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UNCOUPLING ACTIVITY OF A SERIES OF FLAVONES AND FLAVONOLS ON ISOLATED PLANT MITOCHONDRIA

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Phaseolus aureus*; Leguminosae; mung bean; flavones; flavonols; mitochondria; uncoupler; oxidative phosphorylations.

Abstract—Twelve flavonoids tested completely uncoupled oxidative phosphorylation; ombuin was a very poor uncoupler while flavone, 5-hydroxyflavone, kaempferol and 3-hydroxy-7,3',4'-trimethoxyflavone were inactive. Full uncoupling activity was obtained for the twelve other compounds at concentrations between 2 and 500 μM. The presence of a hydroxyl was one of the necessary conditions but not sufficient on its own to cause uncoupling. Hydroxylation at the 7- and 4'-positions induced the greatest uncoupling activity; hydroxylation at the 3- and 5-positions had no effect on uncoupling activity. Glycosylation of active flavone aglycones suppressed activity. A high lipophilic character enhances the uncoupling activity. For instance the presence of an isoprenyl chain greatly increased the efficiency of uncoupling. In spite of its lipophilicity and its hydroxylation pattern, kaempferol was unexpectedly unable to uncouple the oxidative phosphorylation in potato and mung bean mitochondria. It appears that selective binding between kaempferol and some components of the mitochondrial membrane probably prevents this flavonol from being an active uncoupler.

INTRODUCTION

A number of flavonoids have been shown to affect oxidative phosphorylation in plant [1–3] as well as in animal mitochondria [4, 5]. In the case of chalcones, the disappearance of ATP synthesis in intact mitochondria was attributed to uncoupling between electron transfer and phosphorylation process [6], probably through a mechanism corresponding to the simplest scheme described by Terada [7]. Uncoupling by chalcones was not due to an inhibitory action on the mitochondrial ATPase, since experimental findings showed an inhibitory action of other flavonoids on different ATPases [8–10]. The purpose of this paper was to study whether the uncoupling activity was also caused by flavonoids other than chalcones and to obtain a better understanding of the relations between chemical structure and uncoupling activity in the flavonoid series.

RESULTS

The appearance of an uncoupling activity in the flavone–flavonol series: its relation to hydroxylation pattern

Figure 1 shows the effect of flavone and 7-hydroxyflavone on potato mitochondria oxidizing at state IV succinate 6 mM (+ATP 0.3 mM). At a concentration reaching 500 μM (above this concentration flavone precipitated out of the reaction medium) flavone was unable to prevent the increase of O_2 consumption caused by addition of ADP. Moreover, the oxidation rate of succinate at state IV, which was not affected by the addition of 500 μM flavone, was greatly increased by the addition of the well known uncoupler FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone). The only effect observed with 500 μM flavone was a slow inhibition of the electron flow (30%) when succinate was the electron donor [11]. These results demonstrate that flavone is without uncoupling activity on potato mitochondria.

In marked contrast to flavone, it could be shown that the 7-hydroxyflavone at a concentration of 100 μM greatly increased the oxidation rate of succinate at state IV. A further addition of ADP or FCCP was without effect on this consumption. A similar increase in oxidation was obtained when the ADP/ATP transport was inhibited by 10 μM carboxyatractyloside (Fig. 1).

The preceding results, which were obtained polarographically, were confirmed by a spectrophotometric method. A rapid, passive swelling of mitochondria suspended in NH_4Cl or NH_4NO_3 iso-osmotic solutions showed an H^+ transport through the inner membrane when 7-hydroxyflavone (100 μM) was added. With 500 μM flavone, such a swelling did not occur (Fig. 2). Flavone and 7-hydroxyflavone give the same polarographic and spectrophotometric results when using mung bean mitochondria (results not shown). It could therefore be concluded that, in the flavone series, the uncoupling activity was dependent on the presence of at least one hydroxyl group in the molecule.

Changes of the uncoupling efficiency in the flavone–flavonol series: its relation with lipophilicity

In the chalcone series, it was previously demonstrated that glycosylation suppresses the uncoupling properties which characterize the corresponding aglycone [6]. The

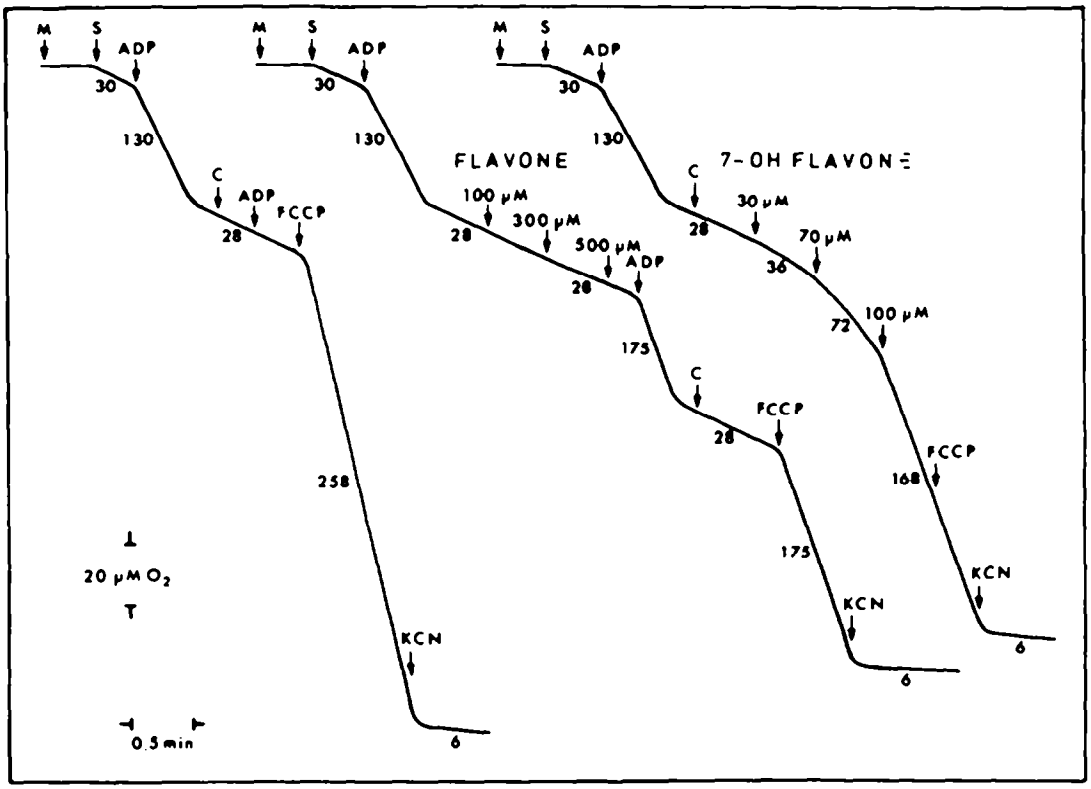

Fig. 1. Polarographic traces showing the full uncoupling activity of 7-hydroxyflavone at 100 μM and the lack of uncoupling effect of flavone. M, Purified mitochondria; S, succinate 6 mM + ATP 0.3 mM; ADP, 200 μM; c, carboxyatractyloside 10 μM; FCCP, 1 μM; KCN, 30 μM. Numbers on traces refer to nmoles O_2 consumed/min mg protein.

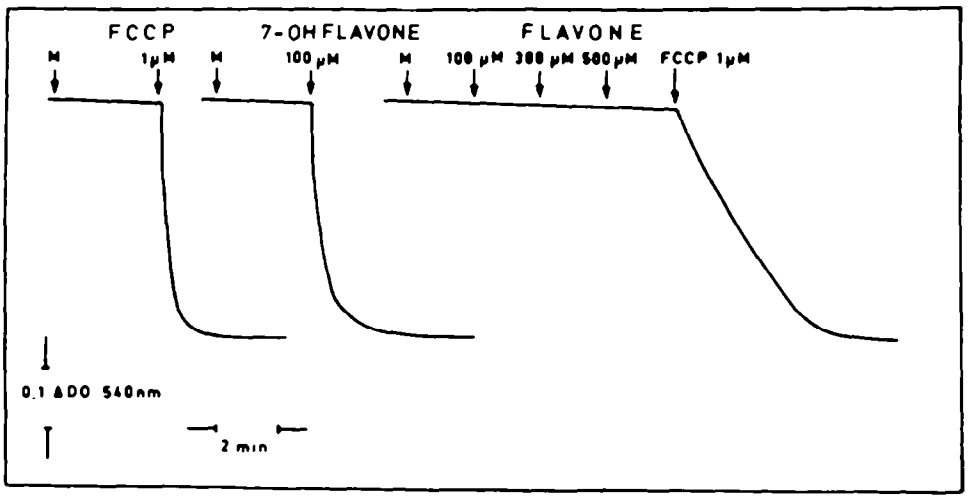

Fig. 2. Spectrophotometric results showing the protonophoric activity of 7-hydroxyflavone, and the ineffectiveness of flavone on the passive swelling mechanism of potato tuber mitochondria suspended in an iso-osmotic NH_4Cl solution. M, 0.25 mg mitochondrial protein/ml.

same has now been shown to be true in both the flavone and flavonol series. For instance, full uncoupling was obtained with 300 M quercetin; however, rutin at a concentration up to saturation had no uncoupling effect (not shown). Such a result is presumably due to the inability of the water soluble glycosides to pass through the inner mitochondrial membrane. With flavones and flavonols, the importance of lipophilic character in re-

lation to the efficiency of uncoupling is nicely illustrated by comparing the activities of 5,7,8,4'-tetrahydroxyflavone (isoscutellarein) and 3,5,7,8-tetrahydroxy-6-isoprenylflavone (platanetin: Fig. 3). The presence of an isoprenyl substituent at the 6-position of the A-ring of the platanetin increases the lipophilic character without affecting the electronic properties [12]. The titration curves of Fig. 3 show the greater uncoupling efficiency of platanetin when compared with isoscutellarein with succinate as substrate. The same results were obtained with both potato and mung bean mitochondria. It seems clear that the presence of the isoprenyl chain is responsible for a 100-fold increase in the uncoupling activity of this type of flavone; platanetin is certainly one of the best natural uncouplers known at the present time, with an efficiency comparable to that of the classical uncoupler FCCP. This result suggests that, in the case of common hydroxylated flavones, uncoupling activity is very probably low because the molecules are not sufficiently lipophilic.

Relationship between efficiency of flavones and flavonols and their substitution patterns

Of the 17 flavonoid aglycones studied, 12 were shown to induce full uncoupling under our experimental conditions (polarographic and spectrophotometric measurements) at concentrations between 2 and 500 μM. It is noteworthy that, when platanetin is excluded, the most active flavones caused complete uncoupling at a concentration near 100 μM. On the other hand with flavone, 5-hydroxyflavone, kaempferol and 3-hydroxy-7,3',4'-trimethoxyflavone, no uncoupling activity could be detected at a concentration of 500 μM (Table 1). In most cases, 500 μM was the highest concentration of flavonoid aglycones used in our experiments. At greater concentrations these compounds tend generally to precipitate out from the reaction medium, which cannot contain more than 3% ethanol without affecting mitochondrial respiration.

When comparing the chemical structures of the flavones used in this study (Table 1), it can be seen that their lipophilicity (except for platanetin) lies within a narrow range, from the most hydrophilic pentahydroxy derivatives (quercetin, isoetin), to flavone itself or its di- or trimethoxy derivatives. All these compounds have a similar solubility in the inner membrane, this solubility being an important parameter for uncoupling activity. A comparison of the results obtained using potato (Table 1) and mung bean mitochondria (results not shown) indicate that hydroxylation at the 7- or 4'-position is necessary for uncoupling activity. Thus 7-hydroxyflavone was very active. The 7-hydroxy group is known to be the most acidic and thus the most readily ionisable hydroxyl group [13]. Also the presence of a *o*-dihydroxylation seemed to increase the uncoupling activity (compare baïkalein, luteolin, scutellarein, isoscutellarein, fisetin, quercetin, isoetin). By contrast, hydroxylation at the 3- or 5-position does not contribute to uncoupling activity. Indeed, both 5-hydroxyflavone and 3-hydroxy-7,3',4'-trimethoxyflavone are inactive.

When considering the lipophilicity and the presence of 'active dissociable' hydroxyl groups (the best positions being the 7 and 4'), almost all the results indicate a common mechanism for the uncoupling activity of these flavonoids. Kaempferol was unexpectedly inactive with potato and mung bean mitochondria. The 3-hydroxyl seems to be the cause of this inactivity since the related flavone apigenin is active. However, it must be pointed out that kaempferol has been described as an uncoupler in etiolated corn seedlings mitochondria [2].

DISCUSSION

The uncoupling activity of flavones and flavonols appears to depend on two factors: on a sufficient lipophilicity permitting a molecular movement from the external face of the inner mitochondrial membrane to the internal face, and a movement of the corresponding anion in the opposite direction; and on the presence of hydroxyl groups which can be dissociated in the environmental conditions pertaining in the membrane. Therefore, the uncoupling activity of the flavones or flavonols does not differ from the uncoupling activity of simple chlorinated phenols [14]. Our previous results concerning the uncoupling effects of these latter substances on isolated plant mitochondria have permitted the establishment of some equations using a quantitative structure–activity relation-

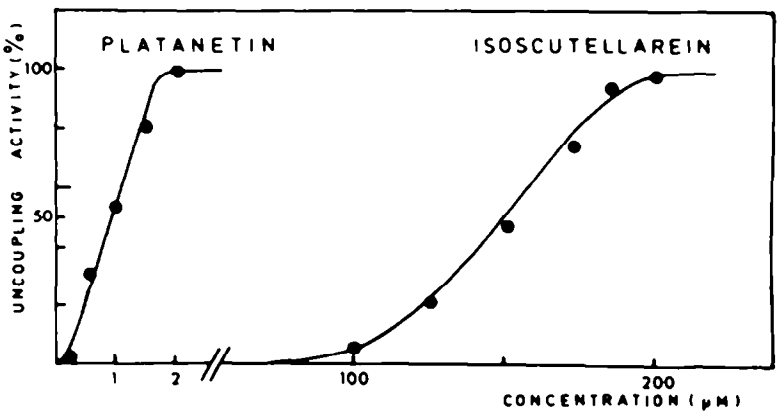

Fig. 3. Comparison of the uncoupling activity of platanetin and isoscutellarein: titration curves showing the percentage of uncoupling activity on potato mitochondria oxidizing succinate.

Table 1. Uncoupling activity of flavones and flavonols on potato tuber mitochondria

Compounds											Uncoupling activity (%)					
	Ring positions										Concentration (μM)					
No	3	5	6	7	8	2'	3'	4'	5'	Common name	2	100	200	300	400	500
1	—	—	—	—	—	—	—	—	—	Flavone						0
2	—	OH	—	—	—	—	—	—	—	—			100			
3	—	—	—	OH	—	—	—	OH	—	—		100				
4	—	—	—	OH	—	—	—	—	—	Chrysin						100
5	—	OH	—	OH	—	—	—	OH	—	Apigenin						100
6	—	OH	OH	OH	—	—	—	—	—	Baïkalein		100				
7	—	OH	—	OH	—	—	OH	OH	—	Luteolin			100			
8	—	OH	OH	OH	—	—	—	OH	—	Scutellarein					100	
9	—	OH	—	OH	OH	—	—	OH	—	Isoscutellarein			100			
10	—	OH	—	OH	—	—	—	OH	—	Kaempferol						0
11	OH	OH	—	OH	—	—	—	OH	—	Fisetin				100		
12	OH	—	—	OH	—	—	OH	OH	—	—						0
13	OH	—	—	MeO	—	—	MeO	MeO	—	Quercetin				100		
14	OH	OH	—	OH	—	—	OH	OH	—	Isoetin			100			
15	—	OH	—	OH	—	OH	—	OH	OH	Ombuin						30
16	OH	OH	—	MeO	—	—	OH	MeO	—	Platanetin	100					
17	OH	OH	iP	OH	OH	—	—	—	—							

Key: iP = isoprenyl substituent.

ship. The best equation for uncoupling activity in the simple chlorinated phenol series is:

$$\log 1/D_{50} = 66.923\,\Sigma D - 12.131\,(\Sigma D)^2 + 0.881\,\sigma_1 - 0.324\,A - 84.284$$
$$r = 0.955 \quad s = 0.22 \quad F = 36.46 \quad n = 19$$

where D_{50} = the concentration giving 50% of the full uncoupling activity obtained with 1 μM FCCP; ΣD = a steric parameter previously described [14] and representing the perimeter of the molecule projected onto the aromatic cycle plane; σ_1 = the Hammett constant; A = a steric parameter representing the angle between the phenolic hydroxyl and the 2 and 6 substituents; r = the coefficient of correlation; s = the standard error; F = the statistical test and n = the number of molecules used to establish the equation.

In this study there is no direct relationship between the lipophilic parameter $\log P$ (logarithm of the partition coefficient octanol–water) and the uncoupling activity. However, $\log P$ and ΣD are partially linked ($r = 0.570$). Thus, it can be suggested from this equation that an appropriate electronic activity, expressed by the parameter σ, and a certain lipophilicity taken into account by ΣD, are necessary, but are not sufficient to explain uncoupling activity. That would mean clearly that the steric character of the molecule, expressed by ΣD and A, has a direct effect on uncoupling efficiency. A similar analysis can be done for kaempferol, which is inactive with isolated potato as well as on mung bean mitochondria [15]. Indeed, apigenin itself (5,7,4'-trihydroxyflavone) appears to be a poor uncoupler when compared to the 7- and 4'-hydroxyflavone. Perhaps this type of hydroxyl substitution gives a partial binding to membrane proteins, which could be greatly increased by a further hydroxyl in the 3-position (kaempferol), causing the disappearance of an uncoupling effect. Thus it may be supposed that not only electronic and lipophilic parameters (respectively as σ and $\log P$) but also steric parameters have to be taken into account in understanding the uncoupling efficiency of flavones and flavonols.

When comparing the uncoupling properties of flavones and flavonols with those of chalcones [6], two major differences can be pointed out. In the chalcone series substitution in the B-ring is unimportant, whereas in the flavone series, 4'-hydroxylation or 3',4'-dihydroxylation enhances uncoupling activity. In the chalcone series, hydroxylation at the 2'- or 4'-positions in the A-ring clearly determines uncoupling activity. In the flavone series, 7-hydroxylation of the A-ring (corresponding to the 4'-position in the chalcone nucleus) is not the only determinant. These differences between chalcones and flavones can probably be explained by variations in the electronic properties of the different hydroxyl substituents.

The case of platanetin is of particular interest. It may be suggested that its enhanced lipophilic properties means that it passes rapidly through the inner membrane. However, it must be noted that increasing the lipophilic properties does not always increase uncoupling efficiency. Thus, the uncoupling activity of quercetin is reduced by methylation of the 7- and 4'-hydroxyl groups (compare ombuin). In the chalcone series, the uncoupling activity of 2'-hydroxychalcone is not increased by methylation in the A-ring (2'-hydroxy-5'-methylchalcone) or in the B-ring (2'-hydroxy-4-methylchalcone). Therefore, we may assume that the steric characters of platanetin are especially favourable to its intramembranar mobility. In this sense, it is analogous in structure with several natural membrane quinones. From a physiological point of view, platanetin is also especially interesting. In contrast with other common flavones, which are usually present as glycosides in cell vacuoles, free platanetin is excreted by the glandular hairs of plane tree bud scales. The uncoupling activity of this compound makes it highly toxic, and its effect on living scales and on young leaves of the planetrees would be interesting to study.

EXPERIMENTAL

Preparation of mitochondria. Mitochondria from potato tubers (*Solanum tuberosum* L.) and etiolated Mung bean (*Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown 5 days in the dark at 26° and 60% relative humidity were prepared and purified by methods previously described [16]. All operations were carried out at 0–4°. Following purification, the mitochondria appeared to be virtually free from extramitochondrial contamination and their membranes were found to be almost fully intact, as judged by electron microscopy 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: average ADP/O ratio for succinate was 1.8 and respiratory control ratio for the same substrate was about 3. Protein was determined using BSA (Sigma, fraction V) as the standard.

O_2 uptake measurements. O_2 uptake was followed polarographically at 25° using a Clark-type electrode system purchased from Hansatech Ltd (Hardwick Industrial Estate, Kings Lynn, U.K.). The reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, known amounts of mitochondrial proteins and in some cases 0.1% defatted BSA. Unless otherwise stated, all incubations were carried out at pH 7.2.

Uncoupling test. Intact mitochondria were suspended in the electrode medium containing a 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 corresponded to an increase in the oxidation rate: 100% uncoupling effect was obtained when the rate of O_2 consumption was not further stimulated by the addition of FCCP (1 μM).

Mitochondrial swelling measurements. Intact mitochondria were suspended in a reaction medium (NH_4Cl, NH_4NO_3 or KCl 150 mM, Tris–HCl 10 mM, pH 7.2). Passive swelling reactions were measured as absorbance decreases at 540 nm in a Kontron spectrophotometer (model UVIKON 810) as previously described [17]. A rapid passive swelling was induced by uncouplers (in NH_4^+ salts) and by valinomycin-like ionophores (in K^+ salts).

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