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EFFECTS OF FLAVONE ON THE OXIDATIVE PROPERTIES OF INTACT PLANT MITOCHONDRIA

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; *Vigna radiata*; Leguminosae; mung bean; flavone; mitochondria; phosphorylation; flavoprotein.

Abstract—The effects of flavone on the oxidative and phosphorylative properties of plant mitochondria from potato tubers and etiolated mung bean hypocotyls were investigated. Flavone inhibited the state 3 oxidation rates of malate, NADH and, to a lesser extent, succinate but was without effect on the ascorbate-TMPD oxidation rate. The inhibition was the same whether the mitochondria were in state 3 or in an uncoupled state 3. When 100 μM flavone was added during the state 4, the tight coupling of succinate or NADH oxidation was not released. In the electron transfer chain, flavone inhibition appeared to be located in the flavoprotein region. All forms of NADH dehydrogenases seemed to be affected but the greatest inhibition appeared when exogenous NADH was used.

INTRODUCTION

Flavone, first isolated from plants at the end of the last century [1], occurs in the genus *Primula* where it is present as a powder covering the aerial parts [2]. Several authors have studied the effects of the flavonoids on the oxidative properties of isolated mitochondria [3–6]. For example, it is well established that the isoflavonoid rotenone, isolated from *Lonchocarpus* and *Derris*, acts at one of the several non-haem iron centres associated with the internal NADH dehydrogenase complex in mitochondria.

In this paper, we report the effect of the parent compound of the flavonoid series, namely flavone, on oxidative and phosphorylative properties of plant mitochondria isolated from potato tubers and etiolated mung bean hypocotyls.

RESULTS

Fig. 1 illustrates the effects of flavone upon the state 3 respiration rates with NADH, malate (+1 mM NAD) and succinate as substrates, in potato mitochondria. It is clear that the respiratory rates decrease more or less rapidly with increasing concentrations of flavone. In addition, the concentration of flavone which brings about a half-maximum inhibition of respiratory rates in state 3 is not identical for all the substrates used. Thus, the concentration of flavone which causes 50% inhibition of NADH driven state 3 respiration is 150 μM whereas a greater concentration of the inhibitor is required to inhibit malate oxidation. Succinate oxidation is particularly resistant to flavone inhibition since, in this case, 300 μM of flavone is insufficient to produce half-maximum inhibition. Fig. 1 also indicates that the inhibition of malate oxidation by flavone is strongly dependent on the pH in the reaction medium. Thus, at pH 7.5, addition of flavone (300 μM) to potato mitochondria supplemented with 15 mM malate (+1 mM NAD), in the presence of ADP, induces a total inhibition of oxygen uptake, whereas, at pH 6.5 the inhibition is weaker. In contrast, the inhibition of succinate or NADH oxidation by flavone is not dependent on the pH (results not shown).

Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1 μM), carbonyl cyanide *m*-chlorophenylhydrazone (1 μM) and 2,4-dinitrophenol (30 μM) are unable to reverse the flavone inhibition of state 3 respiration. As a matter of fact, we have observed that the inhibition by flavone is almost the same whether the mitochondria are in state 3 or in an uncoupled state 3 induced by the addition of 30 μM DNP. In addition, 100 μM flavone added during the state 4 does not release the tight coupling of succinate or NADH oxidation; moreover, the inhibition of ADP-stimulated respiration of potato mitochondria by carboxyatractyloside is not relieved by flavone. Similar experiments performed with mung bean mitochondria give identical results. All these results demonstrate that flavone primarily acts on electron transfer and not on phosphorylation.

In addition and in good agreement with Phillips and Williams [7], we have shown that potato mitochondria swell spontaneously when suspended in an ammonium phosphate solution or in an ammonium malate solution in the presence of Pi. Addition of flavone (200 μM) to the incubation medium does not affect the rate of swelling in ammonium phosphate or ammonium malate. Consequently, these results strongly suggest that flavone does not inhibit the entry of malate or phosphate in potato mitochondria.

Fig. 2 shows that flavone is without effect on the oxidative rate with ascorbate when *N,N,N',N'*-tetramethyl-*p*-phenylenediamine is the substrate. This substrate gives electrons to the respiratory chain at the level of the cytochrome *c* in the electron pathway. Difference spectra of antimycin A treated mitochondria (malate + antimycin A aerobic minus malate aerobic) at liquid

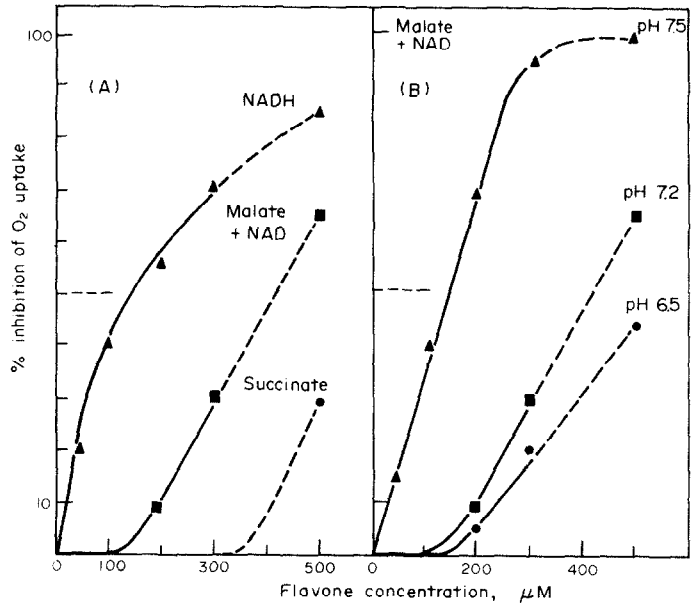

Fig. 1. Effect of flavone on state 3 rate of NADH, malate (+ NAD) and succinate oxidation by potato mitochondria. (A) 2 mM NADH, 15 mM malate (+ 1 mM NAD), 10 mM succinate (pH = 7.2). (B) 15 mM malate (+ 1 mM NAD) pH = 6.5, 7.2 and 7.5. The standard assay solution was used with 1 mg mitochondrial protein and 1 mM ADP. Note that above 300 μM, flavone may precipitate (dotted line).

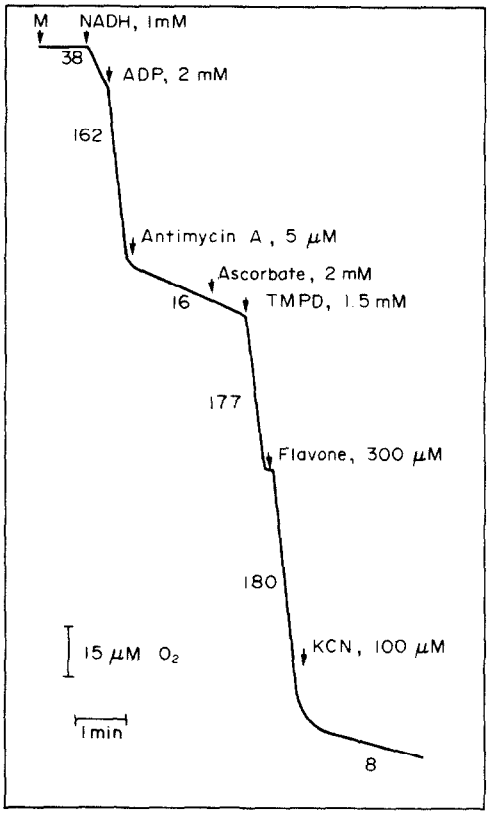

Fig. 2. Effect of flavone on ascorbate–TMPD oxidation by potato tuber mitochondria. Concentrations given are final concentrations in the reaction medium. Numbers on traces refer to nmol O_2 consumed/min/mg mitochondrial protein. M = mitochondria.

N_2 temperature show the classical three α-peaks at 553, 557 and 562 nm, corresponding to the reduced *b* cytochromes. In contrast, difference spectra of flavone-treated mitochondria (malate + flavone aerobic minus malate aerobic) reveal the absence of reduced *b*-type cytochromes (results not shown). Consequently, these results demonstrate that flavone inhibits the electron flow before the antimycin A block.

DISCUSSION

The present results demonstrate that flavone is an inhibitor of the electron flow in potato and mung bean mitochondria which acts before the well known antimycin A block. Mung bean mitochondria are known to possess an alternate pathway of electron transport to molecular oxygen which is insensitive to cyanide and antimycin A. Fig. 3 demonstrates that, in mung bean mitochondria, flavone strongly inhibits this cyanide-insensitive oxidase pathway with NADH or malate as substrates. These results together seem to indicate that the flavone inhibition site is located before the branching point between the cytochrome oxidase pathway and the alternate oxidase pathway. Since succinate oxidation is affected only at extremely high concentrations of flavone, it is very likely that this flavonoid only acts on both the internal and external NADH dehydrogenases. It is noteworthy that rotenone [8] is also a potent inhibitor of the internal NADH dehydrogenase, but, in contrast with the flavone, has no effect on the external NADH dehydrogenase. In addition, the effects of 3,5,7,4′-tetrahydroxyflavone (kaempferol) on the oxidative properties of plant mitochondria contrasts markedly with those of flavone itself [9].

Finally, the fact that the inhibition of malate oxidation by flavone is strongly dependent on the pH in the reaction medium can be readily interpreted since it is well

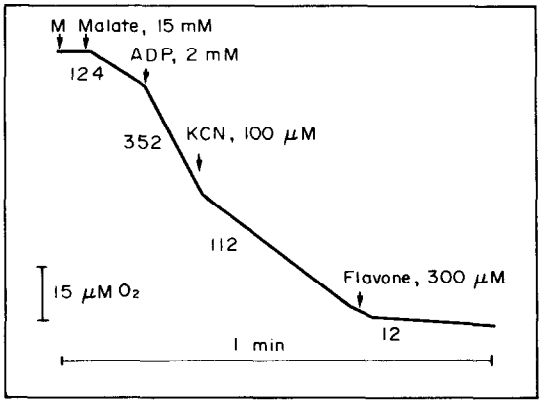

Fig. 3. Effect of flavone on the alternate cyanide insensitive terminal oxidase of mung bean mitochondria. Concentrations given are final concentrations in the reaction medium. Numbers on traces refer to nmol O_2 consumed/min/mg mitochondrial protein. M = mitochondria (pH = 7.2).

established that the rates and products of malate oxidation by plant mitochondria vary in response to changes in pH [10, 11]. Thus, during the course of malate oxidation whenever the NAD^+-linked malic enzyme activity is weakened (alkaline pH), the NADH level will drop and the rate of oxaloacetate production will be much higher than that of pyruvate [9]. Conversely, when the activity of the NAD^+-linked malic enzyme is high (acidic pH) the mitochondrial NADH level will be raised and the rate of oxaloacetate production will be considerably reduced [11]. Consequently, at pH 7.5, only the malate dehydrogenase is operating whereas, at pH 6.5, it is the NAD^+-linked malic enzyme which is operating. Addition of flavone to mitochondria supplemented with malate causes an immediate reduction of the pyridine nucleotide at all the pHs tested because this inhibitor slows down the rate of the electron flow. Under these conditions, the equilibrium of the malate dehydrogenase will move towards malate formation and this could explain the fact that flavone inhibition of malate oxidation is stronger at pH 7.5 than at pH 6.5.

EXPERIMENTAL

Preparation of mitochondria. Mitochondria from potato (*Solanum tuberosum* L.) tubers and from etiolated mung bean (*Vigna radiata* L.) hypocotyls cut from bean seedlings grown for 5 days in the dark at 26° and 60% relative humidity were prepared and purified by methods previously described [12]. All operations were carried out from 0° to 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 the low activities of the inner membrane and matrix marker enzymes (antimycin A sensitive NADH: cytochrome *c* oxidoreductase and malate dehydrogenase) [12]. In addition, the mitochondria were tightly coupled; the average ADP:O ratio for succinate was 1.8 and the respiratory control ratios for the same substrate were approximately 3.

O_2 uptake measurements. O_2 uptake was followed polarographically at 25° using a Clark-type electrode system (Hansatech Ltd., Hardwick Industrial Estate, Kings Lynn, Norfolk). The reaction medium contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate 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.

Mitochondrial swelling. Potato mitochondria (1 mg of protein) were suspended in solns of ammonium phosphate (120 mM) or ammonium malate (100 mM) in a final vol. of 1 ml. Absorbance at 520 nm was recorded with a recording spectrophotometer. Antimycin A (0.15 μg) was added to the incubation medium in order to inhibit malate oxidation. Pi (2.5 mM) was added to activate swelling in ammonium malate [7].

Split beam spectrophotometry. This was performed with the Aminco DW-2 spectrophotometer. The concns of the different cytochromes were measured at room or liquid N_2 temp. (77 K) from reduced minus oxidized difference spectra. The wavelengths selected for measurements were those given by Lance and Bonner [13].

Chemicals. Flavone was purchased from Karl Roth (Karlsruhe, Germany) and was dissolved in EtOH. The concn of EtOH in the reaction medium never exceeded 3%. For this concn, EtOH alone was almost without effect on mitochondrial respiration. When concns of flavone are greater than 300 μM in the reaction medium, this compound tends to precipitate.

REFERENCES

1. Kostanecki, St. V. and Tambor, J. (1895) *Chem. Ber.* **28,** 2302.
2. Müller, H. (1915) *J. Chem. Soc.* **107,** 872.
3. Stenlid, G. (1970) *Phytochemistry* **9,** 2251.
4. Stenlid, G. (1976) *Phytochemistry* **15,** 911.
5. Tissut, M., Chevallier, D. and Douce, R. (1980) *Phytochemistry* **19,** 495.
6. Koeppe, D. E. and Miller, R. J. (1974) *Plant Physiol.* **54,** 374.
7. Phillips, M. L. and Williams, G. R. (1973) *Plant Physiol.* **51,** 667.
8. Ernster, L., Dallner, G. and Azzone, G. F. (1963) *J. Biol. Chem.* **238,** 1124.
9. Ravanel, P., Tissut, M. and Douce, R. (1981) *Plant Physiol.* (in press).
10. Neuburger, M. and Douce, R. (1980) *Biochim. Biophys. Acta* **589,** 176.
11. Tobin, A., Djerdjour, B., Journet, E., Neuburger, M. and Douce, R. (1980) *Plant Physiol.* **66,** 225.
12. Douce, R., Christensen, E. L. and Bonner, W. D. (1972) *Biochim. Biophys. Acta* **275,** 148.
13. Lance, C. and Bonner, W. D. (1968) *Plant Physiol.* **43,** 756.