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UNCOUPLING PROPERTIES OF THREE FLAVONOLS FROM PLANE-TREE BUDS

SOPHIE CREUZET, PATRICK RAVANEL,*† MICHEL TISSUT and MOURAD KAOUADJI*

Laboratoire de Physiologie cellulaire végétale, UA du CNRS 576, Université Joseph Fourier-Grenoble I, B.P. 68, 38402 Saint Martin d'Hères Cedex, France; *Laboratoire de Pharmacognosie, Université Joseph Fourier-Grenoble I, Domaine de La Merci, 38700 La Tronche, France

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Key Word Index—*Platanus acerifolia*; Platanaceae; uncoupling activity; mitochondria; chloroplasts; cells; flavonols.

Abstract—Three new flavonols from *Platanus acerifolia* bud secretions were investigated for their uncoupling activity. They were platanetin (3,5,7,8-tetrahydroxy-6-dimethylallylflavone), platanin (3,5,7,8-tetrahydroxy-6-methylflavone) and 3-hydroxywogonin (3,5,7-trihydroxy-8-methoxyflavone). Their activities were studied on mitochondria (potato tubers, etiolated mung bean hypocotyls and rat livers), thylakoids and chloroplasts (spinach leaves) and *Acer* cells. The uncoupling activity was demonstrated using polarography and spectrophotometry. Platanetin is the most potent uncoupler, inducing a full uncoupling with 5, 10 and 6 μM in potato, mung bean and rat liver mitochondria respectively and 30 μM in spinach thylakoids and in *Acer* cells. The uncoupling activity of the other two flavonoids was lower. The presence of bovine serum albumin in the reaction medium and the variations in pH (between 6.5 and 8) did not affect the uncoupling efficiency. In contrast with other flavonoids, these compounds were only protonophoric and not ionophoric. The pK_a and log P values of the three flavonols studied were near those of reference uncouplers. However, for platanetin and 3-hydroxywogonin in the pK_a and log P values were the same, but the biological activities were not: a binding of 3-hydroxywogonin to a protein component of the membrane might explain this difference. In contrast, the lower uncoupling activity of platanin, when compared to platanetin, is probably due to a one-unit lower log P. Uncoupling activity of these lipophilic flavonols can be explained using the simplest scheme of Terada, but taking into account the protein-binding effects which interfere with the direct transmembrane proton transport.

INTRODUCTION

In a previous study [1] we described some biological properties of platanetin (3,5,7,8-tetrahydroxy-6-dimethylallylflavone), a flavonol isolated in our laboratory from *Platanus acerifolia* bud secretions [2]. Among several interesting activities on isolated mitochondria which were observed, this compound was shown to be a highly potent uncoupler, acting at the same concentration as carbonyl cyanide *p*-trifluorophenylhydrazone (FCCP) which is, at the present time, with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), one of the most widely used reference uncouplers in isolated mitochondria. The aim of this work was to compare the uncoupling efficiency of platanetin to the uncoupling activity of related compounds which were isolated, as was platanetin itself, from plane-tree bud secretions, and to tentatively suggest, from this comparison, the structural features which are directly responsible for this biological activity. For this purpose, two new flavonols: platanin (3,5,7,8-tetrahydroxy-6-methylflavone) and 3-hydroxywogonin (3,5,7,8-trihydroxy-8-methoxyflavone) were isolated from bud secretions in sufficient quantities to allow the biological studies [3].

†Author to whom correspondence should be addressed.

RESULTS

Physical constants of the three flavonols

Hydroxyls bound to the flavone nucleus are susceptible to change into ionized forms $O^- H^+$ depending on the pH. In the flavonol class, the most easily ionizable hydroxyl is the 7-OH, as shown by the classical spectrophotometric identification test, using dry sodium acetate. In contrast, the 5-hydroxyl practically never gives the ionized form.

We have measured the pK_a for the hydroxyl which can ionize at the most acidic pH, ionization occurs for the three products at a pH near 7 (Table 1). However, the pK_a of platantin is *ca* one-unit lower than 3-hydroxywogonin and platanetin. This suggests that both the substitutions on the 6- and 8- positions, and the length of the chain which may be bound to the C-6, greatly influence whether the 7-hydroxyl ionizes or not.

We have also determined the partition coefficient P, expressed by $-\log P$, (a conventional measurement of the lipophilicity of compounds). With a common flavonol nucleus, log P will change, depending on the number and position of hydroxyl, methoxyl, methyl and glycosyl substituents. In the present case (Table 1), it is interesting that the dimethylallyl chain (platanetin) increases log P to a value which remains slightly lower than the value obtained for 3-hydroxywogonin, which is methoxylated

Table 1. pK_a and log P values for the three studied compounds compared to classical uncouplers

	pK_a	log P
Platanetin	7.3	5.1
Platanin	6.5	4.1
3-OH Wogonin	7.5	5.3
FCCP	6.2*	5.6†
CCCP	5.9*	5.1†
2,4-diNO_2 phenol	4.1*	-

*Values from ref. [8].
†Values from ref. [16].

at the 8- position, and unsubstituted at C-6. Replacement of the dimethylallyl chain (platanetin) by a methyl group (platanin) decreases log P by one unit. The water solubility of platanetin has been carefully checked. The solubility limit in distilled water is near 7 μM. However, the presence of bovine serum albumin (BSA, 0.1%) in the medium suspending mitochondria, greatly increases the solubility, showing that the flavonoid is probably able to bind, with a low affinity, to this protein. The same phenomenon can be observed when organelles replace this protein in the medium. The same result has been demonstrated in the case of pentachlorophenol [4, 5].

Uncoupling effect of the three flavonols on isolated and purified mitochondria

Five μM platanetin or 30 μM platanin or 100 μM 3-hydroxywogonin induce complete uncoupling of oxidative phosphorylation in purified potato mitochondria oxidizing succinate in a medium containing 0.1% BSA (Fig. 1). In fact, at these concentrations, the compounds induce, at state IV, a great increase in oxygen consumption and suppress either the increase dependent on the addition of ADP, or the increase obtained by the addition of CCCP (5 μM). The same results are obtained when succinate is replaced by α-ketoglutarate or citrate as substrate. During oxidation of exogenous NADH, the high inhibitory effect of these flavonoids on the electron transfer rapidly masks its uncoupling activity.

The spectrophotometric measurements of mitochondrial swelling confirm, for the three flavonoids studied, the uncoupling activity previously demonstrated by polarography. For example, in an ammonium nitrate iso-osmotic medium, NH_3 and NO_3^- passively enter the internal mitochondrial space [6]. However, this movement is strictly limited, in intact mitochondria, by the transmembrane charge gradient. After the addition of an uncoupler, an uncontrolled movement of anions and protons occurs, which induces an increase in the osmotic pressure followed by mitochondrial swelling. A rapid mitochondrial swelling in a NH_4NO_3 iso-osmotic medium is obtained by the addition of platanetin (5 μM), platanin (30 μM) and 3-hydroxywogonin (100 μM) (Fig. 2). These concentrations are the lowest values which cause complete uncoupling (Fig. 3).

The experimental conditions were checked for their influence on uncoupling efficiency. Between 6.5 and 8 the pH of the medium allows reasonable activity (electron transfer and respiratory controls) of the isolated potato tuber mitochondria. At each pH, the concentrations of the three compounds causing complete uncoupling remained the same. The mitochondrial activities were stud-

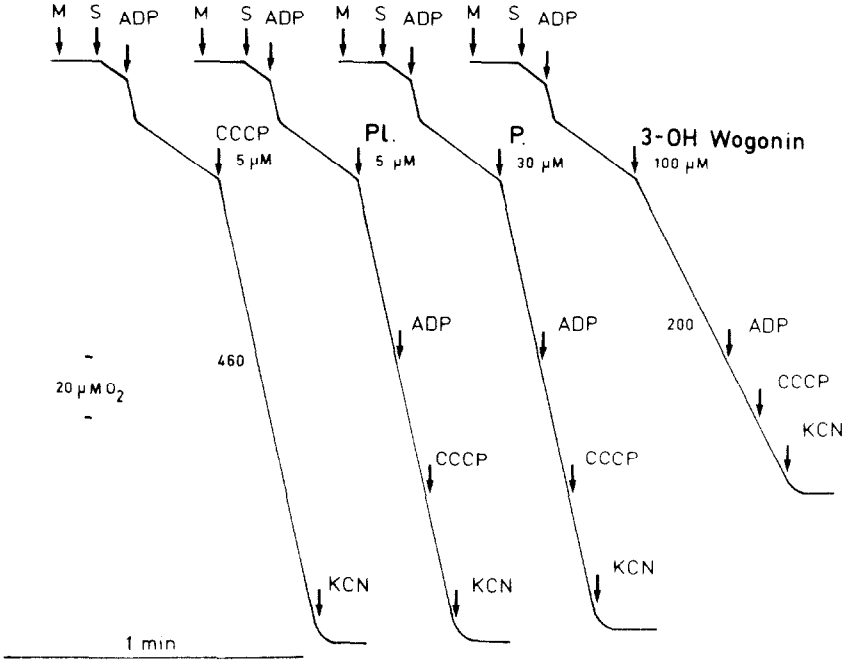

Fig. 1. Polarographic traces showing the full uncoupling activity of the three flavonoids in potato mitochondria. M: purified potato tuber mitochondria, S: succinate 6 mM + ATP 0.3 mM, ADP: 0.2 mM, KCN: 30 μM, Pl.: platanetin, P.: platanin. Numbers on traces refer to nmol O_2 consumed/min mg protein.

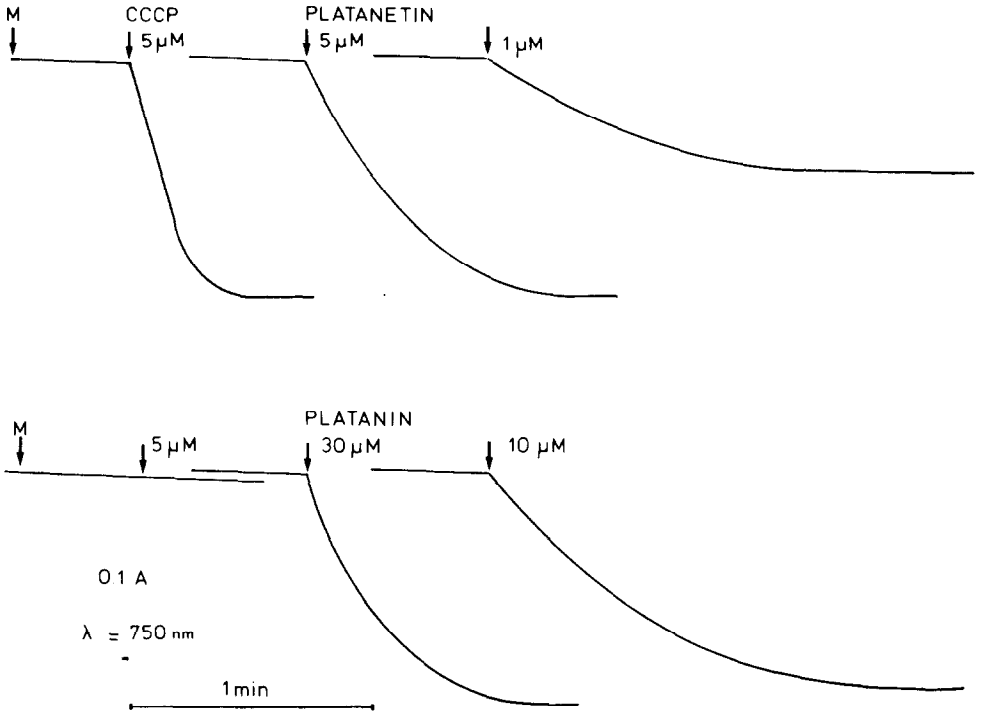

Fig. 2. Spectrophotometric results showing the protonophoric activity on the three flavonoids, as inducing a passive swelling mechanism in potato tuber mitochondria suspended in an iso-osmotic NH_4Cl solution containing 0.1% BSA. M: 0.3 mg mitochondrial protein/ml.

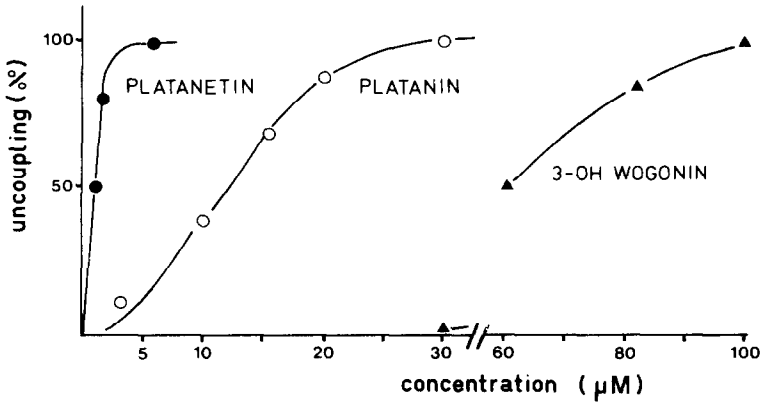

Fig. 3. Comparison of the uncoupling activity of platanetin and related flavonols: titration curves showing the percentage of uncoupling activity on potato mitochondria oxidizing succinate

ied in a medium lacking 0.1% BSA. In such a medium, the uncoupling activity of a classical uncoupler such as pentachlorophenol is 10 times higher than in a medium containing BSA [4]. In contrast, the uncoupling concentrations of the polyphenols studied here are hardly changed. It is likely that the uncoupling concentrations, at least for platanetin and platanin, are lower than the affinity constant of these molecules for BSA itself.

Platanetin and platanin are readily oxidized in an aqueous medium. However, the addition of 1 mM ascorbate is able to maintain these compounds in their reduced state. When added to the medium, 1 mM ascorbate alone is unable to change the mitochondrial activities, because of its high hydrophilicity. Furthermore, under our experimental conditions, the uncoupling activity of platanetin and platanin remains unchanged, even in the presence of ascorbate, showing that, in these short-term spectrophotometric and polarographic experiments, a pool of the flavonoid rapidly enters the inner membrane and is no longer accessible to irreversible oxidation. Swelling experiments were performed not only with NH_4NO_3 and NH_4Cl but also with KNO_3 iso-osmotic medium. A great

Table 2. Comparison of the uncoupling activities of the three studied flavonoids measured on three types of mitochondria

	D_{50}			D_{100}		
	Pl.	P.	3-OH W.	Pl.	P.	3-OH W.
Potato tuber	1	10	60	5	10	100
Mung bean	6	20	120	10	50	200
Rat liver	4	6	60	6	8	100

Pl.: platanetin, P.: platanin, 3-OH W.: 3-hydroxywogonin, D_{50}: dose (μM) required to uncouple activity by 50%, D_{100}: dose (μM) required to uncouple activity by 100%.

number of flavonoids are both uncouplers and also able to transfer K^+ through the mitochondrial inner membrane [7]. With the present compounds, such an effect could not be observed and these flavonoids appear, therefore, to be strictly protonophores.

Uncoupling activity of the flavonols on mitochondria isolated from other species

The uncoupling activities demonstrated in purified potato mitochondria can also be observed in mung bean or rat liver mitochondria (Table 2). However, platanin is slightly more effective on animal than on plant mitochondria. Furthermore, etiolated mung bean hypocotyl mitochondria seem to be less sensitive to uncouplers than potato tuber mitochondria. This discrepancy is probably artefactual: only potato mitochondria are highly purified on Percoll gradients. In the case of mung bean, the loss of some of the applied flavonoid, through binding to contaminating proteins, may be possible.

Uncoupling activity of the flavonols on class C chloroplasts

Cautiously broken class A chloroplasts give rise to true class C chloroplasts which are unable to perform the Mehler reation. After addition of methylviologen, under light, they carry out an electron transfer from water to methylviologen, which catalyses the formation of hydrogen peroxide. This reaction leads to oxygen consumption, which is greatly increased by the addition of platanetin (30 μM)(Fig. 4). This increase remains light-dependent and the further addition of NH_4Cl (5 mM) does not change the rate of oxygen consumption. Platanetin 30 μM is therefore responsible for a total uncoupling of photophosphorylation under our conditions. The same result was obtained when PS I was operating alone, with ascorbate-DCPIP (dichlorophenol-indophenol) as electron and proton donor system, and methylviologen as acceptor. Under the same experimental conditions, complete uncoupling was obtained in spinach class C chloroplasts for 150 μM with platanin and 700 μM with 3-hydroxywogonin (Fig. 5).

This uncoupling activity was responsible for the inhibition of the light-driven O_2 evolution which corresponds to photosynthesis in class A chloroplasts. For 100% inhibition, either 20 μM platanetin, or 100 μM platanin, or 500 μM 3-hydroxywogonin were required (Fig. 6), which is in agreement with the concentration values shown to induce complete uncoupling in thylakoids.

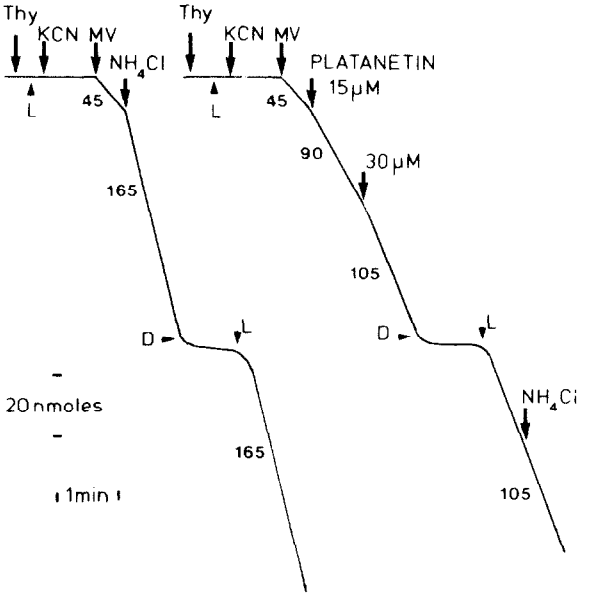

Fig. 4. Polarographic traces showing the uncoupling activity of platanetin on isolated spinach class C chloroplasts. Thy: thylakoids, KCN: 100 μM, MV: methylviologen (1 mM), NH_4Cl: 5 mM. L: light, D: dark.

Demonstration of an uncoupling effect of platanetin and platanin, in situ, on a suspension culture of Acer pseudoplatanus *cambial cells*

A dense suspension of *Acer* cells was put into the reaction vessel of an oxygen electrode. The oxygen consumption of such a suspension, which reached 40 nmol/min mg protein, was greatly increased by 10 μM CCCP and was fully suppressed by 100 μM KCN. This oxygen consumption can therefore be considered as respiration. With such cells, 30 μM, platanetin induced an increase of the respiratory activity comparable with that obtained with CCCP (Fig. 7).

DISCUSSION

Of the three compounds present in plane-tree bud secretions, platanetin appears to be the best uncoupler, acting on plant mitochondria at concentrations as small as the effective concentrations of the classical uncouplers FCCP or CCCP. Thus the easy and reversible ionization

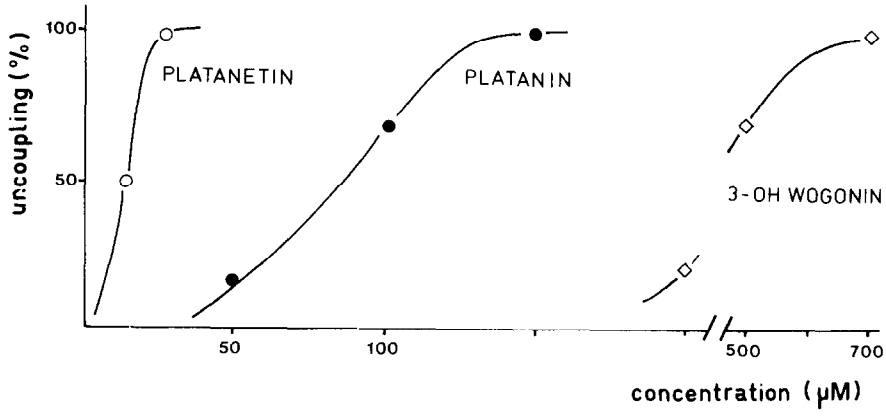

Fig. 5. Comparison of the uncoupling activity of platanetin and related flavonols: titration curves showing the percentage of uncoupling activity on isolated spinach class C chloroplasts using methylviologen as electron acceptor.

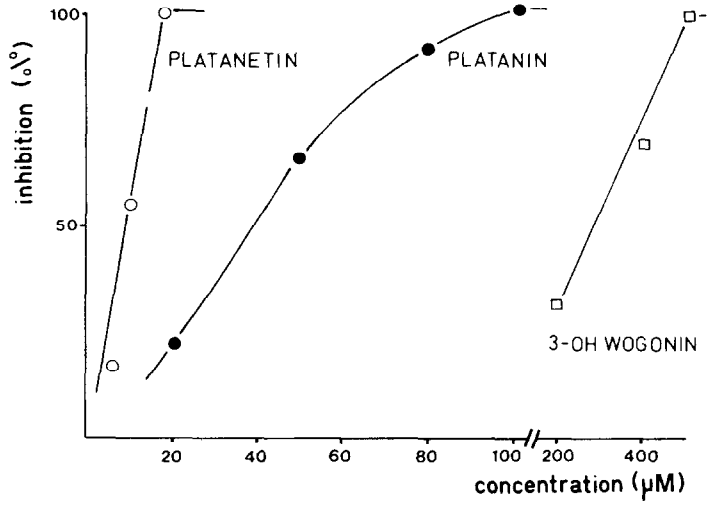

Fig. 6. Inhibitory effect of platanetin, platanin and 3-hydroxywogonin on light-dependent O_2 evolution by spinach class A chloroplasts in the presence of $NaHCO_3$, 1 mM and Na_2HPO_4, 0.1 mM.

of the 7-hydroxyl is responsible for the proton gradient collapse, in both mitochondria and thylakoids. The highly lipophilic dimethylallyl chain so greatly increases the lipophilicity of this flavonoid, in both its neutral and ionized forms, that it moves easily across the inner mitochondrial membrane as across the thylakoid. This view would agree with the simplest scheme established by Terada [8] to explain the uncoupling mechanism. However, determination of pK_a and log P for platanetin, platanin and 3-hydroxywogonin shows that other factors (e.g. steric hindrance) are probably very important for the uncoupling efficiency of each compound. In fact, pK_a and log P are the same for platanetin and 3-hydroxywogonin. However, the uncoupling activity of 3-hydroxywogonin is, at least 20 times smaller than that of platanetin. Possibly an unobserved binding of 3-hydroxywogonin to membrane constituents may take place and thus, prevent transmembrane mobility. Such a binding has been shown to occur in the case of the *N*-phenylcarbamate chlorpropham [5]. In the case of platanin, the fact that uncoupling activity is six times smaller than for platanetin can be understood if we take into account the one-unit smaller log *P*.

One of the interesting points of this study is that the uncoupling activity of these flavonols can affect several different biological membranes (Table 3). The uncoupling process seems, therefore, to be acting in the same way on both plant or animal mitochondria, and on thylakoids. However, thylakoids are six times less sensitive than potato mitochondria. The high sensitivity of rat liver mitochondria to platanin is also noticeable.

In the case of *Acer* cells, the uncouplers have to move across the plasmalemma and cytoplasm to reach their mitochondrial targets. This could explain why the uncoupling concentrations were, in this case, relatively high. In the case of isolated thylakoids, such an explanation

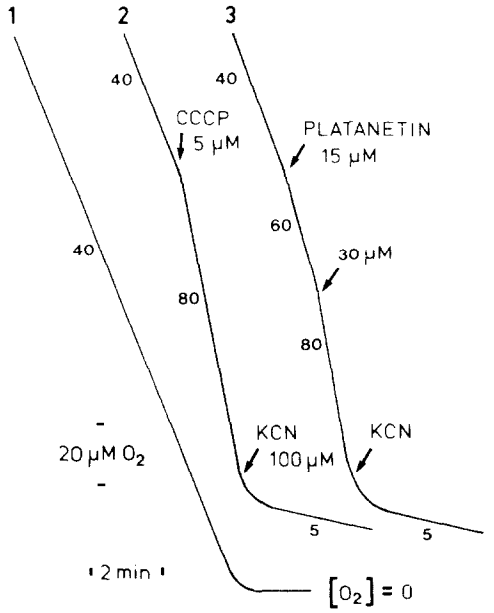

Fig. 7. Uncoupling activity of the platanetin on *Acer* cells. I: respiratory control trace; 2: respiratory control trace in the presence of CCCP; 3: respiratory trace in the presence of platanetin. Numbers on traces refer to nmol O_2 consumed/min mg protein.

Table 3. Values of the smaller concentrations (μM) giving a full uncoupling activity for different types of mitochondria, class C chloroplasts and cells

	Pl.	P.	3-OH W.
Purified potato mitochondria without BSA	5	30	100
Purified potato mitochondria with BSA	5	30	100
Unpurified mung bean mitochondria	10	50	200
Unpurified rat liver mitochondria	6	8	100
Spinach thylakoids	30	150	700
Acer cells	30	80	–

Pl.: platanetin, P.: platanin, 3-OH W.: 3-OH wogonin

cannot be considered and the lowering of the uncoupling activity when replacing mitochondria by chloroplasts would suggest that some protein components of the thylakoids were able to bind with, at least, an average affinity, to these polyphenols. In conclusion, we might suggest that platanetin and platanin, are relatively phytotoxic, when we consider their efficiency as uncouplers. However, in cell culture experiments (not shown), it seems obvious that they were not; these compounds are readily oxidized to form insoluble polymers. Uncoupling of the living cells is only a transient process and normal respiratory and metabolic activities are readily restored in the cell cultures.

EXPERIMENTAL

Solubility of the flavonols. The solubility of the flavonoids in the different media was determined, after centrifugation, by spectrophotometry.

*p*Ka *measurements.* Flavonols were dissolved in soln EtOH–H_2O (1:1) adjusted at different pHs between 3 and 9. The UV spectrophotometric shifts of bands II and I were analysed to determine the pH value for which the equilibrium between the ionized and the neutral forms was 50%.

Log P measurements. 200 ml of an aq. soln saturated in octanol were adjusted at different pHs (3, 7.2 and 9). The flavonol studied was added at a concentration reaching 10μM (in the presence of ascorbate 1mM to prevent auto-oxidation). This solution was extracted by 5 ml octanol. Then, the concentration of the flavonol was measured in each fraction by spectrophotometry. The partition coefficient P is expressed by $-\log P$.

Preparation of mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L.) and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls were prepared by methods previously described [9]. Potato mitochondria were further purified in Percoll gradients [10]. Animal mitochondria were obtained from Wistar rat livers. Animals were decapitated, exsanguinated and the livers quickly removed, washed and sliced in a cold medium (sucrose 0.25 M; EDTA 10μM; MOPS (morpholinopropan sulphonic acid) 10 mM; pH 7.4). Mitochondria were prepared using a standard method [11].

Preparation of chloroplasts. Spinach (*Spinacia oleracea* L.) leaves were homogenized in ice-cold extration medium (0.35 M mannitol, 30 mM sodium pyrophosphate, 0.15% BSA, pH 7.9). The brei was filtered and intact class A chloroplasts were prepared by centrifugation [12] and further purified on Percoll density gradient. Thylakoids (class C) were prepared by gentle osmotic shock of the suspension of intact chloroplasts just prior to experiments.

O_2 *exchange measurements.* O_2 exchange was followed polarographically at 25° using a Clark-type electrode system. For plant mitochondria, the reation medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10mM Pi buffer and in same cases 0.1% BSA. All incubations were carried out at pH 7.2. For animal mitochondria the reaction medium was KCl 0.15 M, KH_2PO_4 10 mM, $MgCl_2$ 6 mM, pH 7.2. For photosynthetic O_2 exchange measurements, the reaction medium contained 0.3 M mannitol, 2 mM EDTA, 50 mM MOPS (pH 7.6). The CO_2-dependent evolution of class A chloroplasts was measured with 0.5 mM $NaHCO_3$ and 0.1 mM Na_2HPO_4. Electron transfer in thylakoids was measured with methylviologen (1 mM) as electron acceptor, NH_4Cl (5 mM) was used as reference uncoupler. KCl 100 μM was added to prevent the activity of an eventual catalase contamination.

Uncoupling test for mitochondria. A suspension of intact mitochondria was energized. After a state III–state IV transition the uncoupling effect of a substance added at this stage corresponds to an increase in the oxidation rate. A 100% uncoupling effect was obtained when the rate of O_2 was not further stimulated by the addition of CCCP (5 μM).

Mitochondrial swelling. Mitochondrial swelling was measured, as previously described [6], by the apparent absorbance decrease at 750 nm. Reaction medium contained 100 mM NH_4NO_3 or NH_4Cl or KCl, 15 mM Tris–HCl, 0.1% BSA, pH 7.2. A rapid passive swelling was induced by uncouplers (in NH_4^+ salts) and by ionophores (in K^+ salts).

Uncoupling test for thylakoids. The uncoupling effect of the

studied products was measured on a light-driven electron flow through photosystems II+I from H_2O to methylviologen (1 mM) and through photosystem I from ascorbate (2 mM) + DCPIP (dichlorophenol indophenol: 0.1 mM) to methylviologen (1 mM) in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea: 10 μM]. The uncoupling effect corresponded to an increase in the O_2 consumption rate. A 100% uncoupling effect was obtained when NH_4Cl (5 mM) did not induce any further stimulation of O_2 consumption.

Isolated cell culture. Acer pseudoplatanus L. cells (cambium) were cultured under sterile conditions on a shaker at 25° under a dim light. The nutrient medium was prepared according to Lescure [13]. The respiratory measurements were carried out by polarography, using 7–9-day-old cells suspended in one ml of fresh nutrient medium.

Protein and chlorophyll measurements. Protein contents were determined according to ref. [14] and chlorophyll concentrations were measured as described in ref. [15].

Chemicals. Platanetin, platanin and 3-hydroxywogonin were isolated and purified from *Platanus acerifolia* Willd. As previously described [3], and were dissolved in EtOH. The concn of EtOH in the reation media never exceeded 3%.

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