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FLAVONOIDS AS INHIBITORS OF THE FORMATION OF ADENOSINE TRIPHOSPHATE IN PLANT MITOCHONDRIA

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Abstract—Various flavonoids inhibit the production of adenosine triphosphate in mitochondria of cucumber hypocotyls. Flavones are more active than flavanones. Substances lacking substituents in the B ring are especially potent inhibitors and aglycones are more active than glycosides. Substituted cinnamic acids are inactive. The structural details regulating the activity against ATP production are different from those determining the activity of flavonoids in the oxidation of indol-3-yl acetic acid and of ascorbic acid.

INTRODUCTION

LIKE many other phenolic compounds, flavonoids of various types affect oxidative phosphorylation in plant mitochondria in a manner indicating an uncoupling effect.[1,2] In previous investigations the formation of adenosine triphosphate (ATP) was measured indirectly through the disappearance of inorganic phosphate. In order to measure the ATP more directly, the formation of ATP by plant mitochondria was followed by means of the firefly–luciferase method. The effect of various flavonoids was tested and special attention was paid to changes in the physiological activity caused by small alterations in the molecular structure of the flavonoid.

RESULTS

Results from experiments with mitochondria from hypocotyls of cucumber seedlings are given in Tables 1–4. Most of the substances tested inhibit the formation of ATP when applied in a concentration of 10^{-4} M. Some of them are inhibitory even at 10^{-5} M and the most inhibitory flavonoids are about as efficient as the classical uncoupler 2,4-dinitrophenol.

As regards the correlation between the structure and physiological activity, some points are obvious:

(1) Flavones are more inhibitory than the corresponding flavanones (compare the pairs apigenin–naringenin, kaempferol–aromadendrin, fisetin–dihydrofisetin and quercetin–taxifolin).

(2) Anthocyanidins, flavones, flavanones and isoflavones without any substituent in the B ring (e.g. 3,7-dihydroxy-flavone, 5,7-dihydroxy-isoflavone and pinocembrin) are very active inhibitors and the presence of substituents at the 3′ and 4′ positions, which is essential for the activity against indol-3-yl acetic acid (IAA) oxidase,[1] is of minor importance for the effect upon ATP formation. In many cases, the activity is diminished through the introduction of a hydroxyl group at 4′ (compare the pairs pinocembrin–naringenin, pinobanksin–aromadendrin and 5,7-dihydroxy-iso-flavone–genistein).

[1] G. STENLID, *Physiol. Plantarum* **16**, 110 (1963).
[2] G. STENLID, *Physiol. Plantarum* **21**, 882 (1968).

TABLE 1. EFFECT OF SOME FLAVONES AND FLAVANONES ON THE FORMATION OF ATP IN MITOCHONDRIA FROM HYPOCOTYLS OF CUCUMBER

Flavonoid added	Type*	Hydroxyl pattern	Conc. 3×10^{-5}	Conc. 10^{-4}	Conc. 3×10^{-4} M
Apigenin	F	5,7,4′		30	
Naringenin	FH_2	5,7,4′	90	56	
Naringin	FH_2G	5,7,4′		84	
Naringenin-7-glucoside	FH_2G	5,7,4′		80	
Hesperetin	FH_2	5,7,3′,4′		101	54
Kaempferol	F	3,5,7,4′	82	19	
Aromadendrin	FH_2	3,5,7,4′		90	
Kaempferol-3-rhamnoside	FG	3,5,7,4′,		79	
Quercetin	F	3,5,7,3′,4′	84	15	
Taxifolin	FH_2	3,5,7,3′,4′		107	93
Quercitrin	FG	3,5,7,3′,4′		88	
Rhamnetin	F	3,5,7,3′,4′		40	
Fisetin	F	3,7,3′,4′	78	27	
Dihydrofisetin	FH_2	3,7,3′,4′	96	83	24
Morin	F	3,5,7,2′,4′		39	
Pinocembrin	FH_2	5,7		38	
Pinobanksin	FH_2	3,5,7		28	
3,7-Dihydroxy-flavone	F	3,7		12	
3,3′,4′-Trihydroxyflavone	F	3,3′,4′		27	

* Key: F = flavone, FH_2 = flavanone, G = glycoside. The values give the formation of ATP as % of formation in control medium.

TABLE 2. EFFECT OF SOME ANTHOCYANIDINS (CONC. 10^{-4} M) ON THE FORMATION OF ATP IN MITOCHONDRIA FROM HYPOCOTYLS OF CUCUMBER

Hydroxylation pattern in the anthocyanidin	ATP formation % of control
6-OH	21
7-OH	93
4′-OH	88
3,4′-$(OH)_2$	69
7,4′-$(OH)_2$	103
3,7,4′-$(OH)_3$	70
3,7-$(OH)_2$	55
5,7-$(OH)_2$	53
3,5,7-$(OH)_3$	40
3′,4′,5′-$(OH)_3$	42

(3) Replacement of a 7-hydroxyl by a methoxyl group decreases the activity in the case of quercetin–rhamnetin, whereas biochanin A with a methoxyl group at 4′ is more active than genistein with a hydroxyl group at the same position. This is in accordance with the results mentioned above showing that a free hydroxyl at 4′ may decrease activity.

TABLE 3. EFFECTS OF SOME ISOFLAVONES AND OF SOME OTHER FLAVONOIDS AND PHENOLICS ON THE FORMATION OF ATP IN MITOCHONDRIA FROM CUCUMBER HYPOCOTYLS

Substance added	Conc. 3×10^{-5}	Conc. 10^{-4} M
Genistein	91	53
Biochanin A	60	32
5,7-Dihydroxyisoflavone	39	16
Coumestrol	51	27
(+)-Catechin		115
Phloretin	83	33
Phloridzin		95
2′,4,4′-Trihydroxychalcone	79	38
Sulphuretin	48	24
Caffeic acid		103
p-Coumaric acid		102
Chlorogenic acid		99
2,4-Dinitrophenol	41	12

The values give the ATP formation as % of formation in control.

TABLE 4. EFFECT OF SOME FLAVONOIDS ON THE FORMATION OF ATP IN TWO TYPES OF MITOCHONDRIA

Type of mitochondria*	Addition of flavonoid: Fisetin 3×10^{-5}	Fisetin 10^{-4}	Dihydrofisetin (10^{-4})	Quercetin 3×10^{-5}	Quercetin 10^{-4}	Taxifolin 3×10^{-5}	Taxifolin 10^{-4} M
Freshly prepared	78	27	83	84	15	107	93
Frozen and thawed	66	13	66	65	4	96	76

* The freshly prepared mitochondria from hypocotyls of cucumbers were used immediately after the preparation. The frozen and thawed mitochondria were stored for 2 weeks at −25° and were thawed immediately before the experiment. The values give the ATP formation as % of ATP formation in control (different controls for the two types of mitochondria).

(4) Glycosides are less active than the corresponding aglycones. This is valid for various types of flavonoids and agrees with tests on other processes.[2-7]

(5) Some isoflavones are active inhibitors. This may have some interest in the interpretation of the effects of isoflavones upon animals, where they may cause oestrogenic effects.[5]

(6) Substituted cinnamic acids (including chlorogenic acid) and catechin seem to be inactive as inhibitors. The aurone sulphuretin is an active inhibitor.

Preliminary experiments with mitochondria from cucumber roots, pea roots and maize coleoptiles indicate that mitochondria from other sources also react in a similar manner to those from hypocotyls of cucumber (Table 5). For some substances the effects were compared

[3] P. BARUAH and T. SWAIN, *J. Sci. Food Agri.* **10**, 125 (1959).
[4] E. V. PARUPS, *Can. J. Biochem.* **45**, 427 (1967).
[5] J. D. BIGGERS, in *The Pharmacology of Plant Phenolics* (edited by J. W. FAIRBAIRN), p. 51, Academic Press, London (1959).
[6] C. A. B. CLEMETSON and L. ANDERSEN, *Ann. N.Y. Acad. Sci.* **136**, 339 (1966).
[7] G. STENLID and G. SAMORODOVA-BIANCI, *Lantbrukshögsk. Ann.* **35**, 837 (1969).

TABLE 5. EFFECT OF SOME FLAVONOIDS (10^{-4} M) ON THE FORMATION OF ATP IN MITOCHONDRIA OF MAIZE COLEOPTILES, CUCUMBER ROOTS AND PEA ROOTS

Substance added	Type of mitochondria: Maize coleoptile	Cucumber root	Pea root	Substance added	Type of mitochondria (pea root)
Kaempferol	23	14	30	6-OH-anthocyanidin	39
Aromadendrin	95	79		7-OH-anthocyanidin	100
Kaempferol-3-rhamnoside	82			4'-OH-anthocyanidin	95
Fisetin	10	10	27	3,4'-$(OH)_2$-anthocyanidin	63
Dihydrofisetin	51	63	59	7,4'-$(OH)_2$-anthocyanidin	106
Apigenin	8		61	3,7-$(OH)_2$-anthocyanidin	57
Naringenin	57		65	5,7-$(OH)_2$-anthocyanidin	59
Naringin	89			3,5,7-$(OH)_3$-anthocyanidin	38
Morin	35		19	Sulphuretin	12
Phloretin			16	*p*-Coumaric acid	97
Phloridzin			96	Coumestrol	31

Values expressed as % of ATP production in control mitochondria. 6-OH-anthocyanidin means 6-hydroxy-anthocyanidin etc.

from experiments with freshly prepared mitochondria and with mitochondria which had been stored frozen at −25° and which were used after thawing (Table 4). The freshly prepared mitochondria were less sensitive to the flavonoids, but the relative efficiency of the various substances was the same with the two types of mitochondria.

An interesting observation is that some flavonoids are very potent inhibitors of the luciferin–luciferase reaction in the firefly preparations used. Only 0·05 ml of the mitochondrial reaction mixture was added to 4 ml containing the firefly enzyme mixture. This means a dilution of 80 times, but in spite of this an inhibition of the firefly enzyme was obtained with several substances. The isoflavones were especially active in this respect and some of them inhibited the firefly enzyme distinctly even when present in a concentration of about 2×10^{-7} M. For such substances, smaller samples than 0·05 ml had to be used in order to diminish the inhibition and make it possible to apply a correction.

DISCUSSION

Clearly, small alterations in the structure of the flavonoids profoundly influence their activity as inhibitors of ATP formation in plant mitochondria. Also as regards other processes affected by flavonoids (enzymatic oxidation of IAA, oxidation of ascorbic acid, growth of plant roots) the details of the chemical structure are very important.[1,2,6,7] It should be emphasized, however, that the molecules regulating each activity are significantly different in structure in the various cases. Flavonoids lacking substituents in the B ring are inactive against IAA oxidase but highly active inhibitors of the ATP formation, and the hydroxyls at positions 3' and 4' which are the main regulators of the activity against IAA oxidase are not important for an effect upon ATP formation. In contrast to the results found for ATP production, there is no regular difference between flavones and flavanones as regards the effects upon IAA destruction. The effects upon the oxidation of ascorbic acid are also very sensitive to changes in the flavonoid molecule.[6,7] This oxidation may be catalyzed in several

ways, and the effects of the flavonoids vary with the oxidative system; in summary, the influence of alterations in the flavonoid molecule in this case is different both from that found for IAA oxidase and that for ATP formation.

It is not clear if the potential capacity of the flavonoids to inhibit ATP production has any physiological significance. But if flavonoids interfere with the ATP production in the intact plant, it is obvious that small alterations in the structure will result in great changes in the activity. In this paper no reports of the quotient P/O are given, but previous experiments[1,2] as well as unpublished data indicate that the inhibition of the ATP formation is combined with an uncoupling effect.

The most strongly inhibitory flavonoids (substances lacking substituents in the B ring) are not common in the plant kingdom; nevertheless, some of the common flavonoids, such as quercetin and kaempferol, are rather inhibitory. The glycosides, which are relatively inactive, certainly form the main pool of the flavonoids in the leaves. The sugars are introduced rather late in the biosynthesis,[8] and it is possible that low concentrations of the aglycones are present in the cytoplasm. The occurrence of transformations of flavanones to flavones in plant leaves[9] and also the possibility that additional hydroxyls may be introduced at late stages of the biosynthesis[10] make the biochemical situation still more complex. Since both IAA and ATP influence almost all processes in the plant, it is not as yet possible to give any definite suggestion about their possible interaction with flavonoids in the plant.[11]

EXPERIMENTAL

Plant Material

Seeds of cucumber (*Cucumis sativus* L., Weibull's cultivar Favör) were soaked in distilled water and grown for 4 days on moist filter papers in the dark at 23°. The hypocotyls (30–50 mm) were used for the preparation of mitochondria according to the method of Bonner and Sikes as described by Bonner.[12]

Experiments with Mitochondria

The production of ATP by the mitochondrial preparation was followed in a medium containing mannitol (0·3 M), disodium succinate (0·005 M), NaF (0·005 M), $MgCl_2$ (5.10^{-4} M), phosphate (2·5 mM) and adenosine diphosphate (5.10^{-7} M). The pH was about 7·2. As a rule 0·1 ml of mitochondrial suspension was added to a total vol. of 2·0 ml. The amount of ATP formed in this control medium was compared to the amount formed when various substances were included in the medium. The time of the experiments (1–3 min) and the activity of the mitochondrial suspension were adjusted so that 50–80% of the ADP were transformed to ATP in the control medium.

Determination of ATP

ATP was determined by means of the firefly–luciferase method. The enzyme was prepared according to Rasmussen and Nielsen[13] from Sigma firefly tails FFT. As a rule samples of 50 μl were removed from the experimental solution containing mitochondria. The samples were rapidly mixed with enzyme and arsenate buffer (containing Na_2HAsO_4, $MgSO_4$, EDTA and glycine) to a total vol. of 4 ml in glass vials. The resulting light flash was measured with a Beckman Scintillation Counter 200. Only one of the photomultipliers was used as this gives better proportionality between ATP and light than measuring with coincidence coupling.[14] The light was measured as the number of counts during periods of 1·2 sec (window set). As the light intensity declines rapidly it is important that the vial is brought into the apparatus as fast as possible and that the whole procedure is strictly standardized.

[8] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, Academic Press, London (1967).
[9] G. SCHULTZ, *Z. Pflanzenphysiol.* **61**, 45 (1969).
[10] P. F. T. VAUGHAN, V. S. BUTT, H. GRIESEBACH and L. SCHILL, *Phytochem.* **8**, 1373 (1969).
[11] H. GRIESEBACH and W. BARZ, *Naturwiss.* **56**, 538 (1969)
[12] W. D. BONNER, JR., in *Plant Biochemistry* (edited by J. BONNER and J. E. VARNER), p. 97, Academic Press, New York (1965).
[13] H. RASMUSSEN and R. NIELSEN, *Acta Chem. Scand.* **22**, 1745 (1968).
[14] P. E. STANLEY and S. G. WILLIAMS, *Anal. Biochem.* **29**, 381 (1969).

For each determination four vials were used, two with control solution and two with addition of the substance to be tested, and at least two determinations were made with each concentration of a substance. The inhibitory effect varied somewhat from day to day, but the relative efficiency of the various substances was not changed.

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