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Platanetin : A Potent Natural Uncoupler and Inhibitor of the Exogenous NADH Dehydrogenase in Intact Plant Mitochondria

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ABSTRACT

Platanetin is a 3,5,7,8-tetrahydroxy, 6-isoprenyl flavone isolated from the bud scales of the plane tree (*Platanus acerifolia* Willd.). Its effects on the oxidative activities of isolated potato and mung bean mitochondria have been studied. The most noticeable effect is the selective inhibitory effect of this compound on the activity of the external NADH dehydrogenase of the inner membrane. A 50% inhibition of the NADH oxidation rate is obtained at a 2 micromolar concentration. This activity is probably due to the flavonoid structure and the high lipophilicity of platanetin associated with the presence of the isoprenyl chain. Another important effect of platanetin is its uncoupling activity on oxidative phosphorylation. The presence of easily dissociable hydroxyl groups and the high lipophilicity of platanetin allow a potent H^+ transfer through the mitochondrial inner membrane. This uncoupling activity is comparable to that of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Platanetin is therefore the most active natural uncoupler known at the present time (full uncoupling at 2 micromolar with succinate as substrate). At higher concentrations (10 micromolar and more), platanetin can transfer electrons from the mitochondrial inner membrane to O_2; the branching point of this KCN-salicylhydroxamic acid insensitive platanetin dependent oxidative pathway is located at the level of flavoproteins, no transfer occurring when succinate is the substrate. The redox properties of platanetin are in accord with such an activity.

It is now well established that flavonoid aglycones are able to modify electron transfer and the oxidative phosphorylation in mitochondria.

All flavonoids which inhibit the electron transfer act in the flavoprotein region of the respiratory chain. The inhibition observed with some particular flavonoids such as rotenone (8) or deguelin (14) is powerful and highly selective toward complex I. In the case of the C15 flavonoids widely distributed in the plant kingdom (such as kaempferol or quercetin), the inhibition of electron transfer appears to be partly selective, affecting first complex I as demonstrated with kaempferol (12), or the external NADH dehydrogenase of the inner membrane, as shown with the flavone (11). At the present time, no flavonoid inhibiting preferentially complex II has been described (20). Moreover, many flavonoids are uncouplers of the oxidative phosphorylations as reported for several chalcones (13).

Another type of interaction between mitochondria and flavonoids is pointed out in the present paper through a study of the effects of the flavonol platanetin (3,5,7,8-tetrahydroxy, 6-isoprenyl flavone; see Fig. 4) which has been extracted from buds of the plane tree (7).

MATERIALS AND METHODS

Preparation of Mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L.) and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown 5 d in the dark at 26°C and 60% RH were prepared and purified by methods previously described (6). All operations were carried out at 0 to 4°C. Following purification, the mitochondria appeared to be virtually free from extramitochondrial contamination and had a high degree of membrane intactness, as judged by EM and by low activities of the inner membrane and matrix marker enzymes (antimycin A-sensitive NADH:Cyt c oxidoreductase and malate dehydrogenase). In addition, the mitochondria were tightly coupled: the average ADP/O ratio for succinate was 1.6 and respiratory control ratio for the same substrate was about 3.

O_2 Uptake Measurements. O_2 uptake was measured at 25°C with a Clark-type O_2 electrode purchased from Hansatech Ltd. (Hardwick Industrial Estate, King's Lynn, Norfolk, U.K.). The reaction medium (medium A) contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, 0.1% (w/v) defatted BSA, and known amounts of mitochondrial protein. Unless otherwise stated, all incubations were carried out at pH 7.2.

Uncoupling test. An amount of intact mitochondria corresponding to 0.30 mg protein was suspended in medium A containing a respiratory substrate. After a state III-state IV transition, 10 μM carboxyatractyloside was added in order to inhibit the nucleotide carrier. The uncoupling effect of a substance added at this stage corresponds to an increase of the oxidation rate; 100% uncoupling effect was obtained when the rate of O_2 consumption was not further stimulated by the addition of 1 μM FCCP.

Mitochondrial swelling. Intact mitochondria were suspended in reaction medium (150 mM NH_4Cl or NH_4NO_3, 10 mM Tris-HCl pH 7.2, 0.1% BSA). Passive swelling reactions were measured by changes at 540 nm in a Beckman spectrophotometer (model 25) as previously described (9). Potato mitochondria show a slow rate of swelling in NH_4^+ salts. In these conditions a rapid passive swelling is induced by uncouplers.

Determination of E'_0[2] Cyclic Voltammetry. Electrochemical

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[2] Abbreviations: E'_0, redox potential measured at pH = 7; E''_0, values expressing the redox potential measured during oxidoreduction transfer of a molecule in the direction oxidation → reduction and in the reverse direction; ΔEp, voltage difference between the anodic and the cathodic peak values; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MOPS, 3-*N*-morpholinopropanesulfonic acid; SHAM, salicylhydroxamic acid; TPP, thiamine pyrophosphate.

measurements were made using a Princeton Applied Research (PARC) potentiostat/galvanostat (model 173D-179) coupled to a PARC model 175 universal programmer. Voltamograms were recorded on a Hewlett Packard recorder 7045 A. The working electrode was made of glass coated with indium oxide. Such an electrode allows the electrochemical study of many compounds on a large range of negative potentials. The reference electrode was an Ag/AgCl electrode in saturated potassium chloride solution. The counter electrode was a simple platinum wire (2, 18).

RESULTS

Platanetin has been shown to induce three types of effects in plant mitochondria: (a) a Cyt-independent O_2 uptake, (b) an inhibition of the electron transfer, and (c) an uncoupling activity.

Appearance of a Cyt-Independent O_2 Uptake. Under our experimental conditions, potato mitochondria oxidizing a substrate such as NADH in the presence of ADP or of an uncoupler did not exhibit a cyanide-insensitive pathway (see reference trace; Fig. 1). However, addition of 10 to 200 μM platanetin to the incubation medium triggered cyanide-insensitive O_2 consumption. The intensity of this pathway was dependent on the concentration of platanetin and represented respectively 0, 10, and 25 nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein when using 1, 10, and 100 μM platanetin with NADH as substrate (Fig. 1). With malate or α-ketoglutarate some comparable observations were made but the activity of this O_2 consumption was clearly lower (Table I). In contrast, with succinate as electron donor, no cyanide-insensitive O_2 consumption could be detected (Table I). It can be suggested that this O_2 consumption, which did not appear with succinate, corresponded to a direct by-pass of electrons from complex I or from the external NADH dehydrogenase to O_2. The lack of effect of antimycin A on this O_2 consumption was in accordance with this hypothesis. Furthermore, this platanetin-dependent O_2 uptake was insensitive to 1 mM SHAM (Fig. 1) and was therefore different from the cyanide-insensitive pathway *stricto sensu* which appears in certain conditions in potato mitochondria (5). Following the hypothesis of a direct electronic by-pass from NADH to platanetin and from platanetin to O_2, these two steps were studied separately.

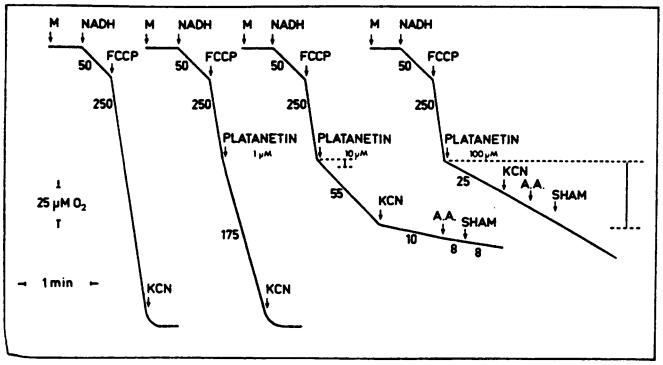

FIG. 1. Evidence for a Cyt-independent O_2 uptake induced by platanetin during the oxidation of exogenous NADH by potato tuber mitochondria. M, purified mitochondria. Substrates: NADH 1mM; KCN 100 μM; FCCP 1 μM; antimycin A (AA) 5·μM; SHAM 1 mM. Numbers on traces refer to nmol O_2 consumed/min·mg protein. The height of vertical lines refer to maximum amount of O_2 consumed by pure autooxidation of platanetin.

Table I. *Rate of O_2 Consumption through the Cyt-Independent Pathway by Potato Tuber Mitochondria Oxidizing Different Substrates in the Presence of Platanetin*

Substrates: NADH 1 mM, pH 7.2; malate 15 mM + NAD 1 mM, pH 6.5 and pH 7.5; α-ketoglutarate 5 mM + TPP 0.15 mM + NAD 1 mM + malonate 2 mM, pH 7.2; succinate 6 mM + ATP 0.3 mM. Results are given in nmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ protein.

Substrates	Platanetin Concentration	
	100 μM	200 μM
NADH	25	28
Malate (pH 6.5)	7.5	10
Malate (pH 7.5)	4	10
α-Ketoglutarate	7	7
Succinate	0	0

(a) Electron Transfer from Platanetin to O_2. In ethanolic solution with or without O_2, platanetin was maintained in the reduced form as shown spectrophotometrically. In aqueous medium, O_2 electrode measurements showed an O_2 consumption with platanetin alone; this O_2 consumption was dependent on the platanetin concentration and stopped when platanetin was completely oxidized. At the end of the reaction, the medium exhibited the appearance of a mauve color corresponding to a bathochromic shift of the platanetin spectrum.

The spectrum of reduced platanetin could be obtained in an aqueous medium only by a continuous stream of H_2. Figure 2 shows that the O_2 consumption required to oxidize platanetin was near 1 natom·O_2 for 1 nmol platanetin. If 1 mM ascorbate was added to the reaction medium, much greater amounts of consumed O_2 were measured, showing that an electron transfer from ascorbate to platanetin took place. Finally, no reduction of the oxidized form of platanetin could be obtained with NADH or malate in the same conditions (*i.e.* in a medium devoid of mitochondria).

As for simple paradihydroxylated phenols (4), the final product of platanetin oxidation was H_2O as shown by the lack of effect of an addition of superoxide dismutase and catalase.

(b) Electron Transfer from Mitochondria in an Energized State to Platanetin. As shown in Figure 1 and Table I, potato mitochondria oxidizing NADH could reduce platanetin, the oxidation of which was responsible for KCN insensitive O_2 consumption. This reaction could be maintained during a long time and the corresponding amount of consumed O_2 was several times

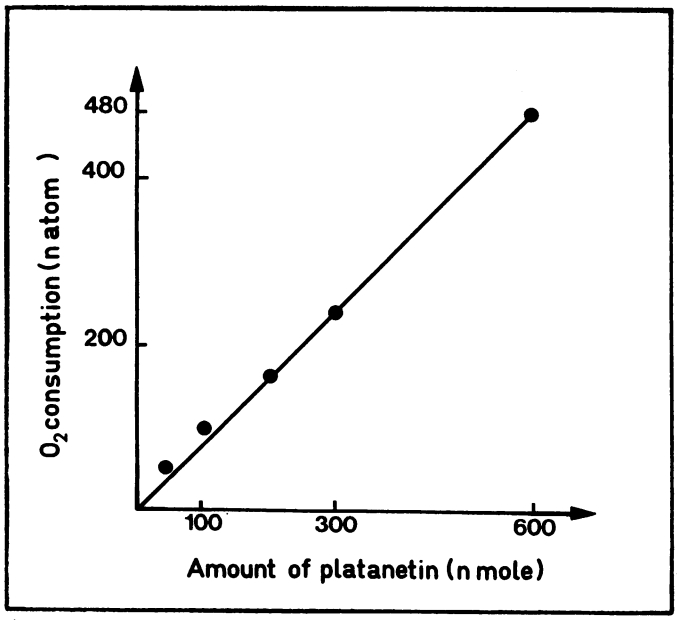

FIG. 2. Relation between O_2 consumption and amount of platanetin added in the reaction vessel. O_2 consumption measurements are carried out polarographically in electrode medium at pH 7.2.

greater than the amount of O_2 necessary to oxidize the platanetin introduced alone in the reaction vessel. The amount of O_2 uptake was only dependent on NADH availability. With malate or α-ketoglutarate, the electron transfer to platanetin was lower and no transfer occurred when succinate was the substrate (Table I). Since NADH alone was unable to reduce platanetin, the electron transfer from mitochondria to platanetin seemed therefore to take place at the level of complex I and of the external NADH dehydrogenase but not at the level of complex II.

For a better understanding of the electrochemical conditions necessary for the electron transfer, a voltametric study of platanetin was caried out (Fig. 3). The cyclic voltametry curve established at 200 $mV \cdot s^{-1}$ showed a reversible change from a reduced to an oxidized state with a cathodic peak at −0.60 V and an anodic peak at −0.05 V, the difference between the two peaks (ΔEp) being 550 mV. In contrast with methyl viologen, which is characterized by a simple electron transfer (ΔEp = 60 mV), the 550 mV ΔEp for platanetin suggested a slow overall transfer of several particles. According to its structure, it must be 2 e^- and 2 H^+. In such a case, it is probably an E.C.E.C. mechanism (1) where a proton transfer is a rate-determining step. In this situation, it is not possible to give a quasi thermodynamic standard potential value E'_0 for platanetin. An E''_0 value can be given, for the reduction of platanetin which is a maximum value: E''_0 = −0.55 V ± 0.03. During homogenous oxidoreduction transfer, a lower value should probably to be taken into account. In the reverse direction, reduction → oxidation, the potential E''_0 is about −0.075 V ± 0.03. In a homogenous phase with different intermediates, owing to proton acceptor or to donor effects, the potential to take into account could be between −0.55 and −0.075 V. These results are in accord with an electron transfer illustrated in Figure 4.

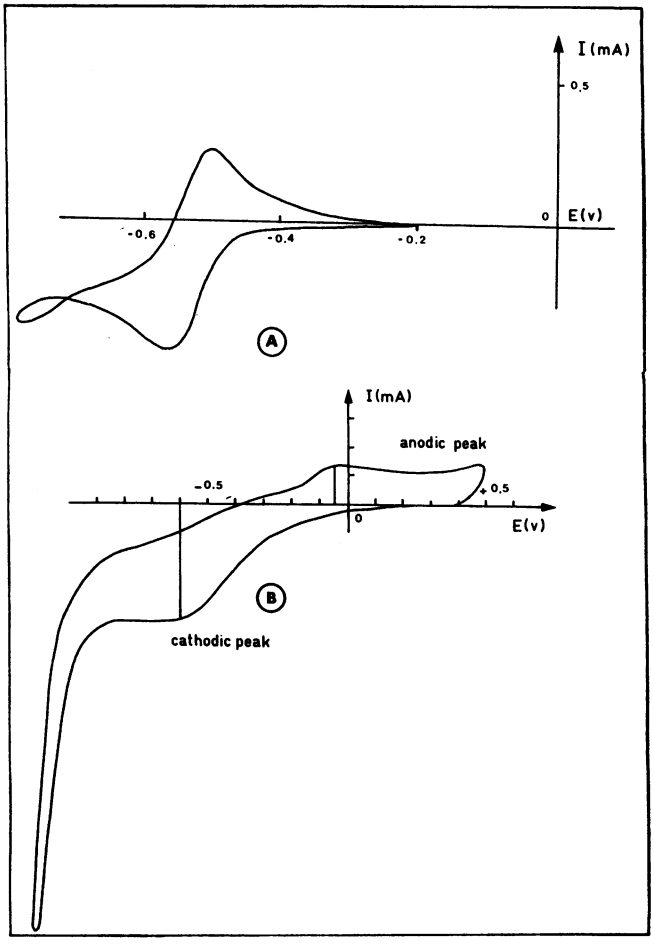

FIG. 3. Cyclic voltammetry of methylviologen (A) and platanetin (B) in aqueous medium at pH 7. Measurements are carried out in the presence of 0.2 mM MOPS, at 20 $mV \cdot s^{-1}$ for (A) and at 200 $mV \cdot s^{-1}$ for (B). Methyl viologen and platanetin were used at 300 μM.

Inhibitory Properties of Platanetin on the Mitochondrial Electron Transfer. Figure 1 shows that the electron transfer was strongly inhibited by platanetin when potato mitochondria oxidized NADH in an uncoupled state induced by the addition of 1 μM FCCP. This inhibition was much lower with other substrates such as malate, succinate or α-ketoglutarate (Table II). Table II shows that platanetin is a selective inhibitor of the external NADH dehydrogenase of the inner membrane for concentrations lower than 100 μM. The concentration of platanetin which brings about half-maximum inhibition of respiratory rates was near 2 μM. This quasi selectivity of platanetin on the exogenous NADH oxidation indicated a site of inhibition probably situated in the flavoprotein region.

The inhibitory properties of platanetin on the mitochondrial

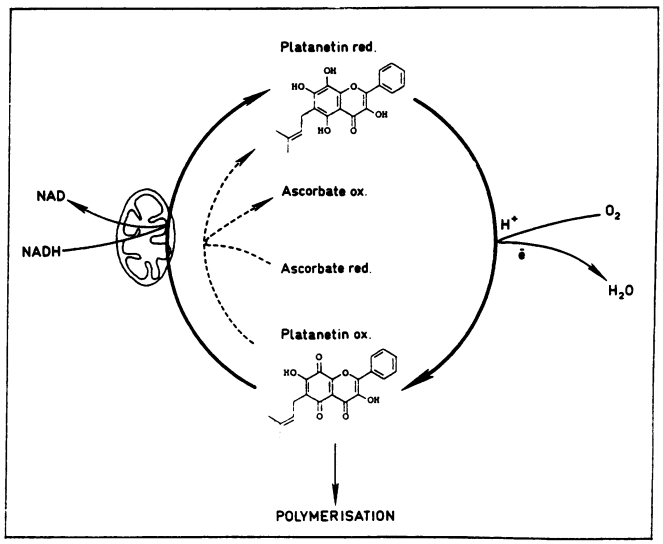

FIG. 4. Illustration of the role played in the inner mitochondrial membrane by platanetin as electron and proton carrier in an electronic by-pass transfer from an electron donor to O_2. Dotted lines show an identical redox mechanism in pure chemical conditions where ascorbate is the proton-electron donor.

Table II. *Inhibition of the Electron Transfer Induced by Platanetin during the Oxidation of Different Substrates by Uncoupled Potato Tuber Mitochondria*

Substrates: NADH 1 mM, pH 7.2; succinate 6 mM + ATP 0.3 mM, pH 7.2; malate 15 mM + NAD 1 mM, pH 7.5; α-ketoglutarate 10 mM + TPP 0.15 mM + NAD 1 mM + malonate 2 mM, pH 7.2; FCCP 1 μM. a, Percentage inhibition of the KCN-sensitive pathway; b, percentage inhibition of the total O_2 uptake.

Substrate		Platanetin Concentration						
		2	5	10	20	50	100	200 μM
NADH	a	48	65	82	90	100		
	b	48	65	78	83	90	90	
Succinate	a	0	0	0	0	0	20	63
	b	0	0	0	0	0	20	63
Malate	a	0	0	0	0	0	30	50
	b	0	0	0	0	0	25	40
α-Ketoglutarate	a	0	0	0	0	0	30	66
	b	0	0	0	0	0	25	60

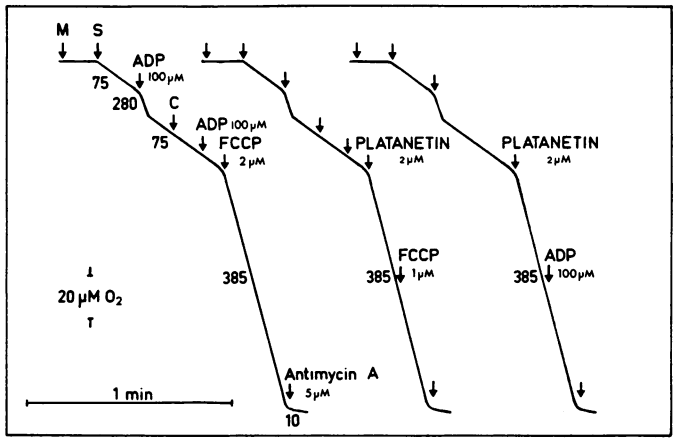

FIG. 5. Uncoupling activity of platanetin on potato mitochondria. *M*, purified mitochondria; S, succinate 6 mM + ATP 0.3 mM; C, carboxyatractyloside 10 μM.

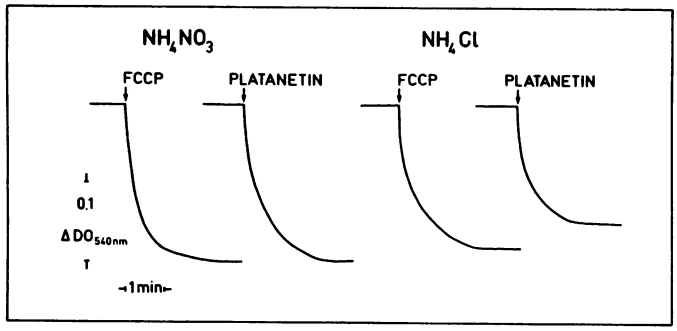

FIG. 6. Uncoupling activity of platanetin shown by mitochondrial swelling in NH_4NO_3 and NH_4Cl isoosmotic solutions. The decrease of the optical density (ΔDO) induced by platanetin for mitochondrial suspensions (0.30 mg protein for 2 ml medium) was measured at 540 nm.

electron transfer remained unchanged when using a reaction medium devoid of BSA.

Uncoupling Properties of Platanetin. Figures 5 and 6 and Table III summarize the experimental results showing the uncoupling activity of platanetin. In the conditions described in "Materials and Methods," 2 μM platanetin induced a 500% increase of the oxidation rate, which remained unchanged after addition of a classical uncoupler (FCCP 1 μM). This result indicated a full uncoupling activity of platanetin at these very low concentrations (only twice the effective concentration of FCCP). The uncoupling activity is also confirmed in Figure 5 showing that after addition of platanetin 2 μM and in the absence of carboxyatractylate, mitochondria were unable to utilize ADP, *i.e.* the tight coupling of substrate oxidation was released. At this fully uncoupling concentration, the amount of O_2 necessary for auto-oxidation of platanetin was very low and not detectable with the Clark-type electrode used.

The same uncoupling effects were obtained using NADH, malate + NAD^+ or citrate + NAD^+ as electron donor (Table III). For all substrates used, the lowest concentrations of platanetin inducing full uncoupling varied between 2 and 4 μM depending on the substrate. It is interesting to note that, at these concentrations, NADH oxidation was already subjected to partial inhibition (Table II). In this case it is, however, difficult to test the uncoupling properties of a compound in a system where this compound itself is a potent inhibitor of electron flow. For this reason, the protonophoric activity of platanetin was studied by following the swelling rate of potato mitochondria suspended in

Table III. *Uncoupling Activity of Platanetin on Potato Mitochondria Oxidizing Different Substrates*

Uncoupling values are expressed in percent of the full uncoupled rate obtained in the presence of 1 μM FCCP. Substrates: NADH 1 mM, pH 7.2; succinate 6 mM + ATP 0.3 mM, pH 7.2; malate 15 mM + NAD 1 mM, pH 6.5; citrate 5 mM + NAD 1 mM, pH 7.2.

Substrates	Platanetin Concentration					
	0.5	1	2	3	4	5 μM
NADH	38	60	100			
Succinate + ATP	32	53	100			
Malate + NAD	15	40	80	100		
Citrate + NAD	5	32	65	80	100	

an isoosmotic medium (see "Materials and Methods"). In the presence of 2 μM platanetin, mitochondria swelled rapidly, indicating a fast transmembrane transfer of H^+, induced by the presence of the flavonol. In the same conditions, platanetin was unable to induce a transmembrane transfer of K^+ (no swelling in isoosmotic KCl medium) indicating that it is a strict protonophoric agent. Identical results were obtained with mitochondria isolated from etiolated mung bean hypocotyls (results not shown).

When the reaction medium was devoid of BSA, the minimal concentration of platanetin inducing a full uncoupling was three times lower. An addition of 1 mg/ml BSA at this stage completely reversed the uncoupling effect, as we have shown previously with pentachlorophenol (16).

DISCUSSION

Our results show that platanetin is the most potent natural uncoupler known at the present time. Its efficiency was comparable to that of FCCP and was clearly better than the efficiency of some classical phenols such as dinitrophenol or pentachlorophenol (15). In the flavone-flavonol series, the comparison between the results obtained with platanetin and other flavonoids was especially interesting. The comparison between platanetin (3,5,7,8-tetrahydroxy, 6-isoprenyl flavone) and isoscutellarein (5,7,8,4′-tetrahydroxy flavone) pointed out that the 5–8 dihydroxylation was not a determining structural feature for potent uncoupling activity. In the flavone series, uncoupling properties seem to depend on the presence of one OH or more, at particular positions. The non hydroxylated flavone does not behave as an uncoupler (11), in contrast with monohydroxylated flavonoids such as the 7-OH flavone (17), the uncoupling activity of which is comparable to that of isoscutellarein. This is also the case with chalcones (13) and simple phenols (15).

The uncoupling activity seems connected with the ability of these molecules: (a) to change from a neutral to an ionized form at the level of the mitochondrial inner membrane and (b) to move in these two forms from one side to the other of the inner membrane. In these conditions, it could be suggested that the especially potent uncoupling activity of platanetin was dependent on its high lipophilic properties (presence of an isoprenyl chain) which allowed platanetin to move rapidly in its neutral or ionized form from one side to the other of the inner membrane, following the simplest scheme of Terada (19).

The ability of platanetin (at a concentration at least 10 times greater than the full uncoupling concentration) to reduce O_2 and form H_2O seems to be directly linked to the para dihydroxylation, because isoscutellarein is responsible for the same electron bypass to O_2 as platanetin in spite of the lack of an isoprenyl chain. Moreover, we have observed that no flavonoid without this para dihydroxylation possesses this property. The oxidation of these two 5,8-hydroxylated flavonoids in oxygenated aqueous medium does not depend on the presence of mitochondria. Nevertheless,

the reduction of the oxidized flavonoid form by one or several redox components of the mitochondrial inner membrane is another step of a complex mechanism responsible for the appearance of the platanetin dependent-pathway. It is possible therefore that platanetin was able to play the same role as ubiquinone itself and compete with the quinone pool for e^- and H^+. However, the fact that electrons coming from the mitochondrial succinodehydrogenase can not reduce oxidized platanetin is not in good agreement with such a hypothesis. When using exogenous or endogenous NADH, the platanetin reduction mechanism is probably directly carried out either from the external NADH dehydrogenase or from complex I.

As a whole, with a powerful uncoupling activity and the possible induction of a particular electron pathway, platanetin represents a quasi selective inhibitory of the exogenous NADH dehydrogenase with one of the best efficiencies ever mentioned for this complex. Only one natural polyphenolic compound (dicumarol) had similar inhibitory properties (3). However, no clear structural analogy appeared between these two compounds. If the structural features needed to obtain the inhibition of the complex I are at least partly known at the present time (14), the situation is much less clear concerning the inhibition of the external NADH dehydrogenase. Nevertheless it could be shown that, among the simple flavonoid series, an increasing lipophilic character decreased the inhibitor activities on complex I and increased those on the external NADH dehydrogenase (14, 20). The potent inhibitory activity of platanetin on the oxidation rate of exogenous NADH is in good agreement with this observation. Owing to the fact that this complex is located on the external face of the inner membrane, the needed lipophily may not be associated with the requirement for the molecule to move through the inner membrane as would be the case for the inhibition of complex I or complex II. It appears therefore that the binding site of the external NADH dehydrogenase for platanetin could be protected by a lipophilic area or that the binding site itself is repulsive to polar molecules. Such a situation has been described in the case of other membrane complexes: a similar structural requirement has been observed by Matsuura *et al.* (10) studying the inhibitory activities of a 3-alkyl-2-hydroxy-1,4-naphthoquinone series acting on the electron transfer at the level of the Rieske iron-sulfur cluster.

Platanetin has been extracted from *Platanus* buds. The presence of so potent an uncoupler forming a part of the glandular hairs and excreted in the bud scales needs further investigations to understand how such a toxin could be synthetized in living cells without immediately killing them. The hypothesis of the accumulation of an inactive precursor in the living hair cells is not excluded. Whatever the mode of biosynthesis of this flavonoid, it appears that its biological significance is that of a true toxin which could be as effective against animals (for instance insects) as against plant seedlings.

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