Phytochemistry, Vol. 21, No. 12, pp. 2845–2850, 1982.
Printed in Great Britain.

0031-9422/82/122845-06$03.00/0

UNCOUPLING ACTIVITIES OF CHALCONES AND DIHYDROCHALCONES ON ISOLATED MITOCHONDRIA FROM POTATO TUBERS AND MUNG BEAN HYPOCOTYLS

PATRICK RAVANEL, MICHEL TISSUT* and ROLAND DOUCE*

Laboratoire de Pharmacognosie et *Laboratoire de Physiologie cellulaire végétale, ERA au CNRS no. 847, Université Scientifique et Médicale de Grenoble, B.P. 53 X, 38 041 Grenoble Cédex, France

(*Revised received* 25 *March* 1982)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Phaseolus aureus*; Leguminosae; mung bean; chalcone; dihydrochalcone; mitochondria; uncoupler; phosphorylation; flavoprotein.

Abstract—The uncoupling properties of 23 chalcones and dihydrochalcones were studied. Twelve compounds completely uncouple oxidative phosphorylation in mung bean hypocotyl and potato tuber mitochondria, four are weak uncouplers and seven are without effect. Usually, mung bean mitochondria are more sensitive to uncoupling agents than potato mitochondria. The uncoupling activity of chalcones and dihydrochalcones appears to be connected with the presence of hydrogen or hydroxyl groups in the 2′-position and hydrogen, hydroxyl or nitrate groups in the 4′-position. The α–β-unsaturated carbonyl system is not essential for activity. For the compounds which are not very lipophilic, substituents on the B-ring are without effect on the uncoupling properties. Phloretin appears to be an active uncoupler; its 6′-glucoside is without effect.

INTRODUCTION

Chalcones and dihydrochalcones are flavonoids with two aromatic rings joined by a three-carbon chain which is unsaturated (chalcones) or saturated (dihydrochalcones). In the plant kingdom, chalcones are well represented in Compositae, Oxalidaceae, Scrophulariaceae, Gesneriaceae, Acanthaceae, Liliaceae and dihydrochalcones in Rosaceae, Ericaceae, Caprifoliaceae. These compounds have been found in different plant parts, particularly in the corolla where they contribute to the pigmentation. Flavonoids used in this work (17 chalcones and six dihydrochalcones) were either natural or synthetic; among the latter are some which do not occur naturally.

Most of the flavonoids previously studied are inhibitors of electron transfer, generally acting at the flavoprotein level. with variable efficiency. Rotenone is a very potent inhibitor acting at one of the several non-haem iron centres associated with the internal NADH dehydrogenase complex [1]. Other flavonoids such as kaempferol or flavone act more or less efficiently at both flavoprotein regions corresponding to the internal and external NADH dehydrogenase complexes [2, 3]. In contrast with what has been described for other flavonoid classes (flavonols, flavones, isoflavones) [4], we have found that some chalcones and dihydrochalcones have uncoupling properties. For this reason, we have attempted to study the relation between chemical structure and the uncoupling activity of these flavonoids.

RESULTS

We have analysed in detail the uncoupling properties of one of the simplest chalcones: 2′-hydroxychalcone. With succinate (6 mM) as substrate, addition of 2′-hydroxychalcone (250 μM) to potato mitochondria at state IV increases the respiratory rate to 230%. After this, addition of either ADP (200 μM) or a classical uncoupler such as FCCP (1 μM) remains without effect on the respiratory rate. Moreover, the acceleration of the respiratory rate induced by 250 μM 2′-hydroxychalcone appears when the nucleotide carrier is inhibited by carboxyatractyloside (Fig. 1). These results demonstrate that 2′-hydroxychalcone (250 μM) is able to completely uncouple the oxidative phosphorylations of potato mitochondria with succinate as substrate. Similar results are obtained with α-ketoglutarate (5 mM) or citrate (5 mM) as substrate (Fig. 1). However, with NADH (1 mM) or malate (15 mM), 2′-hydroxychalcone (250 μM) causes an important inhibition of the electron flow which prevents the appearance of the uncoupling effects (results not shown). In these conditions, FCCP (1 μM) or DNP (30 μM) also remain without effect on the oxygen consumption rate.

Figure 2 shows that the uncoupling effect on potato mitochondria appears at concentrations between 100 and 200 μM; a 50% effect is obtained with 200 μM. The respiratory rates obtained with FCCP(1 μM)

Abbreviations: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; FCCP, carbonyl cynaide *p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol.

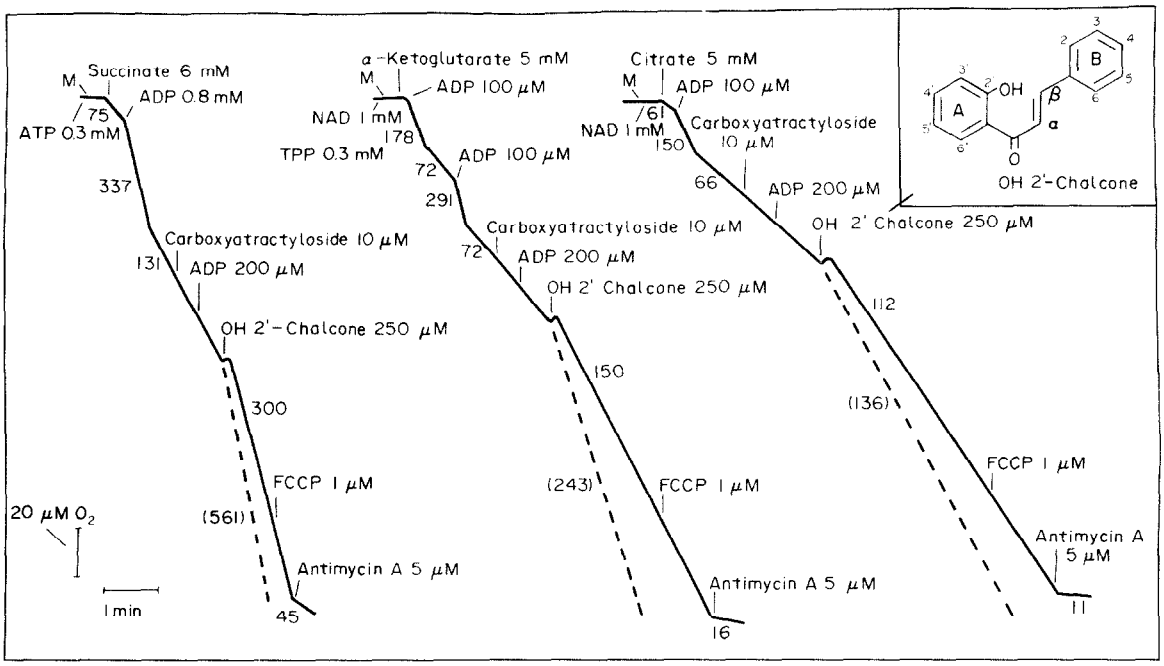

Fig. 1. Uncoupling activity of 2'-hydroxychalcone on potato mitochondria oxidizing succinate, α-ketoglutarate and citrate. Dotted lines show uncoupling effects obtained with FCCP (1 μM) on a state IV after action of 10 μM carboxyatractyloside (= reference traces). Results are the same with or without bovine serum albumin (0.1%) in the electrode medium. Numbers on traces refer to nmol O_2 consumed/min/mg protein. M = purified mitochondria.

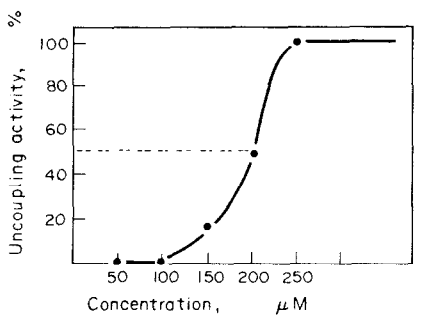

Fig. 2. Titration of 2'-hydroxychalcone: % of uncoupling activity on potato mitochondria oxidizing succinate.

alone are greater than with 2'-hydroxychalcone (250 μM) plus FCCP. This result shows that even with succinate as substrate, a small inhibition of the electron flow is induced by the chalcone. Similar results are obtained with mung bean hypocotyl mitochondria. However, potato tuber mitochondria are less sensitive to 2'-hydroxychalcone than mung bean hypocotyl mitochondria (results not shown). We have investigated the uncoupling properties of 22 other chalcones and dihydrochalcones using succinate as substrate with which inhibition by chalcones is generally very low. Among the 23 compounds studied, 12 have full uncoupling properties at a concentration range of 70–500 μM; seven are completely inactive in our conditions; four are more or less good uncouplers; they give a 100% uncoupling effect with mung bean mitochondria but at the same concentration only give a 15–30% uncoupling effect on potato mitochondria. A much higher concentration would certainly be needed for a 100% effect but the compounds precipitate out from the reaction medium. Similarly, for the 12 fully uncoupling compounds, the sensitivity of mung bean mitochondria is always stronger than that of potato (Table 1). Using the results obtained with 2'-hydroxychalcone, we have studied the possible changes in uncoupling efficiency following the introduction into this structure of different substituents.

The study of 17 different chalcones shows the following results.

(*a*) *Modifications in the B-ring.* If the 4-position is hydroxylated (no. 9) or methylated (no. 2), the uncoupling activity is maintained as well as for 2'-hydroxychalcone. It is also true when 2'-hydroxychalcone is substituted with a methylenedioxy group in the 3–4-position plus a hydroxyl in the β-position (**4**). On the other hand, a proton in the 2'-position plus methoxylation at 4 (**6**) considerably decreases the uncoupling efficiency.

(*b*) *Modifications in the A-ring.* When a nitrite or a methyl group are linked at the 4'-position (**14**) or 5'-position (**15** and **3**), no important modification occurs in the uncoupling activity of 2'-hydroxychalcone. In contrast, if methoxylations occur at the 4'-(**13**) or 4'- and 6'-positions (**16**), the uncoupling activity decreases. 4'-Methoxylation plus a proton at the 2'-position (**5**) also leads to a decrease in uncoupling efficiency. Finally, uncoupling activity disappears

Table 1. Uncoupling activity of chalcones and dihydrochalcones on potato tuber and mung bean hypocotyl purified mitochondria

	Compounds											Uncoupling activity			
	Ring positions											Potato		Mung bean	
No.	2′	3′	4′	5′	6′	3	4	α	β	Common name	O	(C) · 10^{-4}M	%	(C) · 10^{-4}M	%
Chalcones															
1	OH	—	—	—	—	—	—	—	—	—	S	2.5	100	1	100
2	OH	—	—	—	—	—	Me	—	—	—	S	3	100	1	100
3	OH	—	—	Me	—	—	—	—	—	—	S	2.5	100	1	100
4	OH	—	—	—	—	O–CH_2–O	—	—	OH	—	S	2.5	100	1	100
5	—	—	MeO	—	—	—	—	—	—	—	N	5	30	2	100
6	—	—	—	—	—	—	MeO	—	—	—	S	4	20	3	100
7	OH	Me	MeO	—	MeO	—	—	—	—	—	S	3	0	3	0
8	OH	—	MeO	—	MeO	MeO	Meo	MeO	—	—	S	4	0	3	0
9	OH	—	—	—	—	—	OH	—	—	—	S	1.5	100	0.75	100
10	OH	—	OH	—	—	—	OH	—	—	Isoliquiri-tigenin	N	2.5	100	2	100
11	OH	—	OH	—	—	OH	OH	—	—	Butein	N	2.5	100	2.5	100
12	OH	OH	OH	—	—	OH	OH	—	—	Okanin	N	2.5	100	1.5	100
13	OH	—	MeO	—	—	—	—	—	—	—	S	3	25	2.5	100
14	OH	—	NO_2	—	—	—	—	—	—	—	S	2	100	1.5	100
15	OH	—	—	NO_2	—	—	—	—	—	—	S	2.5	100	2	100
16	OH	—	MeO	—	MeO	—	—	—	—	—	N	4	15	3	100
17	OH	—	NH–CO–Me	—	—	—	—	—	—	—	S	3	0	3	0
Dihydrochalcones															
18	OH	—	OH	—	OH	—	OH	—	—	Phloretin	N	5	100	5	100
19	OH	—	—	—	—	—	MeO	—	O	—	S	2	100	1	100
20	OH	—	MeO	—	—	—	MeO	—	O	—	S	5	0	2	0
21	OH	—	–CH_2 \ $(CH_2)_2$ / SO_3Na	—	—	OH	MeO	—	—	—	S	10	0	10	0
22	OH	—	MeO	MeO	MeO	–O–CH_2 | Ph	MeO	—	O	—	S	2	0	2	0
23	OH	—	OH	—	O–Gl	—	OH	—	—	Phlorizin	N	10	0	10	0

O, origin; S, synthetic compound; N, natural compound.

if 2'-hydroxychalcone is substituted with NHCOMe at the 4'-position (**17**) or methoxylated at the 4' and 6'-positions plus methylation at the 3'-position (**7**).

(*c*) *Modifications in the A- and B-rings.* Hydroxylation of 2'-hydroxychalcone in the 4'- and 4- (**10**), in 4'- , 3- and 4- (**11**) or in 3'-, 4'-, 3- and 4-positions (**12**) do not modify its uncoupling activity. By contrast, methoxylation at the 4', 6'-, 3- and 4-positions (**8**) causes a complete loss in uncoupling activity.

Among the five dihydrochalcones tested, three have no effect as uncouplers (**20–22**). Of the two dihydrochalcones with uncoupling activities, one (**19**) has the same A-ring as 2'-hydroxychalcone and the same uncoupling efficiency. By contrast, a great concentration (500 μM) of phloretin (**18**) is needed to effect uncoupling completely. Glycosylation at the 6'-position of phloretin, causes a loss of uncoupling activity (**23**).

In addition, we have studied the inhibitory effects of chalcones and dihydrochalcones on the electron flow of mitochondria, using malate, succinate and NADH as substrates in states III or IV. Most of the chalcones, and dihydrochalcones analysed more or less inhibit oxidation of malate, NADH and succinate in mung bean hypocotyl and potato tuber mitochondria. Generally the inhibition of succinate oxidation is very low in contrast with those obtained with malate or NADH. Some chalcones or dihydrochalcones give maximal inhibition with NADH (**2–7**, **13**, **14**, **16** and **19**) and for others, the best inhibition is obtained with malate. (**1**, **10–12**, **17** and **18**). For some other compounds, oxidation rates of malate, NADH or succinate are practically not affected (**8**, **15** and **20–23**).

All these flavonoids are without effect on the oxidative rates when ascorbate plus *N*, *N*, *N'*, *N'*-tetramethyl-*p*-phenylenediamine is the substrate. This substrate fits in the respiratory chain at the level of the cytochrome *c* in the electron pathway. The chalcones and dihydrochalcones tested do not affect this part of the respiratory chain between cytochrome *c* and cytochrome oxidase. In the same way, difference spectra of chalcone- and dihydrochalcone-treated mitochondria (mitochondria plus malate plus flavonoid aerobic minus mitochondria malate aerobic) at liquid N_2 temperature do not show the classical three α-peaks in the *b*-region at 553, 557 and 562 nm (Fig. 3). These results demonstrate that these flavonoids inhibit the electron flow before the well-known antimycin A block. Moreover, in some cases, the inhibitions differ greatly with the nature of the substrate used. Succinate is often particularly resistant to flavonoid inhibition. Such inhibitions also appear when the alternate pathway of electron transport to molecular oxygen, which is insensitive to potassium cyanide, is operating alone (mung bean). Consequently, the inhibition seems to take place before the branching point between the cytochrome oxidase pathway and the alternate oxidase pathway, probably at the level of complex I or of the flavoprotein involved in external NADH oxidation.

DISCUSSION

As with the other flavonoids previously studied [3–4], chalcones and dihydrochalcones are respiratory inhibitors for plant mitochondria. Inhibitions are probably located at the flavoprotein level. Most of the flavonoids tested preferentially inhibit either the internal or the external NADH dehydrogenase. However, none of the flavonoids used here are as efficient or as specific as rotenone [1].

Among phenolics, some phenols and substituted anthracene derivatives have been described as uncouplers [6–7]. Stenlid first showed that several flavonoids strongly inhibit ATP formation in isolated cucumber mitochondria [8–11]. Two reports describe kaempferol as an uncoupler [11–12]. In our conditions we found that neither kaempferol nor flavone increase mitochondrial respiration in the presence of carboxyactractyloside. In contrast, among the chalcones and dihydrochalcones tested here, 16 compounds clearly uncoupled oxidative phosphorylation at concentrations between 70 and 500 μM. These concentrations are higher than those used with FCCP (1 μM) or DNP (30 μM) which are powerful uncouplers commonly employed.

Among the 23 chalcones and dihydrochalcones studied, seven are without uncoupling properties even for concentrations reaching saturation in the reaction medium. The uncoupling properties seem to be linked to some structural characteristics of the chalcones and dihydrochalcones. The nature and position of the substituents in the A-ring seem to play an important role: when hydrogen or hydroxyl groups are present at the 2'-position and when hydrogen, hydroxyl or nitrite groups are linked to the 4'-position, the compound is a powerful uncoupler. A 4'-methoxyl lowers the effect but the presence of a 5'-methyl does not change the uncoupling properties. Compounds such as 2'-hydroxy,-4', 6', 3, 4, α-pentamethoxychalcone (**8**), which are strongly lipophilic, are without effect. In contrast, more hydrophilic compounds may be good uncouplers and, in this case, the effect remains unchanged whatever substituents are on the B-ring (e.g. 2'-hydroxy-4-methylchalcone and 2'-hydroxy-4-methoxy-β-carbonyldihydrochalcone). As for chalcones, suitable substitutions on the 2'- and 4'-positions of dihydrochalcones induce an uncoupling effect. Therefore, the α, β-double bound is unnecessary. Nevertheless, the intensity of the effects is generally better when the double bond is present. For example, a 500 μM concentration is needed for a 100% effect with 2', 4', 6', 4-tetrahydroxydihydrochalcone (**18**) whereas a 200 μM concentration is sufficient for the same effect with 2', 3', 4', 3, 4-pentahydroxychalcone. The spatial arrangement of the dihydrochalcone molecule is perhaps important. Thus, the 2'-hydroxydihydrochalcone with a β-carbonyl is one of the most efficient compounds studied. Only one glycoside has been studied at present; 6'-glucosylation of the 2', 4', 6', 4-tetrahydroxydihydrochalcone nullifies its uncoupling activity.

Among the 16 uncoupling chalcones or dihydrochalcones tested, at least six are natural products, [13–14]. Thus, it appears that some plants are able to synthesize compounds which could probably be toxic to their own mitochondria. Consequently, there must be physiological or structural compartmentation. Nevetheless these results shown that a relatively high concentration is required for the uncoupling effect; moreover, glycosylation would induce the disappearance of the uncoupling property. For several plants, free aglycones with uncoupling properties are

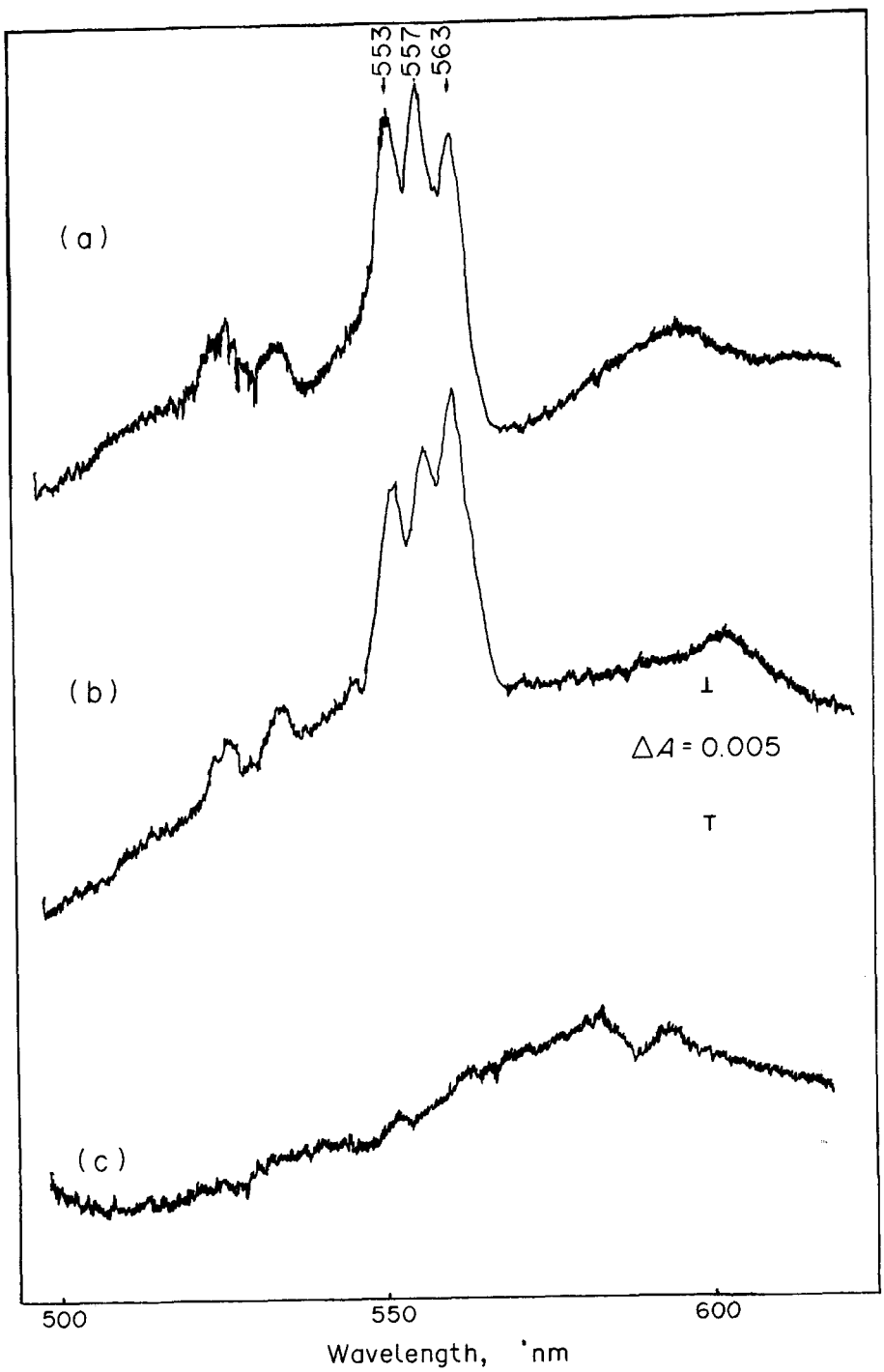

Fig. 3. Difference spectra of potato mitochondria at liquid N_2 temperature (77K). (a) Malate (20 mM) + antimycin A (10 mM) aerobic minus malate (20 mM) aerobic. (b) Malate (20 mM) + antimycin A (10 μM) + 2'-hydroxychalcone (100 μM) aerobic minus malate (20 mM) + 2'-hydroxychalcone (100 mM) aerobic. (c) Malate (20 mM) + 2'-hydroxychalcone (100 μM) aerobic minus malate (20 mM) aerobic. Mitochondria were suspended in the reaction medium. Optical path, 2 mm (Plexiglass cuvettes). Mitochondrial protein 5 mg/ml.

produced in relatively large quantities; these substances could be considered in this case as true toxins. For example, this is the case of phloretin in the apple tree roots [15] and of okanin (**12**) in the heartwood of *Cylicodiscus gabunensis* [16]. These products could have an ecological significance, acting on surrounding plants, micro-organisms or animals.

EXPERIMENTAL

Preparation of mitochondria. Mitochondria from potato (*Solanum tuberosum* L.) tubers and etiolated mung bean (*Phaseolus aureus* Roxb.) hypocotyls cut from bean seedlings grown from 5 days in the dark at 26° and 60% r.h. were prepared and purified by methods previously described [17]. All operations were carried out at 0–4°. Following purification, the mitochondria appeared to be virtually free from extra-mitochondrial contamination and had a high degree of membrane intactness, as judged by electron microscopy and by low activities of the inner membrane and matrix marker enxymes (antimycin A sensitive NADH: cytochrome *c* oxidoreductase and malate dehydrogenase) [17]. 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 *ca.* 3.

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 Pi buffer, 0.1% defatted bovine serum albumin and known amounts of mitochondrial proteins. Unless otherwise stated, all incubations were carried out at pH 7.2.

Split beam spectrophotometry. This was performed with the Aminco DW-2 spectrophotometer between 500 and 600 nm, corresponding to the α- and β-bands of the *b* cytochromes. The concns of the different *b* cytochromes were measured at liquid N_2 temp. (77°K) from reduced minus oxidized difference spectra. Plexiglass cells (0.2 cm light path) were used [18].

Uncoupling test. An amount of intact mitochondria corresponding to 0.30 mg protein was suspended in the electrode medium containing 6 mM succinate and 300 μM ATP. 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 FCCP (1 μM). After each assay, the incubation medium was rapidly centrifuged at 10 000 *g* for 3 min (Beckman Microfuge B). Concn of flavonoids was determined in the supernatant by spectrophotometry. We have also verified that our compounds are not oxidized by the mitochondria.

Chemicals. Phloretin (**18**) and phloridzin (**23**) were purchased from Karl Roth (Karlsruhe, Germany). All other products were generous gifts from Dr. A. Grouiller (Lyon, France) (**1–3**, **9**, **10**, **13–15** and **17**), from Professor J. Chopin, and Dr. M. Chadenson (Lyon, France) (compounds **7**, **8**, **12**, **16**), from Professor N. Saito (Tokyo, Japan) (**11**), from Professor L. Farkas and Dr. J. Strelisky (Budapest, Hungria) **4–6** and **19–22**). All these products were dissolved in EtOH. The concn of EtOH in the reaction medium never exceeded 3%. At this concn EtOH alone is almost without effect on mitochondrial respiration under our conditions (presence of bovine serum albumin). Usually, 500 μM was the upper concn of flavonoids used in our expts. For greater concns these compounds tend generally to ppt out from the reaction medium.

REFERENCES

1. Ernster, L., Dallner, G. and Azzone, G. F. (1963) *J. Biol. Chem.* **238**, 1124.
2. Ravanel, P., Tissut, M. and Douce, R. (1981) *Phytochemistry* **20**, 2101.
3. Ravanel, P., Tissut, M. and Douce, R. (1982) *Plant Physiol.* **69**, 375.
4. Tissut, M., Chevallier, D. and Douce, R. (1980) *Phytochemistry* **19**, 495.
5. Neuburger, M. and Douce, R. (1980) *Biochim. Biophys. Acta* **589**, 176.
6. Van Sumere, C. F., Cottenic, J., De Greef, J. and Kint, J. (1972) in *Recent Advances in Phytochemistry* Vol. IV p. 165. Appleton–Century–Crofts, New York.
7. Verhaeren, E. H. C. (1980) *Phytochemistry* **19**, 501.
8. Stenlid, G. and Saddik, K. (1962) *Physiol. Plant.* **15**, 369.
9. Stenlid, G. (1963) *Physiol. Plant.* **16**, 110.
10. Stenlid, G. (1968) *Physiol. Plant.* **21**, 882.
11. Stenlid, G. (1970) *Phytochemistry* **9**, 2251.
12. Koeppe, D. E. and Miller, R. J. (1974) *Plant Physiol.* **54**, 374.
13. Muti, P. B. R. and Seshadri, T. R. (1941) *Chem. Zentralbl.* **II**, 621.
14. Bate-Smith, E. C. and Swain, T. (1953) *J. Chem. Soc.* 2195.
15. Rochleder, F. (1866) *J. Praktische Chem.* **98**, 205.
16. King, F. E. and King, T. J. (1951) *J. Chem. Soc.* 569.
17. Douce, R., Christensen, E. L. and Bonner, W. D. (1972) *Biochim. Biophys. Acta* **275**, 148.
18. Lance, C. and Bonner, W. D. (1968) *Plant Physiol.* **43**, 756.