Phytochemistry, 1968, Vol. 7, pp. 2131 to 2137. Pergamon Press. Printed in England

FLAVONOIDS IN GENOTYPES OF *TRIFOLIUM SUBTERRANEUM*—II.

MUTANTS OF THE GERALDTON VARIETY

E. WONG

Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand

and

C. M. FRANCIS*

Western Australian Department of Agriculture, Perth, Western Australia

(*Received* 17 *April* 1968)

Abstract—Flavonoid patterns in five mutants of the Geraldton variety of subterranean clover have been determined and compared with that of the parent Geraldton. Differences observed can be interpreted in terms of blockages at various points of the biosynthetic scheme for flavonoids deduced from tracer studies.

INTRODUCTION

IN PREVIOUS work,[1] the normal flavonoid pattern in the Geraldton variety of subterranean clover was established in detail. Three chemically induced mutants of the Geraldton clover, L858, B763 and A258 have been described with respect to their isoflavone concentrations, and their simply inherited monogenic character established.[2] Two other flavonoid mutants, N4285 and N3761, were also found during this work but have hitherto not been reported. The latter mutants were distinguished by deficiencies in anthocyanin pigments both in their leaves and seeds, which in contrast to the black seeds of the Geraldton parent, are white and pale-pink respectively.

Effects of genetic factors on anthocyanin pigments in genotypes of cultivated plants have been extensively studied.[3] Other classes of flavonoid have been taken into account less systematically in studies of this type,[4] and no chemical–genetical studies linking isoflavones and other flavonoids have been reported. In this work, mutation effects on the complex of flavonoid constituents in Geraldton clover have been studied. Quantitative comparisons

* Present address: Institute of Agriculture, University of Western Australia.

[1] Part 1, E. WONG and C. M. FRANCIS, *Phytochem.* 7, 2123 (1968).
[2] C. M. FRANCIS and A. J. MILLINGTON, *Australian J. Agri. Res.* **16**, 565 (1965).
[3] J. B. HARBORNE, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 593, Pergamon Press, Oxford (1962); R. E. ALSTON, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 171, Academic Press, London (1964).
[4] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoid Compounds*, p. 250, Academic Press, London (1967).

were made of flavonoid patterns in the mutants with that of the parent Geraldton. The differences observed are explicable in terms of postulated blockages at various points of the biosynthetic scheme for flavonoids deduced from tracer studies.

RESULTS

The flavonoid constituents of subterranean clovers exist predominantly as glycosides.[1,5] In this study these compounds were examined as the free phenols. Extracts were first hydrolysed with acid, then analysed by two-dimensional paper chromatography. Visual scanning of the chromatograms, under u.v. light or after spraying with diazotized sulphanilic acid, revealed much variation in the patterns of flavonoids in the various mutants. For constituents existing in high enough concentrations, it was possible to express their variations in quantitative terms. The various spots were cut out, eluted with aqueous ethanol, and the concentration determined spectrophotometrically. Results obtained are summarized in Table 1.

The main anthocyanin in Geraldton clover has been identified as a cyanidin glycoside,[6] but the concentrations in the samples studied here were too low to be measured spectrophotometrically.

From the values given in Table 1, and from visual comparisons of the other constituents, the flavonoid pattern for Geraldton, and for the mutants relative to Geraldton, can be summarized as follows:

Geraldton. The three isoflavones, genistein, biochanin A and formononetin together constitute the bulk of the flavonoid constituents. Kaempferol is present in comparable quantity, but all other flavonoids are present in trace amounts only. Visually, however, some of these minor components, particularly those of the 5-deoxy series, manifest themselves much more prominently than their actual amounts would indicate, because of their strong colour reactions under u.v. light.

L858. In this mutant the isoflavones and most of the minor constituents are greatly reduced. Values for kaempferol, quercetin and isorhamnetin, however, indicate that the flavonols are only slightly affected. Anthocyanins are also reduced relative to Geraldton as judged by leaf coloration.

N4285 (white seed). Isoflavones in the mutant are again reduced but to a lesser extent than in L858. All other constituents including flavonols are reduced compared to Geraldton. There appears to be a complete inhibition of anthocyanins in this mutant as shown by the white seed and the apparent absence of red coloration in the leaves at all stages of growth.

B763. Isoflavones are reduced[2] but all other flavonoids are greatly enhanced. This mutant was originally distinguished by the presence of two bright-blue fluorescent spots on TLC plates.[2] These spots are now identified as the 5-deoxyflavones.[1] Visually this mutant is not distinguishable from the parent Geraldton.

A258. As previously shown,[2] the methylated isoflavones are almost completely absent from this mutant and the free phenols daidzein and genistein are greatly increased in amount. The other flavonoids appear to be little changed. This mutant is visually indistinguishable from Geraldton.

N3761 (pale seed). The pattern of flavonoids on the whole is not much different from Geraldton. The only significant effect appears to be a visible reduction of anthocyanin pigments in the seeds and in leaves.

[5] A. B. BECK, *Australian J. Agri. Res.* **15**, 223 (1964).
[6] E. WONG and C. M. FRANCIS, *Phytochem.* **7**, 2139 (1968).

TABLE 1. CONCENTRATION OF FLAVONOID COMPOUNDS IN LEAVES OF GERALDTON AND MUTANT CLOVERS (mg% fresh weight)

	$E^{1\%}_{1cm} \times 10^{-3}$	Genotype					
		Geraldton	L858	N4285	B763	A258	N3761
Isoflavones							
Genistein	1·32	14·6	3·0	9·3	+	60·5	32·9
Biochanin A	1·23	23·4	0·6	6·3	1·6	0·4	52·4
Pratensein	1·13	1·6	+	+	+	+	4·1
Daidzein	1·00	<0·2	+	+	+	76·5	+
Formononetin	1·01	42·7	7·7	21·8	22·1	4·0	85·6
Flavonols							
Kaempferol	0·67	38·8	30·0	21·3	61·3	43·8	30·0
Quercetin	0·69	7·6	7·6	3·6	15·7	5·6	6·6
Isorhamnetin	0·66	0·9	0·9	0·7	2·2	1·0	1·1
4′,7-Dihydroxyflavonol	0·78*	0·8	+	+	2·5	+	+
Fisetin	0·94	0·4	+	+	2·4	+	+
Geraldol	0·82*	<0·2	+	+	1·4	+	+
Flavones							
Luteolin	0·67	0·7	+	+	1·7	+	+
4′,7-Dihydroxyflavone	1·03*	0·4	+	+	0·9	+	+
3′,4′,7-Trihydroxyflavone	0·79	<0·2	+	+	2·4	+	+
Geraldone	0·78*	<0·2	+	+	0·8	+	+
Chalcones							
Isoliquiritigenin	1·21*	<0·1	+	+	1·5	+	+
Homobutein	1·10*	<0·1	+	+	0·8	+	+

+ Present in traces, comparable to or less than in Geraldton, not measured.
* Determined in this work; for sources of the other values see Experimental.

DISCUSSION

The biosynthetic interrelationships for the different classes of flavonoid can, on the basis of present knowledge,[7–10] be represented by the scheme shown in Fig. 1. With regard to the individual compounds found in clover,[1] these all belong to one of four types of substitution pattern, viz. (1) 5,7,4′-, (2) 5,7,3′,4′-, (3) 7,4′-, and (4) 7,3′,4′-. Patterns (1) and (2) may be

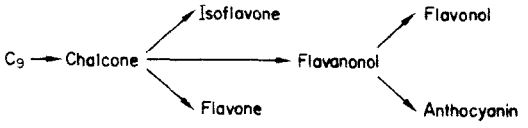
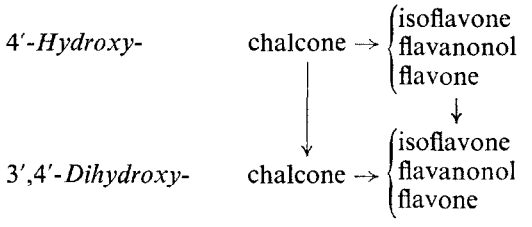


FIG. 1. PROBABLE BIOSYNTHETIC RELATIONSHIPS OF FLAVONOIDS.

termed "normal" with respect to ring A, and (3) and (4) are the corresponding 5-deoxy derivatives. The removal of a 5-OH group during biosynthesis most probably occurs at the poly-keto acid stage,[11] prior to chalcone formation.[12]

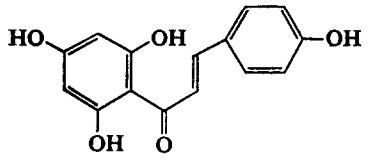
Compounds of types (2) and (4) differ from those of (1) and (3) respectively in having an additional 3′-OH in ring B. The sum of available evidence points to this hydroxylation taking place after chalcone formation.[4, 7, 13] Precisely at what point this takes place is, however, not certain. It is possible that hydroxylation of the B ring can take place at various points of the pathway, forming a "metabolic grid"[14] to 3′,4′-dihydroxy compounds, as exemplified in the scheme below:



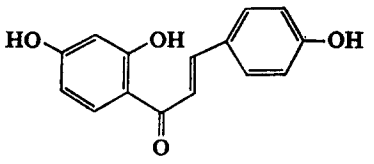
For ease of presentation in this paper we will assume that hydroxylation takes place at the chalcone stage.

The above considerations enable us to assign all the known compounds of clover to two parallel biosynthetic series, arising from chalconaringenin (I) and isoliquiritigenin (II) (Fig. 2). It can be seen from the results presented that in the Geraldton parent, the normal

[7] H. GRISEBACH, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN) p. 279, Academic Press, London (1965).
[8] E. WONG, *Chem. Commun.* in press; E. WONG, *Phytochem.* submitted April, 1968.
[9] T. A. GEISSMAN, E. C. JORGENSEN and B. L. JOHNSON, *Arch. Biochem. Biophys.* **49**, 368 (1954).
[10] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966); L. PATSCHKE and H. GRISEBACH, *Phytochem.* **7**, 235 (1968).
[11] A. J. BIRCH, in *Progress in the Chemistry of Organic Natural Products* (edited by L. ZECHMEISTER), Vol. 14, p. 198, Springer Verlag, Vienna (1957).
[12] H. GRISEBACH and G. BRANDNER, *Z. Naturforsch.* **16b**, 2 (1961).
[13] H. GRISEBACH and H. J. GRAMBOW, *Phytochem.* **7**, 51 (1968).
[14] J. D. BU'LOCK, *The Biosynthesis of Natural Products*, p. 82, McGraw-Hill, London (1965).



(I)



(II)

pathway (1) is quantitatively the most important. In this pathway, as also in pathway (3), isoflavones are the most abundant compounds formed. The results also show that in the "isoflavone deficient" mutants L858, B763 and N4285, the patterns of all the other flavonoids have been affected in conjunction with the isoflavones. The effects in the other two mutants, A258 and N3761 and more localized. The biochemical effects in the mutants will now be discussed individually in terms of the biosynthetic schemes in Fig. 1 and Fig. 2.

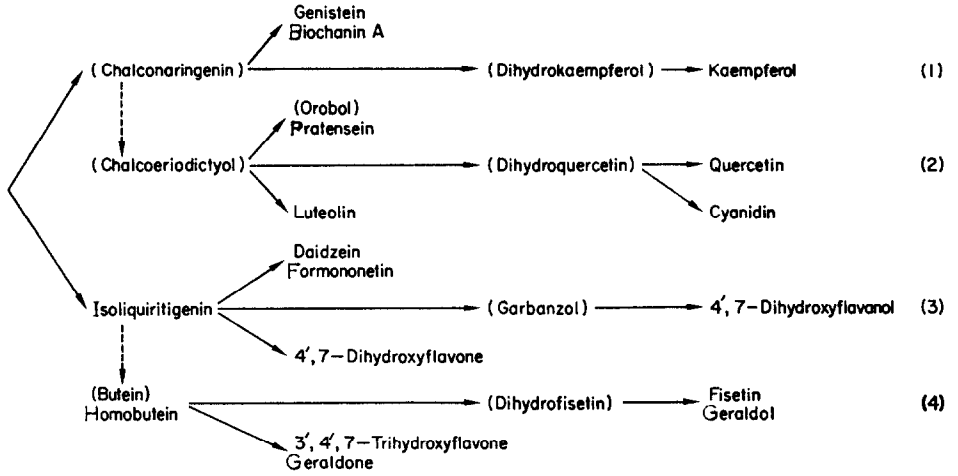


FIG. 2. PROBABLE BIOSYNTHETIC INTERRELATIONSHIPS OF THE CLOVER FLAVONOID COMPOUNDS. Compounds in brackets not detected in clover.

L858. Since all flavonoids are reduced in this mutant, the obvious explanation is that a partial blockage has taken place at an early step which is common to the biosynthesis of all the compounds. In terms of the scheme in Fig. 3 this step would have to be prior to chalcone formation.

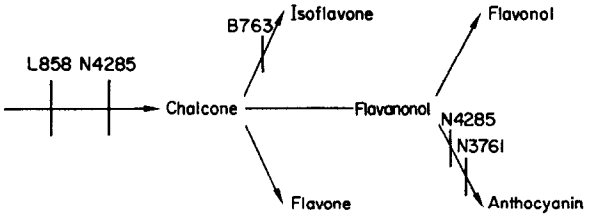


FIG. 3. POSTULATED SITES OF BLOCKAGE IN THE MUTANTS.

B763. The concomitant increase in other flavonoids with decrease in isoflavones can be rationalized in terms of a partial blockage at the stage in the pathway specific for isoflavone formation (Fig. 3). Since isoflavones are quantitatively the most important products in the pathway, and decrease in their synthesis would enable a large pool of common intermediates (e.g. chalcone) to be used for the synthesis of other compounds.

N4285. A similar early blockage as in L858 would explain the decrease in all flavonoids. It is not clear however, why such a blockage in this case should cause an apparent complete interference with anthocyanin production. It is possible that we may be dealing with the effects of two closely linked genes, one acting at a pre-chalcone stage, the other specifically inhibiting anthocyanins (Fig. 3). Another possibility is that a single gene affects the two sites indirectly, for example, via a common co-factor for the two enzymic steps. Any such indirect effect would have to be more important for the anthocyanin step to explain the different degrees of inhibition observed.

N3761. The effects of this mutant appear to be confined to anthocyanins. Interference therefore must be at the specific anthocyanin stage (Fig. 3). Since anthocyanins are quantitatively not important as flavonoid constituents in Geraldton, interference in this area of biosynthesis should have little indirect effects on other flavonoids as a whole. This situation is opposite to that in the isoflavone-deficient mutant B763.

A258. This provides a very good example of blockage at a single end step. The inhibition of biochanin A and formononetin with concurrent accumulation of genistein and daidzein must mean that the methylation step is blocked. Since both the normal and 5-deoxy isoflavones are similarly affected, this *O*-methylation must be non-specific with respect to the isoflavone substrate. Furthermore, since the other methyl ethers (e.g. homobutein, geraldone) are not affected, methylating systems other than this one must be responsible for their formation. The gene action here can therefore be regarded as affecting the enzyme system specific for 4′-*O*-methylation of isoflavones.

Crosses of the different mutants have been made producing progeny possessing various combinations of these genetic blocks. These will form the subject of a subsequent paper in this series.

EXPERIMENTAL

Plant Material

All the clovers were grown at 20° in the glasshouse and harvested when 9–10 weeks old.

Extraction

30-g samples of leaves of each clover were extracted with hot 95 per cent ethanol and the extract washed several times with petrol. ether. The extract was concentrated to dryness and then hydrolysed at 100° with 60 ml N HCl in 50 per cent ethanol for 45 min. The mixture was concentrated *in vacuo*, made up to 30 ml with water and extracted with ethers (4 × 60 ml). The residue, after evaporation of ether, was taken up in 6 ml ethanol. This solution was used for paper chromatography.

*Paper Chromatography**

100–400 μl of the extracts were analysed by two-dimensional paper chromatography in the solvent systems BeAW and 30 per cent HOAc on Whatman 3MM paper, previously washed with 5 per cent HOAc. Most of the clover flavonoids are well resolved in this system; the three pairs of common and 5-deoxyflavonols (e.g. kaempferol and 7,4′-dihydroxyflavonol) run as contiguous spots, but due to the strong fluorescence of the 5-deoxy compounds, these were easily distinguishable although present in much smaller amounts than the common flavonols. The isoflavones, however, particularly formononetin, streak when chromatographed in 30 per cent HOAc. For the quantitative determination of these compounds additional two-dimensional chromatograms in BeAW and 2N NH_3 were necessary.

* For composition of solvent systems, see E. WONG, P. I. MORTIMER and T. A. GEISSMAN, *Phytochem.* **4**, 89 (1965).

Quantitative Determinations

The individual spots, after visualization under u.v. light, were cut out and eluted with 85 per cent ethanol. Appropriate areas were eluted as blanks. The u.v. spectrum for each compound was determined after elution as a further criterion of identity. The absorbance at the wavelength maximum was used to calculate the concentration, using the $E^{1\%}_{1cm}$ values listed in Table 1. These $E^{1\%}_{1cm}$ values were determined experimentally in this work, or taken from the literature (isoflavones,[15] isorhamnetin,[16] the trihydroxyflavone,[17] others[18]). Results given are means from two or more different chromatographic and spectrophotometric determinations; deviations from the means were in general within 10 per cent.

Acknowledgements—Financial assistance from the Australian Wool Board is gratefully acknowledged. We thank also Professor W. K. Stern, Department of Agronomy, University of Western Australia, for the use of glasshouse and other facilities.

[15] E. WONG, *J. Sci. Food Agr.* **13**, 304 (1962).
[16] G. E. INGLETT, *J. Org. Chem.* **22**, 189 (1957).
[17] A. L. LIVINGSTON and E. M. BICKOFF, *J. Pharm. Sci.* **53**, 1557 (1964).
[18] T. A. GEISSMAN, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. V. TRACY), Vol. 3, p. 450, Springer Verlag, Berlin (1955).