycin. $\Delta\psi$ was calculated from the steady-state level of TPP⁺ uptake. Values were corrected for concentration-dependent binding of TPP⁺ to the membranes [25, 28]. The internal volume of the COVs was 1.5 μ L \times (mg of phospholipid)⁻¹ [25].

ΔpH , inside alkaline, was measured with the membrane-impermeable fluorescence dye PHPS [29]. COVs were loaded with PHPS (100 μ M) during the freeze/thaw sonication step [18–20]. External PHPS was removed by gel filtration using a Sephadex G-75 column (30 cm [height] \times 1 cm [diameter]). The column was equilibrated and eluted with 50 mM potassium phosphate, pH 6.0. For fluorescence measurements, PHPS-loaded COVs (86 pmol oxidase) were diluted into a solution containing: 50 mM potassium phosphate, pH 6.0, 2.5 mM MgCl₂, 50 nM valinomycin, 10

* Abbreviations: DPH, diphenylhexatriene; COVs, cytochrome *c* oxidase vesicles; $\Delta\psi$, transmembrane potential difference (V); ΔpH , transmembrane pH difference (V); TPP⁺, tetraphenylphosphonium; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylene diamine; PHPS, pyranine (8-hydroxy-1,3,6-pyrenetrisulfonate); CCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; and U₅₀, the concentration of flavonoid necessary to uncouple the COVs by 50%.

TABLE 1. Structure, relative hydrophobicity, uncoupling efficiency, and affinity for vesicles of some flavonoids

Subclass Common name	Substitution						RH* %	Uncoupling efficiency U ₅₀ (μM)	K _d [†] (μM)
	3	5	7	2'	3'	4'			
Flavones									
Flavone							100		
5OH-flavone		OH					99	500	
7OH-flavone			OH				83	110	
Chrysin		OH	OH				96	90	
Apigenin		OH	OH			OH	81	350	
Luteolin		OH	OH		OH	OH	34	300	
Flavonols									
Quercetin	OH	OH	OH		OH	OH	53	NA‡	1.9
Morin	OH	OH	OH	OH		OH	4		7.3
Flavanones									
Pinocembrin		OH	OH				95	110	170
Naringenin		OH	OH			OH	73	900	120
Naringenin-Glu		OH	O-Glu			OH	0	NA‡	75
Eriodictyol		OH	OH		OH	OH	29	800	130
Eriodictyol-Glu		OH	O-Glu		OH	OH	0	NA‡	40
Hesperitin		OH	OH		OH	OMe	73		130

* RH, relative hydrophobicity, expressed as the percentage ratio of the partitioning of flavonoids between a water and olive oil phase.

† K_d, dissociation constant between vesicles and flavonoids.

‡ NA, not active as uncoupler; no uncoupling activity could be observed below a concentration of 1 mM.

μM cytochrome *c* with a final volume of 2 mL. Energisation was started by the addition of 200 μM TMPD and 10 mM ascorbate-KOH, pH 6.0. Excitation and emission of PHPS was at 460 and 510 nm, respectively. The fluorescence of PHPS was partially quenched by reduced cytochrome *c*. Calibration of the intravesicular PHPS fluorescence intensity versus the pH was achieved by adjusting the external pH in the presence of 100 nM nigericin under conditions whereby cytochrome *c* remained reduced [24–26].

Other Analytical Procedures

Oxygen consumption by COVs was measured with a Clark oxygen electrode in a 3.0-mL reaction vessel thermostatted at 25° [20]. COVs (108 pmol oxidase) were suspended into oxygen-saturated solution of 50 mM potassium phosphate, pH 6.0, supplemented with 2.5 mM MgCl₂, 0.1 μM valinomycin, and 1 μM CCCP. The rate of oxygen consumption was determined after the additions of 10 μM cytochrome *c*, 10 mM potassium ascorbate, and 200 μM TMPD. The cytochrome *c* oxidase concentration in the COVs was estimated from the heme content. An absorption coefficient of 13.5 mM⁻¹ × cm⁻¹ was used for the reduced-minus-oxidised heme at the wavelength couple 605 nm/630 nm. Protein was assayed as described [30].

Chemicals Used

Pinocembrin and hesperitin were from Extrasynthese; eriodictyol and eriodictyolglucosides were from Roth GmbH. All other flavonoids were purchased from Sigma Chemical Co. All other chemicals were of analytical grade and obtained from commercial sources.

RESULTS

Water–Oil Partitioning

To characterise the hydrophobic nature of the flavonoids studied, the partitioning of these compounds between water and olive oil was determined. The affinity of flavonoids for olive oil has been shown to be a reliable standard for their relative hydrophobicity [31]. Previously, it was shown that the pH value of the water phase affects the partitioning of the flavanone naringenin [19]. An increase in pH value of the water phase favours the hydrophilic character of naringenin, due to protonation of the hydroxyl groups. The pK values of some flavonoids have been published by Jovanovic *et al.* [32]. The partitioning coefficient of flavonoids was determined at pH 6.0 for the water phase. This pH value was also used to study the effect of flavonoids on the membrane potential of the COVs (see below).

As expected, the relative hydrophobicity of the flavonoids decreased as the amount of hydroxylation increased (see Table 1). Comparing the relative hydrophobicity of the flavones chrysin, apigenin, and luteolin with the structurally analogous flavanones pinocembrin, naringenin, and eriodictyol, the decrease in relative hydrophobicity was obvious upon an increase in the number of hydroxyl groups. However, comparing the flavones and flavanones with the same number of hydroxyl groups, the former always appeared slightly more hydrophobic than the latter, possibly due to the difference in their three-dimensional configuration. X-ray analysis showed that the flavonol quercetin is planar [33]. However, the exocyclic phenyl group of the flavanon naringenin lies extensively twisted outside the plane of the rest of the molecule. This difference in three-dimensional configuration, together with the

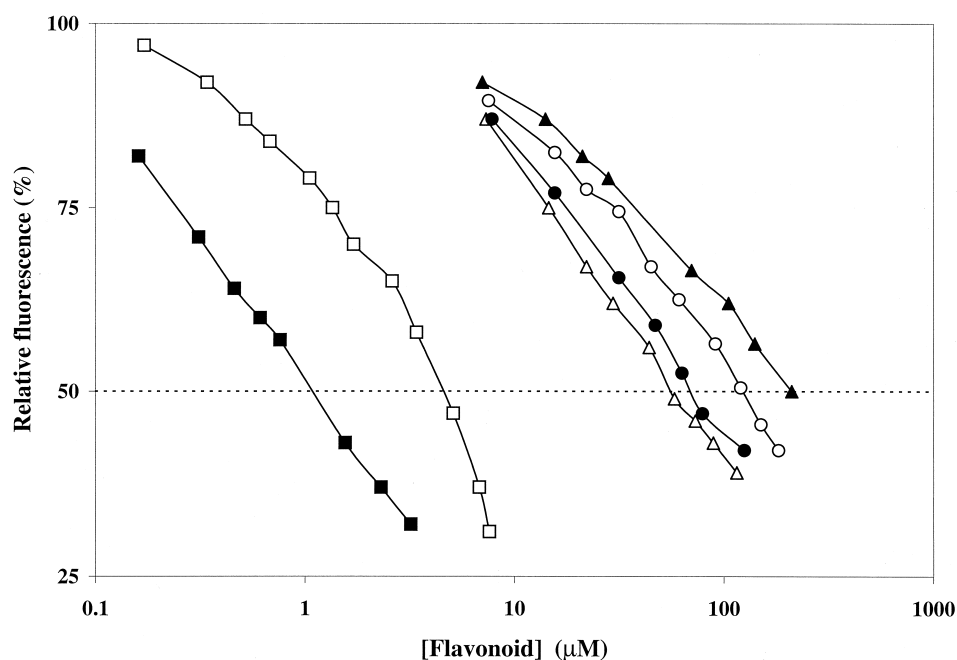


FIG. 1. The affinity of flavonoids for vesicle membranes as measured by the fluorescence quenching of the membrane probe DPH. Liposomes composed of phosphatidylcholine were charged with the intrinsic membrane probe DPH. The concentration-dependent quenching of DPH fluorescence by flavonols and flavanones was used as a measure of the affinity of flavonoids for membranes. Quercetin; ■, morin; □, hesperitin; △, pinocembrin; ●, eriodictyol; ○, naringenin; ▲. The 50% quenching level is indicated by the dashed line (···).

effect of the configuration on the pK values of the hydroxyl groups, might explain the observed difference in partitioning between flavonols and flavanones.

Affinity of Flavonoids for Liposomes

To further study the affinity of flavonoids for a biological, hydrophobic environment, their affinity for lipid vesicles was quantified. This was done using lipid vesicles charged with the intrinsic membrane probe DPH. The fluorescence quenching of this probe by flavonoids [20, 21] was used to assess the affinity of a set of flavonoids for these artificial membranes (see Fig. 1). The flavonoids quercetin and morin were the most efficient quenchers of the DPH fluorescence. Using the Stern–Volmer equation, adapted according to Verkman [21], yielded a dissociation constant, K_d , for quercetin and morin of 1.9 and 7.3 μM , respectively. The K_d values for the flavanones pinocembrin, naringenin, eriodictyol, and hesperitin were 170, 120, 130, and 130 μM , respectively (see Table 1). Despite the fact that the flavanones tested, at the same degree of hydroxylation, were more hydrophobic than the flavonols tested (see Table 1), the affinity of flavonols for liposomes was substantially higher than that of flavanones. The difference in the K_d value between the flavonols and flavanones tested was at least one order of magnitude.

From the results of this affinity study, it is obvious that the planar configuration of flavonols [33] strongly favours, in contrast to the tilted configuration of flavanones, the intercalation of the flavonols into the organised structures

of the phospholipids within the vesicle membranes. The partitioning of flavonoids over water–olive oil refers to the overall hydrophobic character of the flavonoids and their interaction with randomly oriented phospholipids. For their interaction with vesicles, a planar configuration of flavonoids appears of far greater importance than the relative hydrophobicity index as determined by the water–olive oil partitioning.

In addition to the flavonoid aglycons, the 7-glucosides of both eriodictyol and naringenin were tested with reference to their efficiency to quench the DPH fluorescence (data not shown). Their K_d values were 40 and 75 μM , respectively. Surprisingly, glucosylation of both naringenin and eriodictyol at the 7 position resulted in a higher affinity for the vesicle membrane compared to the corresponding aglycons. The reason for this increase in affinity upon glucosylation of flavonoids might be a decrease in torsion between the exocyclic phenyl ring and the rest of the structure, favouring a more planar configuration [33]. Due to this more planar configuration, the affinity for vesicles of the glucosylated flavonoids is higher as compared with their corresponding aglycons.

Dissipation by Flavones and Flavanones of the $\Delta\psi$ in Cytochrome *c* Oxidase Vesicles

To exclude any indirect effect of high flavonoid concentrations on cellular physiology, the protonophoric properties of flavonoids were tested on the steady-state Δp in COVs. When supplied with reduced cytochrome *c* and the

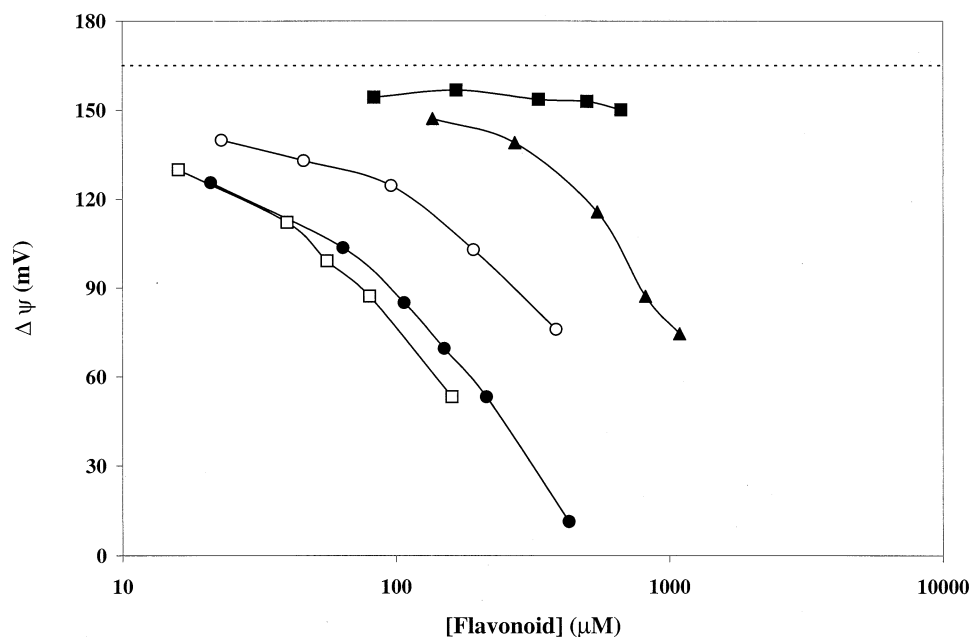


FIG. 2. Flavones and flavanones dissipate the $\Delta\psi$ in cytochrome *c* oxidase vesicles. The concentration-dependent dissipation of the $\Delta\psi$ (inside negative) in the presence of various flavonoids was measured with a TPP^+ -selective electrode. Flavonoids were added as an ethanolic stock before energisation. The following flavones were tested: chrysin, (\square); apigenin, (\circ); also tested were 5-hydroxy-, 7-hydroxy-, and 5,7,3',4'-tetrahydroxyflavone (data not shown). The flavanones tested were: pinocembrin, (\bullet); naringenin, (\blacktriangle); also tested was eriodictyol (not shown). The flavonol tested was quercetin (\blacksquare). The ethanolic control value is indicated by the dashed line (\cdots).

ionophore nigericin, COVs generate an $\Delta\psi$ of up to -165 mV. $\Delta\psi$, inside negative, was continuously monitored using a TPP^+ -selective electrode [27]. Increasing concentrations of both flavones and flavanones caused a progressive dissipation of the $\Delta\psi$ (Fig. 2). The flavonoid concentrations at which half-maximal dissipation of the $\Delta\psi$ is observed, U_{50} , are given in Table 1.

For the flavones tested, the 7-hydroxy and the 5,7-dihydroxy (chrysin) flavanones in particular exhibited an efficient protonophoric activity ($U_{50} \approx 0.1$ mM). The 5-hydroxy flavone ($U_{50} \approx 0.5$ mM) was substantially less efficient as uncoupler as compared with the 7-hydroxy flavone. Most likely, the 5-hydroxy group is less acidic in comparison with the 7-hydroxy group, due to the interaction of this 5-hydroxy with the oxygen atom at the 4 position, probably causing an increase in pK value. This assumed increase in pK value goes at the expense of the protonophoric efficiency. Hydroxylation of the aromatic β -ring of flavones (apigenin, luteolin) decreases both the uncoupling efficiency and the relative hydrophobicity. Almost no difference was observed in the uncoupling efficiency of apigenin (5,7,4'-trihydroxyflavone) and luteolin (5,7,3',4'-tetrahydroxyflavone) ($U_{50} \approx 0.3$ mM), suggesting that the 3'-hydroxyl group does not contribute much to the protonophoric behaviour of these flavanones.

In addition to flavones, the uncoupling efficiency of the flavanones pinocembrin, naringenin, and eriodictyol was determined. The flavanon pinocembrin and the flavone chrysin, both containing a 5- and a 7-hydroxy group at the α -ring, exhibited the same uncoupling efficiency ($U_{50} \approx 0.1$ mM). Naringenin and eriodictyol were weak uncouplers ($U_{50} \approx 0.9$ mM), again suggesting that neither the 3'- nor the 4'-hydroxy group contributes to the uncoupling activity. Their U_{50} values were about twice the U_{50} values of the structural analogues apigenin and luteolin. This difference

in protonophoric efficiency is assumed to be caused by the above-mentioned difference in planarity. Glucosylation of both naringenin and eriodictyol abolishes the uncoupling effect of these flavanones; no uncoupling was observed below a concentration of 1 mM (data not shown).

Based on the efficiency of DPH fluorescence quenching, flavonols exhibit a more efficient interaction with liposomes than flavanones. For this reason, the protonophoric activity of quercetin was also tested. However, no protonophoric activity could be observed below a concentration of about 1 mM. This suggests that the efficiency of association with vesicle membranes, as quantified by DPH fluorescence quenching, and the uncoupling performance, as quantified by the dissipation of the $\Delta\psi$, are not related to each other in the sense that an efficient DPH fluorescence quenching by flavonoids does not directly relate to an efficient protonophoric effect. As reference, the uncoupler CCCP was used; the $\Delta\psi$ collapsed with a U_{50} of 50–70 nM.

Dissipation by Flavones and Flavanones of the ΔpH in Cytochrome *c* Oxidase Vesicles

The effect of flavones and flavanones on the dissipation of the ΔpH , with emphasis on the flavone naringenin, was studied with COVs, pretreated with valinomycin at a solution pH of 6.0. The ΔpH , inside alkaline, was measured with the membrane-impermeable fluorescent dye PHPS, trapped in the COVs during the freeze/thaw sonication step. The ΔpH was dissipated by naringenin in a manner analogous to its effect on $\Delta\psi$. The U_{50} value was approximately 0.6 mM (Fig. 3). Although examined in less detail, the flavonoids apigenin, chrysin, and pinocembrin had similar effects on the ΔpH (data not shown). To exclude the possibility that the uncoupling effect may have been caused by a direct inhibitory effect of these compounds on

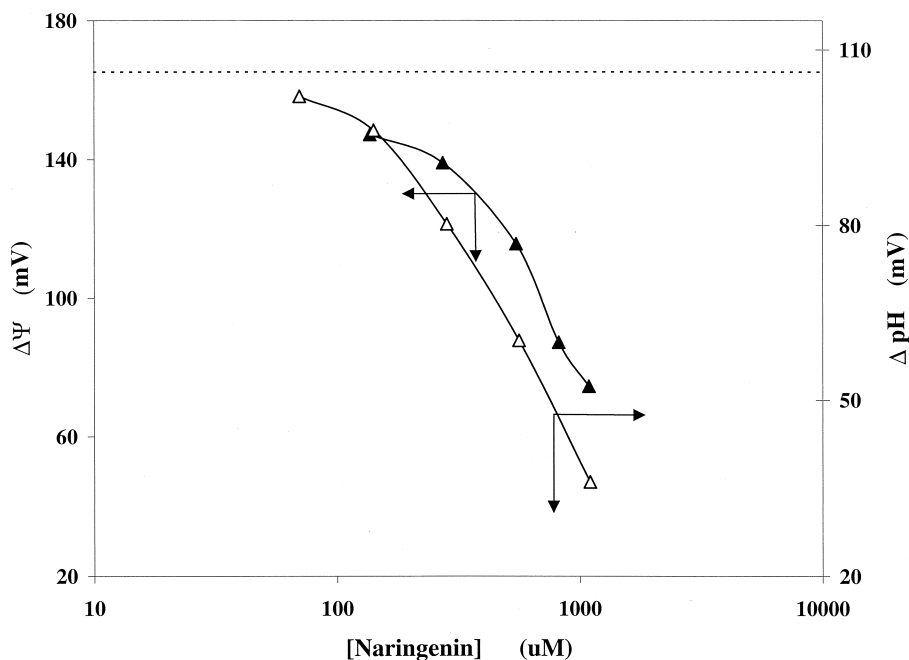


FIG. 3. Naringenin dissipates both the ΔpH and the $\Delta\psi$ in cytochrome *c* oxidase vesicles. The concentration-dependent dissipation of the ΔpH of COVs by naringenin was monitored by the fluorescence of PHPS. Naringenin (Δ) was added as an ethanolic stock solution. ΔpH , inside alkaline, was calculated from the level of DPH fluorescence and the extravesicular pH. For comparison, the dissipation of the $\Delta\psi$ by naringenin (\blacktriangle) is also shown (data derived from Fig. 2). The ethanolic control value for both the ΔpH and $\Delta\psi$ is indicated by the dashed line (\cdots).

cytochrome *c* oxidase, respiration rates in uncoupled (valinomycin/CCCP-treated) COVs were measured in the presence and absence of the different flavonoids. At their effective concentrations, flavonoids exhibited only a moderate (less than 10%) inhibitory effect on the rate of respiration (data not shown). Therefore, any direct effect of naringenin on the activity of cytochrome *c* oxidase can be excluded.

Further support for the dissipation of the ΔpH by naringenin was obtained by studying the effect of the solution pH, at a fixed added naringenin concentration, on the ΔpH . The effect of the solution pH on the ΔpH , in the absence and presence of naringenin, is shown in Fig. 4. The difference in the measured ΔpH in the absence and presence of 0.54 mM naringenin at pH 5, 6, 7, 8, and 9 was 34, 29, 6, 6, and 7 mV, respectively. From pH 7 onward, hardly

any effect of naringenin on the ΔpH was observed. From the spectral information of Recourt *et al.* [19], a pK value for naringenin of 7.0 can be calculated. This pK value coincides with the 50% value for the partitioning of naringenin [19], which is also at about pH 7. No attempts were made to correct for the pH effect on the concentration of the active, protonated form of naringenin in relation to the dissipation of the ΔpH . From this information, it is obvious that the protonated form of naringenin in particular, as well as of the other flavonoids researched, cause the effect on both the $\Delta\psi$ and ΔpH .

DISCUSSION

The research presented focuses on the relation between the relative hydrophobicity of flavonoids, their binding with

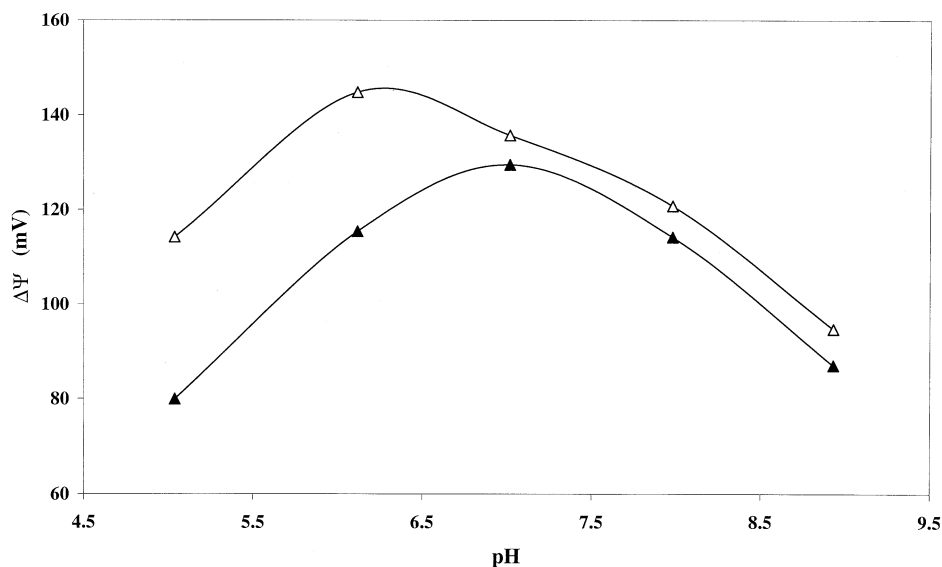


FIG. 4. An acidic pH is required for efficient dissipation of the $\Delta\psi$ in cytochrome *c* oxidase vesicles by naringenin. The effect of naringenin on the $\Delta\psi$ in COVs was tested at different pH values. Naringenin (\blacktriangle) was added to a final concentration of 220 μM before energisation. Controls (Δ) received only the solvent ethanol. Further experimental details are as described in the Materials and Methods section.

the artificial membranes of vesicles as quantified by DPH fluorescence quenching, and their uncoupling activity. Our *in vitro* studies with COVs indicate that flavonoids bear a weak uncoupling activity. This uncoupling activity of flavonoids is probably the result of a cycle involving the following steps: (i) diffusion of the uncharged form of the flavonoid across the membrane; (ii) dissociation of ionisable hydroxyl group(s) in the cytoplasm (deprotonation of these groups is facilitated by an alkaline intracellular pH which is maintained by the ΔpH); (iii) repulsion of the ionised form in response to the $\Delta\psi$; and (iv) protonation of the ionised molecule at the outside of the membrane. Important for an efficient uncoupling seems the 7-hydroxyl group, present in 7OH-flavones, chrysin, and pinocembrin: the U_{50} value of these three compounds is around 0.1 mM. Additional hydroxylation at the 4' (apigenin, naringenin) or 3' and 4' (luteolin, eriodictyol) goes at the expense of both the uncoupling efficiency and relative hydrophobicity. Glucosylation at the 7OH group (naringenin, eriodictyol) completely abolishes both the uncoupling effect as well as the hydrophobic character. The best-known protonophores such as 2,4-dinitrophenol, CCCP, and 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazol are all weak acids ($\text{pK}_a \approx 5$). They are all soluble within the membrane both in their protonated and unprotonated forms. Proton-conducting uncouplers induce a collapse of both the $\Delta\psi$ and ΔpH [34], as is the case for flavonoids. Apparently, a neat balance between the chemical structure and conformation and, as a consequence of this, the hydrophobic character and pK value(s) of the ionisable hydroxyl group(s) exists which determines the protonophoric properties of flavonoids.

In his work, Ravel [12] determined the uncoupling efficiency of a series of flavonoids using isolated plant mitochondria. He determined the flavone and flavonol concentration at which full uncoupling was obtained based on the rate of oxygen consumption, using a polarographic method. In essence, the uncoupling efficiencies determined in this study and those determined by Ravel are similar, with some exceptions. We determined chrysin to be an efficient uncoupler and quercetin to be a very inefficient uncoupler. However, Ravel showed that chrysin was a weak and quercetin a moderate uncoupler. This difference in uncoupling activity could be caused by the difference in the tools used to quantify the uncoupling activity of flavonoids, i.e. vesicles versus intact mitochondria.

Limited binding studies, based on the fluorescence quenching of the membrane probe DPH, reveal that (planar) flavonols exhibit a substantially higher affinity for the phospholipid bilayer of the membrane vesicles than the (non-planar) flavanones. In the case of quercetin, no uncoupling could be detected, although a strong binding was observed. For the flavanones (aglycons) tested, a weak binding was observed ($K_d \approx 0.15$ mM), while the U_{50} value ranged from 0.1 mM (pinocembrin) to about 1 mM (naringenin, eriodictyol). Surprisingly, the binding of the 7-glucosides of both naringenin and eriodictyol to the vesicle membranes was higher than that of their corre-

sponding aglycons. Based on this (limited) amount of information, it is tempting to speculate that steric considerations in particular are of importance to explain the substantially higher affinity of (planar) flavonols than (non-planar) flavanones to the membrane vesicles.

In their study, Hollman *et al.* [35] determined the quercetin plasma concentration. The maximal quercetin plasma value they determined was around 0.7 μM . Based on this value and the determined K_d value of 1.9 μM , it can be speculated that part of the quercetin could be associated *in vivo* with biological membranes to function there as an antioxidant. In plant tissue, most of the flavonoids are present as glycosides [36]. Due to this glycosylation, these flavonoids most probably lose their uncoupling ability, thereby effectively preventing the plant cell from becoming depolarised by its own secondary metabolites. With reference to their weak uncoupling activity, such as determined in this study and in the study of Ravel [12] and with the exception of platanetin [15], none to only a very weak uncoupling activity of flavonoid aglycons can be anticipated *in vivo*.

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