Phytochemistry, 1970, Vol. 9, pp. 1745 to 1749. Pergamon Press. Printed in England.

ISOLATION OF RHIZOSPHERE BACTERIUM CAPABLE OF DEGRADING FLAVONOIDS*

W. BARZ

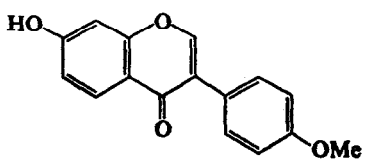
Biological Institute II, Plant Biochemistry, Schaenzlestr. 9/11
University of Freiburg/Br., Germany

(*Received* 30 *September* 1969)

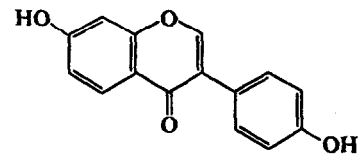
Abstract—The isolation from the roots of *Cicer arietinum* of a rhizosphere bacterium which can utilize formononetin as substrate is described. The organism was only capable of degrading isoflavones and flavones with common hydroxylation patterns, i.e. those substituted at the 3-,5-,7-,3′- or 4′- positions.

INTRODUCTION

IN THE course of our investigations[1] on the turnover of isoflavones in *Cicer arietinum* L. and *Phaseolus aureus* Roxb. it became apparent that the experimental plants grown in hydroponic solutions had microorganisms absorbed to their roots which effectively degraded the isoflavones formononetin (I) and daidzein (II). The preferential occurrence of such microbes on the roots seemed understandable because it had earlier been shown[2] that the plants excrete isoflavones through the roots into the medium thus forming a suitable medium for microbial enrichment.



Formononetin (I)



Daidzein (II)

These results prompted us to attempt the isolation of such rhizosphere microbes and the present communication describes the isolation and partial characterization of a bacterium degrading isoflavones and flavones.

Several reports on the fungal and microbial assimilation of flavonoids have been published[3,4] showing that flavonols such as quercetin and kaempferol are degraded to hydroxybenzoic acids (ring B), phloroglucinol carboxylic acid (ring A), phloroglucinol (ring A) and carbon monoxide (C-3). Similarly, degradation of the dihydrochalcone phloretin led to the formation of 4-hydroxybenzoic acids and phloroglucinol.[4] In degradative studies with

* Part I in a proposed series "Bacterial degradation of plant products".

[1] W. BARZ, CH. ADAMEK and J. BERLIN, *Phytochem.* **9**, 1735 (1970).
[2] W. BARZ, *Z. Naturforsch.* **24b**, 234 (1969). H. GRISEBACH H. ZILG, *Z. Naturforsch.* **23b**, 494 (1968).
[3] G. H. N. TOWERS, in *Biochemistry of Phenolic Compounds*, (edited by J. B. HARBORNE) p. 249, Academic Press, London (1964).
[4] N. P. JAYASANKAR, R. J. BANDONI and G. H. N. TOWERS, *Phytochem.* **8**, 379 (1969).

mammals[5,6] ring B and the carbon atoms of the heterocyclic ring of flavonoids are liberated as phenylpropionic or phenylacetic acid, respectively. In some of these investigations the observed assimilation of flavonoids seems to have been effected by the intestinal microflora. Common and coworkers[7] working with the non-laying hen presented evidence that formononetin, daidzein, biochanin A (II), genistein (I) and coumestrol were all converted into equol (I) as the main metabolite isolated from urine.

RESULTS

Isolation of Rhizosphere Microorganism

When the roots of hydroponically grown[8] *Cicer arietinum* and *Phaseolus aureus* plants were kept in phosphate buffer solution at pH 8·5 for 24 hr appreciable amounts of microorganisms could be detected in the solutions by plating onto nutritional agar and microscopic inspection. Suspensions of these microbes isolated by centrifugation effectively degraded formononetin-(methyl-^{14}C) as indicated by the high percentage of $^{14}CO_2$ liberated and by the almost complete disappearance of the isoflavone from the solution. Such microbial preparations from the roots of *Cicer arietinum* were used for enrichment cultures, with formononetin as sole source of carbon. Due to the very limited solubility of formononetin in neutral aqueous solution the enrichment and all degradative procedures were carried out at pH 8·5 where a concentration of the isoflavone in the region of 2×10^{-3} % can be achieved. Though growth in this medium is rather poor because of the low concentration of the carbon source available, the rapid disappearance of formononetin could be demonstrated by spectrophotometric measurements. After repeated subcultures at 30°, suspensions of the microbes were serially diluted and plated on nutrient agar. Two distinct types of colonies were observed. Individual colonies were selected and compared for growth on nutrient agar and for isoflavone degradation on formononetin plates. In the latter case, assimilation could easily be observed by inspection of the plates in u.v. light because isoflavone degradation is accompanied by the disappearance of the characteristic fluorescence of formononetin. Colonies which grew the fastest and readily decomposed formononetin were replated twice to check their purity. A homogeneous culture was readily obtained in this way.

The isolated bacterium forms slender, rod-shaped cells which show considerable branching and form a small mycelium. The cells are non-motile, gram-positive and acid-fast and the organism failed to grow anaerobically. The colonies have a paste-like consistency on agar slants, a smooth surface, a white to yellowish colour and they do not fluoresce. The properties observed so far indicate that this organism belongs to the order Actinomycetales and that it is a *Mycobacterium* or more probably a *Nocardia*. Until its exact identification is ascertained this bacterium will be referred to as "Cicer M1". The limited solubility of formononetin prevented the cultivation of Cicer M1 in liquid media on any larger scale with the isoflavone as the only carbon source. Of all the other media tested, the organism grew well only on standard nutrient bouillon (Merck), yeast extract and soya peptone (Oxoid). Maximum growth was observed with soya peptone (0·5%) supplemented with trace metals in phosphate buffer (pH 8·5, 0·08 M).

[5] F. DEEDS, in *Comprehensive Biochemistry* (edited by M. FLORKIN and E. STOTZ), Vol. 20, p. 127, Elsevier, Amsterdam (1968).
[6] N. P. DAS and L. GRIFFITHS, *Biochem. J.* **110**, 449 (1968).
[7] G. TANG and R. H. COMMON, *Biochem. Biophys. Acta* **158**, 402 (1968) and earlier papers cited.
[8] W. BARZ and B. ROTH-LAUTERBACH, *Z. Naturforsch.* **24b**, 638 (1969).

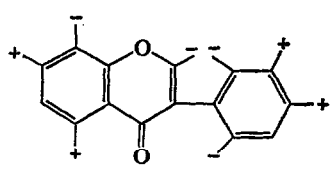
Substrate Specificity of Cicer M1

Apart from the observed degradation of formononetin it seemed of interest to determine the range of isoflavonoids and flavonoids which can be assimilated by this bacterium. Cells grown in soya peptone medium were harvested in the late logarithmic phase of growth and inoculated into solutions of various compounds. Degradation was followed by determining the decrease in absorbance at wavelengths of maximal absorption. Both isoflavonoids and flavonoids were found to be assimilated (Table 1). However, a characteristic substrate

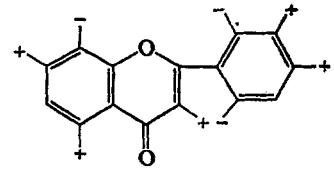
TABLE 1. ISOFLAVONOID AND FLAVONOIDS TESTED FOR DEGRADATION BY CICER M1

Assimilated by Cicer M1	Not assimilated by Cicer M1
Isoflavonoids	*Isoflavonoids*
Daidzein	7,2′,4-trihydroxyisoflavone
Formononetin	5,7-dihydroxy-2′,4′-dimethoxyisoflavanone (homoferreirin)
Biochanin A	Coumestrol
Genistein	3-(4-methoxyphenyl)-4,7-dihydroxycoumarin
5,7,3′,4′-tetrahydroxyisoflavone (orobol)	Eucomin
	5,7-dihydroxy-4′,8-dimethoxyisoflavone
Flavonoids	*Flavonoids*
5,7,4′-trihydroxyflavone (apigenin)	2′,4′-dihydroxy-4-methoxychalcone
3,5,7,4′-tetrahydroxyflavone (kaempferol)	2′,4′,4-trihydroxychalcone
3,5,7,3′,4′-pentahydroxyflavone (quercetin)	7-hydroxy-4′-methoxyflavanone
	7,4′-dihydroxyflavanone
3,5,7-trihydroxyflavone (galangin)	2′,4′,6′,4-tetrahydroxychalcone-4′-β-D-glucoside
Rutin	5,7,4′-trihydroxyflavanone
Phloretin	(±)-catechin
3-methoxy-5,7-dihydroxyflavone	(−)-catechin
5,7,3′,4′-tetrahydroxyflavone (luteolin)	3,5,7,2′-tetrahydroxyflavone (datiscetin)
Apigenin-7-β-D-glucoside	Apiin
5,7,-dihydroxy-4′-methoxyflavone (acacetin)	3,5,7,4′-tetrahydroxyflavanone (dihydrokaempferol)
	Saponaretin
	Saponarin

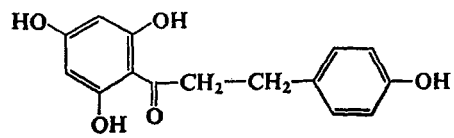
specificity of this organism is quite obvious. Only compounds with the basic skeleton of an isoflavone and a flavone (see Fig. 1) are degraded. Compounds with substituents in the 2-2′- and 8-positions in the case of isoflavones and in the 2′- and 8-positions of flavones



Isoflavon



Flavon/Flavonol



Phloretin

FIG. 1. BASIC SKELETONS OF FLAVONOIDS DEGRADED BY CICER M1.
+: Substituent does not prevent degradation.
−: Substituent completely inhibits degradation.

respectively, are not degraded. The only exception is the dihydrochalcone phloretin which is rapidly decomposed. The organism seems to be the first example of a microorganism found to degrade both isoflavonoids and flavonoids.

Observations on Isoflavone Degradation

In replacement cultures with freshly harvested cells, formononetin and daidzein are very rapidly degraded. No indication of any lag phase could be observed even with preparations which had repeatedly been cultured in yeast extract without isoflavones. During the incubation, the solutions first turned purple and finally became light yellow. When aliquots of the supernatant were extracted into ether and chromatographed on thin layer plates, numerous fluorescent and quenching compounds were observed in u.v. light. Comparison with controls showed that most of these metabolites were not derived from isoflavones. From present knowledge of microbial degradation of flavonoids,[3-5] 2,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid or *p*-methoxybenzoic acid would be expected to be formed but they were all absent from the cultures. From experiments with formononetin-(methyl-^{14}C), formononetin-(4-^{14}C) and daidzein-(4-^{14}C)[1] (Table 2), it is clear that the bulk of the radio-

TABLE 2. DISTRIBUTION OF RADIOACTIVITY AFTER COMPLETE DEGRADATION OF LABELLED ISOFLAVONES

Substrate / Fraction	Formononetin-(methyl-^{14}C) (%)	Formononetin-(4-^{14}C) (%)	Daidzein-(4-^{14}C) (%)
CO_2	40·9	38·2	32·7
Washed cells	4·0	7·2	8·2
Supernatant	49·6	49·4	52·7
Ether extract of supernatant	47·3	48·3	39·1
Aqueous phase after ether extraction	1·8	1·1	8·3

activity appears in the ether fraction and as CO_2. However, even after use of autoradiography, none of the expected conversions (e.g. formononetin → daidzein) in isoflavone degradation could be detected.

Investigation of Phloretin Degradation

During studies on the fungal decomposition of the dihydrochalcone phloretin, Towers and collaborators[3,4] found that this compound was degraded by various strains of *Aspergillus* and *Penicillium* to phloroglucinol, *p*-hydroxyphenylacetic acid and *p*-hydroxybenzoic acid. These results led us to examine phloretin degradation in Cicer M1. In replacement studies with phloretin in a concentration of 0·3 mg/ml, rapid decomposition was observed. However, none of the above metabolites could be detected at any stage, indicating that the degradation followed a different pathway from that produced by fungi.

DISCUSSION

The discovery of an isoflavone-degrading microorganism on roots of *Phaseolus aureus* and *Cicer arietinum* provides additional evidence for a relation between plants and root microorganisms[9] because it has earlier been shown that isoflavones are excreted from the roots.[2] From the substrate specificity of the organism (see Table 1) and the observation that

[9] A. D. ROVIRA, *Ann. Rev. Microbiol.* **19**, 241 (1965).

coumestrol F and homoferreirin[10] are also excreted from the roots, it is clear that other F microorganisms capable of degrading isoflavonoids may be associated with the roots. The data presented in this and the preceding paper[1] clearly demonstrate that biosynthetic or metabolic feeding experiments should be carried out either under sterile conditions or that the participation of microbial contaminations should otherwise be minimized.

The observation that isoflavones as well as flavones are decomposed by Cicer M1 shows that the enzyme system or systems involved may be different from that observed in *Aspergillus*.[11] This is further indicated by the fact that only the unsaturated compounds (e.g. kaempferol) but not the related saturated compounds (e.g. dihydrokaempferol) are decomposed.

EXPERIMENTAL

Isolation of Cicer M 1

Roots of hydroponically grown *Cicer arietinum* plants[8] were kept in potassium phosphate buffer (pH 8·5, 0·08 M) for 48 hr at 25° and microorganisms were isolated by centrifugation. Suspensions of these microbes were used for enrichment cultures. For the enrichment the following medium was used: K_2HPO_4, 3 H_2O (1·5 g), $(NH_4)_2SO_4$ (400 mg), $MgSO_4.7\,H_2O$ (240 mg) salts A[12] (3 ml), salts B[12] (0·6 ml), formononetin (150 mg), tap water (300 ml), pH 8·5. The cultures were shaken at 30° on a rotary shaker at 160 c/min. Subcultures were inoculated every 48 hr. Nutrient agar plates: a solution of the inorganic salts mentioned above with formmononetin omitted was supplemented with standard I nutrient agar (Merck, 2%) and the pH adjusted to 8·5. Formononetin agar plates: The same quantities of the inorganic salts as used above were dissolved in tap water (100 ml), supplemented with agar (Difco, 2%) and sterilized (121°, 20 min). Formononetin (100 mg) was dissolved in ethanol, 1 N NaOH (0·4 ml) added and the solvent removed under vacuum at 30°. The residue was dissolved in phosphate buffer (pH 8·5, 0·08 M) and the pH readjusted to 8·5. Any precipitate was removed by filtration and the solution sterilized by filtration. The two solutions were combined at approx. 50° and poured into petri dishes.

*Cultivation of Cicer M*1. The organism was routinely grown on soya-peptone (Oxoid, 0·5%) in potassium phosphate buffer (0·08 M, pH 8·5) with salts A and B.[12]

Degradative Studies with Isoflavones and Flavones

Freshly harvested and washed cells (300 mg fr. wt.) were suspended in 50 ml of a conc. solution (not exceeding 3 mg/ 50 ml) of the substrate in potassium phosphate buffer (pH 8·5, 0·08 m) and shaken under air. Aliquots were diluted 1:1 with buffer, centrifuged and the absorption spectra of the supernatant measured. Radioactive substrates were similarly treated except that the incubations were carried out in stoppered flasks. $^{14}CO_2$ was collected in a mixture of ethanolamine–ethylene glycol monomethyl ether[13] after injection of conc. H_2SO_4. Cells were isolated by centrifugation and the supernatant continuoulsy extracted with ether for 24 hr. Paper chromatograms were developed with (1) C_6H_6–HOAc–H_2O (125:72:3) and (2) 30% HOAc. Silica gel plates were developed with (1) C_6H_5Me–HCO_2Et–HCO_2H (5:4:1) and (2) C_6H_6–EtOAc–MeOH–petrol- ether (60–70°) (6:4:1:3). Phenolic compounds were detected by spraying with diaz. sulphanilic acid.

Radioactivity Assay

Radioactivity was measured as described.[1]

Acknowledgements—The financial support by the Deutsche Forschungsgemeinschaft and the helpful suggestions and discussion by Dr. H. Reichenbach, Freiburg, are gratefully acknowledged. Prof. Hörhammer, Munich, and Dr. P. M. Dewick, Freiburg kindly provided several flavonoid samples.

[10] W. BARZ, unpublished.
[11] J. PADRON, K. L. GRIST, J. B. CLARK and S. H. WENDER, *Biochem. Biophys. Res. Commun.* 3, 412 (1960).
[12] D. H. HARKNESS, L. TSAI and E. R. STADTMAN, *Arch. Biochem. Biophys.* 108, 323 (1964).
[13] H. JEFFAY and J. ALVAREZ *Anal. Chem.* 33, 612 (1961).