BIOCHEMICAL GENETICS OF PIGMENTATION IN *PISUM*

by

Carmel M. Statham, B. Sc. (Hons.), Tas.

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requirements for the degree of
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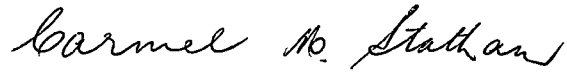
This thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no copy or paraphrase of material published or written by any other person except where due reference is made in the text of the thesis.



Carmel M. Statham

University of Tasmania
Hobart

October, 1974

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NOTES AND ABBREVIATIONS

In presenting any genotypes in this thesis, only one of each pair of alleles will be given, such that a plant homozygous dominant or recessive for a gene will only be given one sumbol, and heterozygotes will be described like homozygote dominants except in special circumstances where heterozygosity requires to be made clear.

Throughout this thesis, no attempt has been made to differentiate between the species *Pisum arvense* and *P. sativum*. However, the line S 1256 is always referred to as *P. fulvum*.

HOAc	=	acetic acid
HCl	=	hydrochloric acid
MeOH	=	methanol
EtOH	=	ethanol
F_1	=	first filial generation
F_2	=	second filial generation, obtained by self-fertilization of the F_1
T C	=	testcross; a cross involving multiple recessive genes in one parent
C 1	=	Cross 1
C 133	=	Cross 133
S 1017	=	line number 1017, of Swedish origin
L 60	=	line number 60, of Tasmanian origin
Mv	=	malvidin
Pt	=	petunidin
Dp	=	delphinidin
Pn	=	peonidin
Cy	=	cyanidin
Pg	=	pelargonidin
3G	=	3-O-glucoside
3G,5G	=	3-glucoside-5-glucoside
3GG	=	3-O-sophoroside
3GX	=	3-O-sambubioside
3GalX	=	3-O-lathyroside
3R,5G	=	3-rhamnoside-5-glucoside

R.C.V.	=	recombination value
P	=	probability
*	=	$P < 0.05$
**	=	$P < 0.01$
***	=	$P < 0.001$
X_1^2	=	Chi-squared, with one degree of freedom

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SUMMARY

The biochemical genetics of flavonoid pigmentation has been investigated for many species and genera over a long period. In the case of the genus *Pisum*, a great many genetical studies have been carried out, but very little biochemistry has been undertaken. The present investigation was designed to use the large amount of genetical material available as a basis for an investigation of the biochemistry of pigmentation in *Pisum*.

Six loci were known to affect flower colour in *Pisum*, these being *A*, *Am*, *Ar*, *B*, *Ce* and *Cr*, and a pair of complementary loci, *Pur* and *Pu* had been shown to be responsible for the anthocyanin colouration of pod tissue. No specific information was available as to the mode of action of these loci in the biosynthesis of flavonoids in *Pisum*, and also there were no reports of the interaction of the flower colour loci with the loci responsible for the flavonoid pigmentation in vegetative tissues.

The results presented in this thesis outline the mode of action of the six loci, *A*, *Am*, *Ar*, *B*, *Ce* and *Cr*, in the production of the range of flower colours found in the genus *Pisum*. Three of these loci, *A*, *B* and *Cr*, have a direct qualitative effect on the nature of the floral anthocyanins, *A* being responsible for the production of all classes of flavonoid in the flowers and for the production of anthocyanins in pod and leaf axil tissue; the *B* gene is responsible for the 5'-hydroxylation of anthocyanins in the flowers, pods and leaf axils, while the *Cr* gene was found to be responsible for the 3'- and 5'-methylation of floral anthocyanins and for the determination of rhamnose as the glycosyl substituent at position 3 of such anthocyanins. Further investigations showed that the biochemical effect of the *Cr* gene is confined to floral tissues, and is not evident in the vegetative tissues such as pods and axils.

The other three loci which were known to affect flower colour in *Pisum*, *Am*, *Ar* and *Ce*, were shown to have quantitative effects on floral anthocyanins, the precise complement being determined by the genotypic constitution with respect to the previously mentioned loci, *A*, *B* and *Cr*, The three quantitative flower colour loci have varying effects in vegetative tissue, which is described in the text. No definite conclusions as to the mode of action of the quantitative loci were reached, but the results obtained suggested that the loci, *Ar* and *Ce*, may have their effects by modifying the action of one of the qualitative loci, *Cr*.

During the investigation, a new gene, *Cgf*, was described, C-glycosyl flavones being present in the standard petals of *Pisum* in the presence of the dominant allele. Several new combinations of this gene with the previously described flower colour genes were obtained from the crossing programme undertaken, and the chemical screening of these new genotypes showed the dominant *Cgf* gene to be hypostatic to the recessive *a* gene. The structure of the standard petal flavones was found to be the same for all genotypes investigated, and the qualitative flower colour loci do not affect the chemical structure of the floral flavones.

INTRODUCTION

The colour of most flowers and fruits is imparted by a complement of chemicals belonging to two major groupings; lipid soluble plastid pigments, the carotenoids, and water-soluble sap pigments, the flavonoids. Chlorophylls provide the prevailing green colour of plant leaves and are generally distributed throughout the plant kingdom. The flavonoids are widely distributed in nature; anthocyanins being most abundantly present in flower petals and fruits, but also occurring in leaves, stems and tubers; flavonols and flavones are also common leaf constituents, but their pale yellow or cream colours are masked by the ubiquitous chlorophyll. In contrast to chlorophylls, carotenoids and quinones, flavonoid pigments are characteristic only of higher plants, being virtually absent from the lower phyla.

1:1 Biochemical Genetics of Flavonoids

Flavonoid compounds have been the subject of investigation since the beginnings of modern organic chemistry, the greatest attention having been paid to those compounds known since antiquity as dyestuffs. Investigations into the inheritance of flower colour began with the classical studies of Mendel in 1865, who crossed purple and white flowered peas, and obtained a single gene difference, purple being dominant to white. No further progress was made in this field until the independent rediscovery of his work by de Vries, Correns and Tschermak in 1900. During the subsequent growth of the study of genetics, the possibilities of chemical-genetical studies of flower pigments were first formally recognized and investigated by Wheldale, using *Antirrhinum* (Wheldale, 1907, 1914). However, it was Scott-Moncrieff (1931) who first reported a specific chemical difference correlated with a flower colour difference governed by a single Mendelian

locus. She found that the flower colour difference in *Pelargonium zonale* involved a change in the hydronylation pattern of the anthocyanins while the glycosyl residues of these anthocyanins was the same in both colour varieties.

Most chemical-genetical studies have been concerned with the inheritance of flower colour in garden plants where artificial selection has produced a wide variety of colour mutants, e.g. *Antirrhinum, Dahlia, Lathyrus*. Flower colour genetics has been studied in about 100 plants (Paris et al., 1960) but the number for which accompanying chemical data is available is considerably less, and Harborne (1967) lists only 14 species for which satisfactory results are available. Anthocyanin colour variation is not restricted to floral organs, and in the case of the French bean, potato and radish, colour variation in seeds, tubers and roots respectively, is of primary interest, and these are the organs in which colour selection has occurred.

The quick qualitative tests devised by the Robinsons (1931, 1932) were more accurate for anthocyanins than for other flavonoids, with the result that anthocyanins were better documented in the earlier chemical-genetical studies, e.g. Scott-Moncrieff (1936). The introduction of paper chromatography (Bate-Smith, 1948) and absorption spectroscopy (Harborne, 1958, Jurd, 1956, 1962) has allowed the rapid separation and identification of complex mixtures of flavonoids on a reasonably small scale, with the result that modern studies have allowed flavone, flavonol, chalcone, aurone and cinnamic acids to be analysed along with anthocyanins.

1:2 Biosynthesis of Flavonoids

Despite the chemical-genetical studies on floral pigments, little evidence was accumulated as to the biosynthetic origin of the flavonoids.

Knowlege of the biosynthetic route to the basic flavonoid structure has been provided through studies with radioactive tracers, and it is possible to derive a scheme as presented in Fig. 1 with some confidence. Excellent reviews of flavonoid biosynthesis have appeared (e.g. Bogorad. 1958; Neish, 1964; Grisebach, 1965). Tracer studies have shown flavonoids to be secondary matabolites referable to the flavylium nucleus, the most direct links between flavonoid biosynthesis and intermediary metabolism being most direct links between flavonoid biosynthesis and intermediary metabolism being between flavonoids and aromatic compounds having C_6-C_3 subunits (Underhill, Watkin and Neish, 1957; Grisebach, 1958) and between flavonoids and acetate (Birch and Donovan, 1953, Birch et al., 1953, 1955; Watkin, Underhill and Neish, 1957, Grisebach, 1957).

1:3 Chemical Classification of Flavonoids

Classification of flavonoids into major chemical groups is based on the configuration and state of oxidation of the connecting C_3 portion of the C_6-C_3-C_6 molecule, as illustrated in Fig. 2. Numbering conventions shown are also important. The benzene ring to which the pyran ring (anthocyanin) or pyrone (flavone, etc.) is fused is designated the A ring, and the other benzene ring is the B ring.

Characterization of individual flavonoids within each major chemical group is based on analysis of the pattern of substitution and on the nature of the substituents. Hydroxyl substituents are the most common, and frequently one or more of them is utilised in the formation of ethers. Among the alchohols which participate in ether formation are methanol (methoxyl substituents resulting) and sugars. Flavonoids occur in plant tissue as glycosides, with at least one glycosyl substituent. Examples of flavonoids are given in Fig. 3.

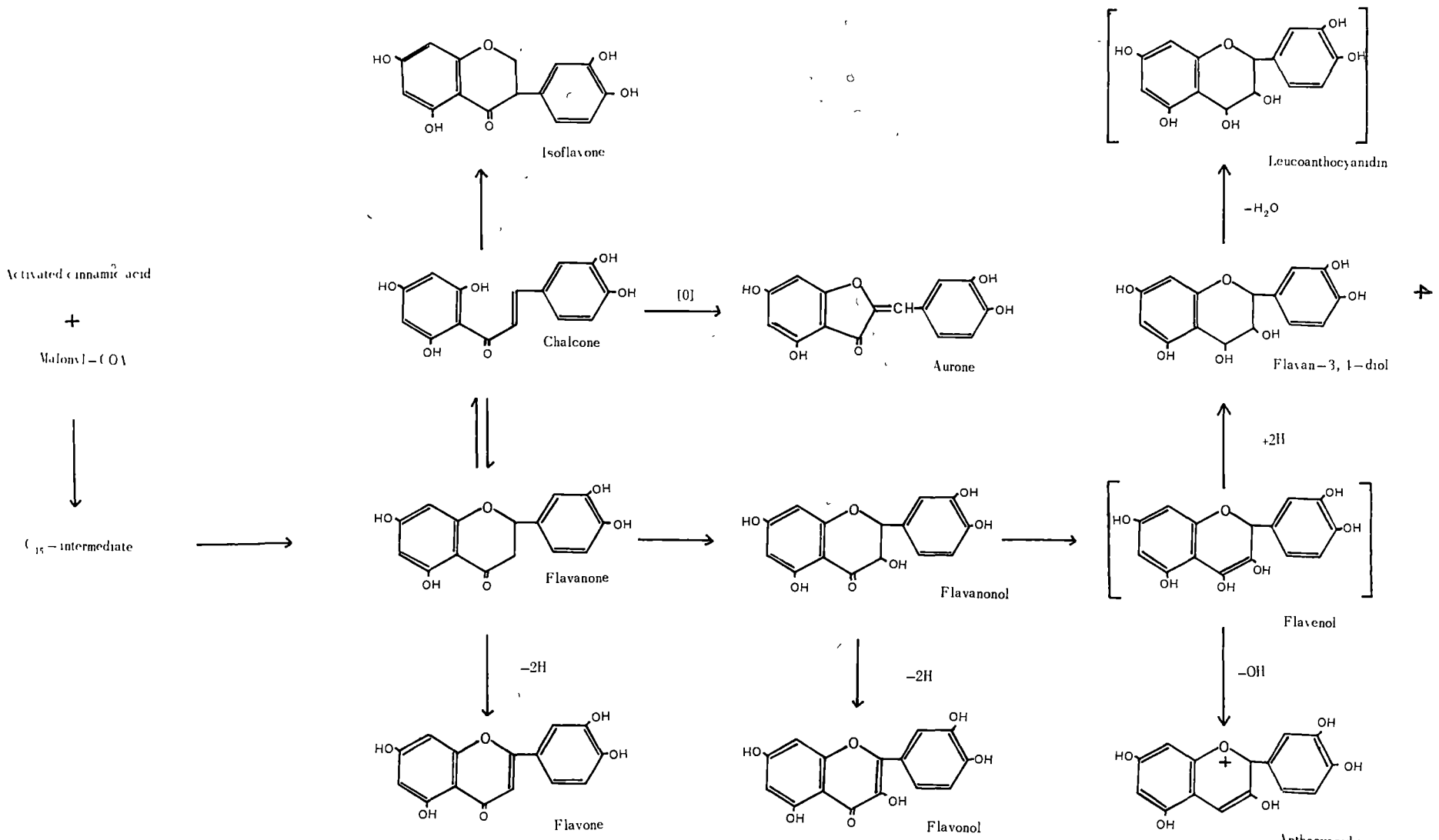
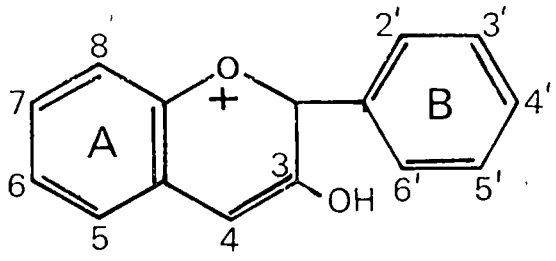
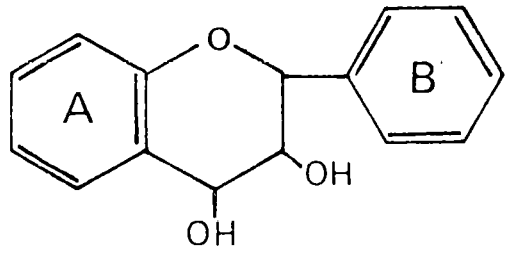


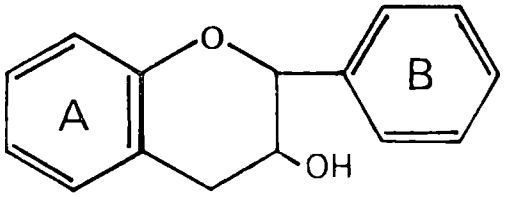
FIGURE 1. POSSIBLE BIOSYNTHETIC PATHWAYS TO THE FLAVONOIDS



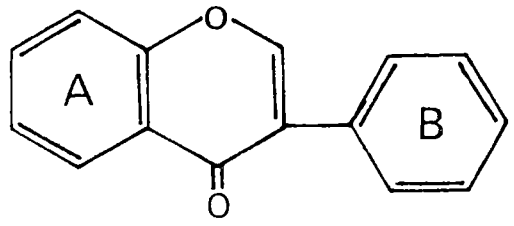
Anthocyanidin



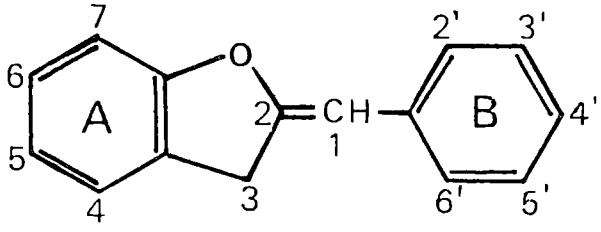
Leucoanthocyanidin



Catechin

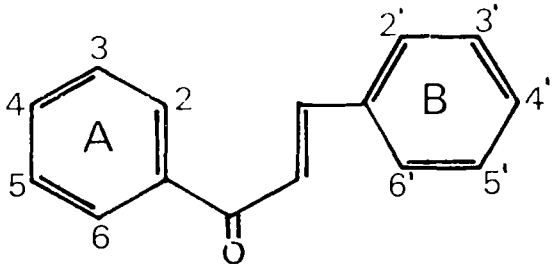


Isoflavone

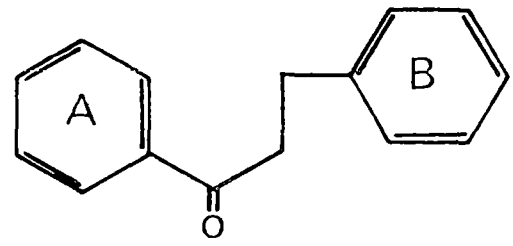


Aurone

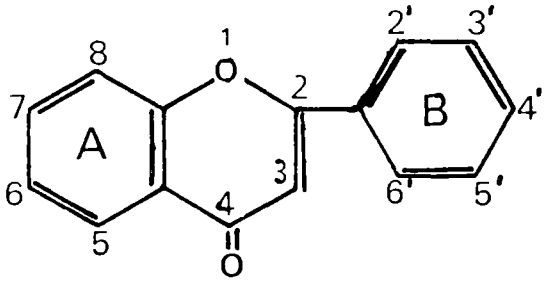
FIG. 2



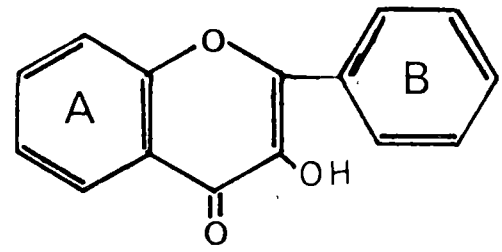
Chalcone



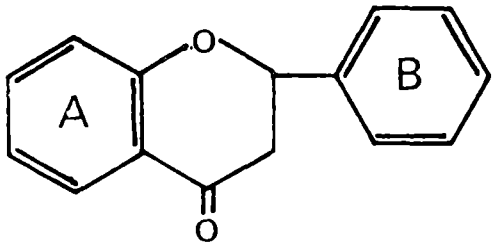
Dihydrochalcone



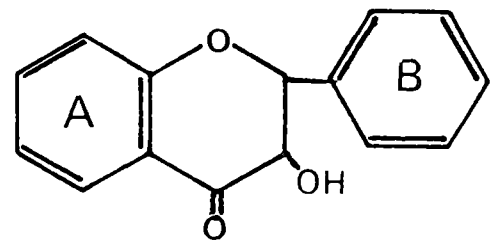
Flavone



Flavonol



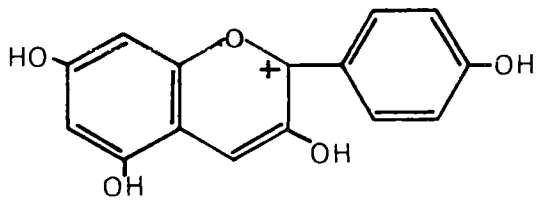
Flavanone



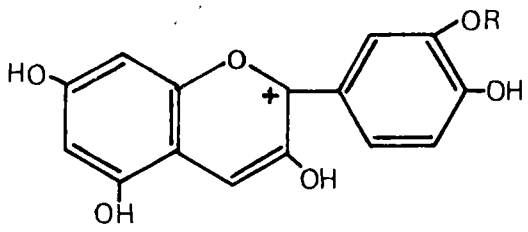
Flavanonol

FIG.2

Anthocyanidins

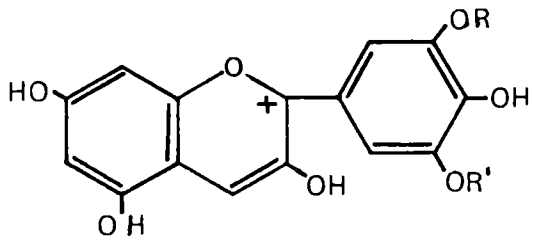


Pelargonidin



Cyanidin, R = H

Peonidin, R = Me

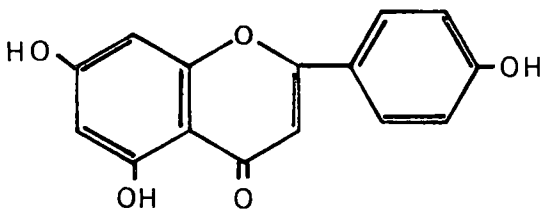


Delphinidin, R,R' = H

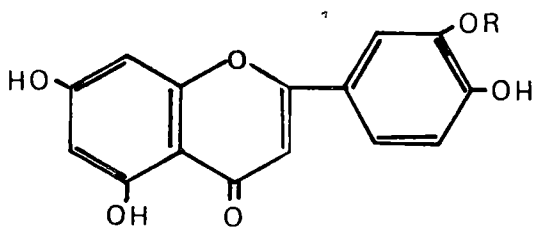
Petunidin, R = Me R' = H

Malvidin, R,R' = Me

Flavones



Apigenin

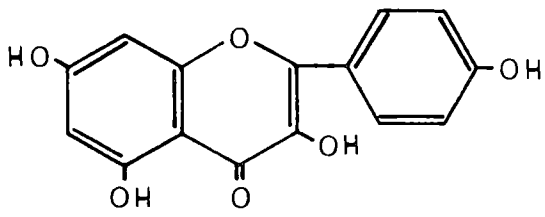


Luteolin, R = H

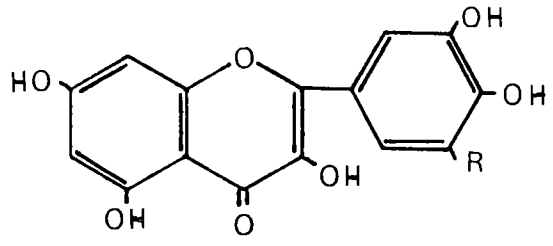
Chrysoeriol, R = Me

FIG 3

Flavonols



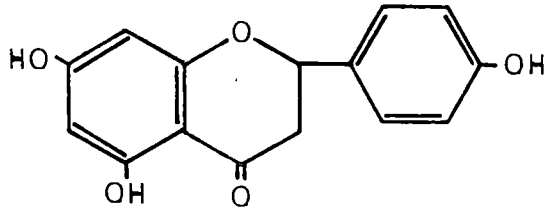
Kæmpferol



Quercetin, R = H

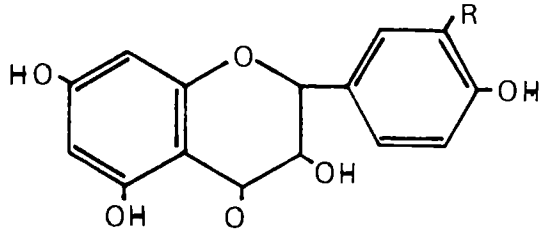
Myricetin, R = OH

Flavanone



Naringenin

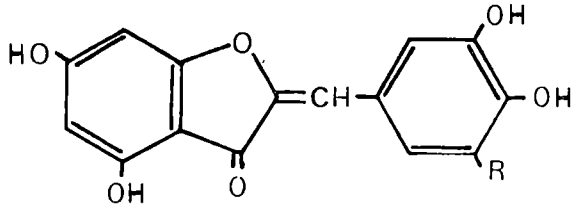
Leucoanthocyanidins



Leucopelargonidin, R = H

Leucocyanidin, R = OH

Aurones



Aureusidin, R = H

Bracteatin, R = OH

FIG 3

1:4 Flavonoids and Flower Colour

Reviews dealing with flavonoids and flower colour have been presented from time to time and include Scott-Moncrieff, 1936; Blank, 1947, 1958; Lawrence, 1950; Paech, 1955; Reznik, 1956 and Harborne, 1965. From these reviews it would appear there are very many different ways in which flavonoids contribute to plant colouration.

The contribution of anthocyanins to flower colour is basically simple. Three main pigment groups are based on pelargonidin, cyanidin and delphinidin, which differ in structure only by the number of hydroxyl substituents in the B ring. Broadly speaking, pink, scarlet and orange-red flowers have pelargonidin anthocyanins, crimson and magenta flowers have cyanidin anthocyanins and blue flowers have delphinidin anthocyanins, i.e. the addition of hydroxyl substituents to the B ring of anthocyanins tends to increase blueness. This is well illustrated by reference to the colour mutants of *Lathyrus odoratus*. Genotypes *E Sm and E sm* have purple flowers pigmented by delphinidin (and petunidin and malvidin) anthocyanins; *e Sm* genotypes possess red flowers and cyanidin (and peonidin) anthocyanins, while the double recessive, *e sm*, has salmon flowers pigmented with pelargonidin anthocyanins (Beale et al. 1939; Harborne, 1960).

Methylation of some of the hydroxyl groups of the anthocyanidin molecule has a small reddening effect on colour, which is apparent from a consideration of the absorption spectra of the known methylated pigments. Few examples of this effect can be quoted because mostly it is obscured by other factors, particularly copigmentation. However, the relative amounts of various methylated pigments have been measured in several mutants of *Primula sinensis*, all of which are recessive for the copigment gene *B*, and the results support the thesis that methylation has a reddening effect (Harborne and Sherratt, 1961).

Although glycosylation of the 3-hydroxyl group of anthocyanidins has a relatively large hypsochromic effect (-15nm) in the visible region of the spectrum, glycosylation is not thought to be an important factor in flower colour, because, as mentioned previously, flavonoids always occur as glycosides, i.e. and anthocyanins have at least one sugar attached to the 3-hydroxyl. From the point of view of colour tone, the nature of the 3-substituted sugar is immaterial, as all 3-glycosides of a particular anthocyanidin have the same visible spectrum. Substitution of a sugar at the 5-position of anthocyanins does have a small effect on colour, although 3-glycosides and 3,5-diglycosides have almost identical visible maxima. The 3,5-diglucosides of pelargonidin, peonidin and malvidin differ from the corresponding 3-glucosides by being fluorescent in solution.

Variations in the amounts of anthocyanin in plant tissue have profound effects on colour. Rather few quantitative measurements have been made, but Harborne and Sherratt (1961) found that the orange "Dazzler" mutant of *Primula sinensis* has three times as much pelargonidin glucoside as the coral form, this difference in anthocyanin concentration being under monogenic control.

The phenomenom of co-pigmentation, the blueing of anthocyanin *in vivo* by flavones and related substances was discovered independently by the Robinsons (1931) and by Lawrence (1932), and is easily demonstrated *in vitro* At room temperature, aqueous acid extracts of co-pigmented flowers are bluer in tone than those of unco-pigmented petals. On heating, the loose co-pigment-pigments complex is dissociated and there is a reddening in colour; on cooling, the colour reverts to the original blue shade. Blueness in flowers of *Lathyrus* is due to co-pigmentation of delphinidin, petunidin and malvidin 3-rhamnoside, 5-glucoside (Harborne, 1963) with kaempferol and

quercetin glycosides. Pecket and Selim (1962) have demonstrated this by hybridizing a species with red flowers, which lack flavonols, with one having cream flowers, possessing flavonols. The resulting hybrid *Lathyrus clymenum* x *ochrus*, had the expected mauve coloured flowers.

Another major factor thought to be responsible for blueing of flower colour in higher plants is the chelation of anthocyanins with metal ions. These metal complexes are unstable *in vitro* and are dissociated by acid or ion-exchange columns, but are more stable *in vivo* than non-complexed pigments. The pure blue pigment from the cornflower, *Centaurea cyanus* has been the most studied. Bayer (1958) and Bayer et al. (1960) showed that it contained cyanidin 3,5-diglucoside, complexed with ferric iron and aluminium. It was also shown that this possessed spectral properties very similar to those of synthetic anthocyanin metal complexes. Study of metal complexes of other plants has shown that an essential factor for formation of stable metal complexes is the presence in the anthocyanin of a free o-dihydroxyl grouping.

As solutions of anthocyanins are red in acid and blue in alkali, pH of the cell sap was considered to be an important factor in flower colour. However, a survey by Shibata et al. (1949) of the cell sap of 200 plants showed that flowers were all acidic (pH about 5.5) irrespective of their colour. Nevertheless, small changes in pH within the acid range appear to affect flower colour. Scott-Moncrieff (1936) noted differences of 0.5 - 1 pH units between red and mauve colour forms of *Primula sinensis*, *P. acaule* and *Papaver rhoeas*. In *Primula sinensis*, the change of pH from 5.4 to 6.2 has a blueing effect and is controlled by the gene *R* which is independent of factors controlling co-pigmentation and methylation.

Flower colour effects are also produced by interaction of anthocyanin and other pigments, as exemplified by the colour range in the garden snapdragon *Antirrhinum majus*, which contains yellow aurone pigments, but no carotenoids.

(Wheldale, 1907, Jorgensen and Geissman, 1955a and b). When anthocyanins and lipid-soluble yellow pigments co-occur, the resulting flower colour may be brown rather than the orange observed with water-soluble yellow pigments. For example, the brown colour formed by magenta cyanidin on yellow carotenoid background in the wallflower, *Cheiranthus cheiri*, as compared with cyanidin and water-soluble, but unidentified, yellow pigments of yellow-red petals of *Lapeyrousia cruenta*, (Harborne, 1965).

Inidvidual flowers may exhibit a change in colour as they develop. Such changes are very pronounced in some instances and have therefore attracted the interest of pigment chemists. The assumption that such colour changes are due to the interconversion of one kind of pigment into another has not been verified experimentally. Tracer experiments and enzymatic studies have shown, in fact, that rather than being end products of metabolism, flavonoids and carotenoids are regularly turned over in the plant. (e.g. Grisebach and Bopp, 1959). Reznik (1961) using *Primula obconica*, and Hess (1963) considering flavonoids of *Petunia hybrida*, have shown that the initiation and rate of synthesis of flavonols are quite independent of anthocyanin synthesis. Such studies of flavonoids formed at different stages of floral development have indicated which are the later stages in pigment synthesis. Generally as tissues mature, there is a progression from less complex to more complex molecular structures. Reznik (1961) provided evidence on the accumulation of glycosides of both flavonol and anthocyanin; Hagen (1966) reported acylation of anthocyanins as a terminal stage of floral pigmentation, and Hess (1963) using three genetically homozygous lines of *Petunia* showed that anthocyanidins appeared during floral development in the sequence cyanidin → delphinidin → peonidin → petunidin → malvidin, which reflects the proposed biosynthetic sequence.

The commonly occurring flavonols and flavones do not contribute significantly to flower colour, except when present as co-pigments. Chalcones and aurones not only affect flower colour when present with anthocyanins, but also contribute the yellow flower colours of *Dahlia* and *Antirrhinum*. Quercetagetin (6-hydroxy-quercetin) and related compounds are the principal pigments in the primrose, *Primula vulgaris*, and the former is also a major pigment of *Rhododendron*. The isomeric 8-hydroxy-quercetin, gossypetin, is an important pigment in yellow cotton flowers. The relative contribution of flavonoid and carotenoid to yellow flower colour is difficult to determine, due to the paucity of quantitative data, but the general situation was tabulated by Harborne in 1965 who noted that there was a relatively large number of plants which are coloured yellow by both flavonoid and carotenoid, but the results available at the time showed that the carotenoids contribute more to yellow colour in higher plants than do flavonoids. However, it was suggested that future studies may bring flavonoids into equal prominence.

A large proportion of cream, ivory and white flowers of higher plants possess flavones and/or flavonols. These chemicals are not visible to the human eye, but they absorb strongly in the ultraviolet and can be "seen" by bees and, presumably, other insects. The presence of these flavonoids adds to the reflectance of such flowers, and the albino mutant of *Antirrhinum majus* is readily distinguished in appearance from ivory flavone-containing flowers of the species (Harborne, 1965). White flowered plants, completely lacking in flavonoids, are rare and appear to be less hardy than flavonoid-containing plants, being difficult, in some instances, to preserve in cultivation.

1:5 Biochemical Genetics of *Pisum*

Flower colour

In the studies which led to the birth of genetics, Mendel (1865) used flower colour as one of the characters in his crosses. He crossed a wild type purple flowered pea with a white flowered pea, and obtained a monofactorial ratio in the F_2 generation with dominance of purple flowers. Tschermak (1912) one of the rediscoverers of Mendel's work, ascribed to this locus the symbol *A* on confirming the monofactorial difference between purple and white. Also Tschermak was responsible for the discovery of the *B* locus controlling flower colour in peas, the colour being pink when the gene is in the recessive state.

The gene responsible for the recessive colour mutant, violet, was first described by Tedin in 1920, from the following results:

Light purple *Abc*	x		White *aBC*	
		Purple *AaBbCc*		
27 Purple *ABC*	9 Violet *AbC*	9 Rose *ABc*	3 Light purple *Abc*	16 White *a--*

The trifactorial nature of this cross was confirmed by results in F_3.

It can be seen from above that the early workers did not use the same symbols for hereditary differences, i.e. Tschermak had described the *B* locus such that *Ab* plants possessed rose coloured flowers. In Tedin's work rose coloured plants are described as *ABc*. Thus the Tedins and Wellensiek (1925) agreed to standardize nomenclature and to indicate the three factors determined for flower colour in peas by *A*, *B* and *Ar*. The formulae for the then known colours was thus:-

Purple	*A B Ar*
Rose	*A b Ar*
Violet	*A B ar*
Light purple	*A b ar*
White	*a - --*

A further mutant locus was characterized by de Haan (1930) as *Ap* for the uni-coloured variety called apple blossom. The existence of this gene had already been demonstrated by Fedotov (1930), and symbolized *Cr* for crimson flowers, but this was not known to de Haan at the time, and thus the symbol *Cr* took precedence.

In the same publication, de Haan (1930) described an additional new gene controlling flower colour in peas. This mutant appeared to deviate from the rule that "coloured flowers and a coloured seed-coat go together". The mutant plants appeared not to be pure white flowering, the flowers showing a red colour after treatment with an acid, which was not the case for acyanic flowers of *Pisum*. Sometimes when in the bud stage, these mutant flowers showed a faint pink colour at the margins of the wings, and it was in connection with this latter point that the new flower colour was called pinkish-white. The symbol *am* was proposed for this gene which inhibited anthocyanin formation in a recessive condition.

Wellensiek (1947) published data to support another locus affecting flower colour in peas, which he symbolized *Aw*. He suggested that "there is much agreement between *Am* of de Haan and my *Aw*, so much that I have supposed them to be identical." However, both de Haan and Wellensiek used acid to distinguish the genotypes of white flowered plants, with conflicting results.

de Haan: Pinkish-white flowers (*A am*) on treatment with HC1 showed a red colouration, while pure white flowers (*a Am*) produced no colour.

Wellensiek: *A aw* flowers treated with HC1 showed no distinct change, *a Aw* flowers, on the other hand, produced a distinct rose or red colour after treatment with acid.

Lamprecht (1954) had access to material from both de Haan and Wellensiek. He found that the two genes were identical by genetic analysis,

since the linkage of both genes with marker gene *I*, in group 1, was identical, and in crossing the two lines, the F_1 was pinkish-white, and not purple as would have been expected should the two genes not have been identical. Also in these experiments, no crossovers resulted in the F_2 generation. Lamprecht suggested the symbol *am* should replace *aw*, which would be omitted from the gene list of *Pisum*. Unfortunately, Lamprecht did not report any chemical testing of the flowers.

Wellensiek (1951) reported a new *Pisum* flower colour, cerise, governed by the recessive gene *ce*. From the data he concluded that *Ce* and *Cr* were linked with a crossing over value of approximately 26.5%.

New flower colours have been reported from time to time, as new combinations of genes occurred, e.g. Lamprecht, (1957). Blixt (1962) in reviewing colour mutations of peas, lists the combined colour effect on the wing petals of *Pisum* flowers as given below:

A Am Ar B Ce Cr	Purpureus, a dull dusky purple (highly variable)
A Am ar B Ce Cr	Coeruleoviolaceus, nigrosin violet
A Am Ar b Ce Cr	Clariroseus, deep pink rose
A Am ar b Ce Cr	Roseialbus, pale rose pink
A Am Ar B ce Cr	Roseus, cerise
A Am Ar B Ce cr	Fuscopurpureus, Indian lake
A Am ar B Ce cr	Malvaceous, light mauve
A Am Ar B ce cr	Palleopurpureus, pale rose purple

Lamprecht (1961) undertook a critical analysis of the species characters of *Pisum fulvum* Sibth. and Sm. On the basis of descriptions and dried plants, only one qualitative character could be found separating *P. fulvum* from *P. arvense* -- the yellow colour of the flowers. In four crosses, the segregation of 15 genes was studied. Two genes for flower colour, *Cit* for clariluteus (citron-yellow) and *Cm* for claricorallinus (pale coral red) were detected which jointly determine the ochraceus flower colour of *P. fulvum* (see Fig. 4). Together with the further six genes for flower colour described above, (i.e. *A*, *Am*, *Ar*, *B*, *Ce* and *Cr*), *Cit* and *Cm* give a very complicated segregation, producing at least twelve different flower colours in F_2. However, no chemical analysis of these

flowers colours was attempted by Lamprecht, and it would appear that his segregates have not been preserved.

Several other flower colour loci have been described for *Pisum*, including B_1 and *Cv*, first described by Fedotov (1936), but information on these loci is sparse since the genetic material has not been maintained.

Flower colour nomenclature in *Pisum* (ii)

Flower colour nomenclature in *Pisum* is somewhat confused, since most of the reports are in German, and give the names of the ascribed colours in German, Latin and English; up to four different colour charts have been consulted in each case, and this has led to the profusion of names for flower colours of any one genotype. Most of the colour charts referred to in the literature are difficult to procure, and in the rest of this thesis, the following names for flower colours are used.

Purple (wild type)	*A Am Ar B Ce Cr*	Fig. 5
Pinkish-white	*A am Ar B Ce Cr*	Fig. 6
Violet	*A Am ar B Ce Cr*	Fig. 7
Pink	*A Am Ar b Ce Cr*	Fig. 8
Cerise	*A Am Ar B ce Cr*	Fig. 9
Crimson	*A Am Ar B Ce cr*	Fig. 10
Pale pink	*A Am ar b Ce Cr*	Fig. 11
Pale lilac	*A Am ar B ce Cr*	Fig. 12
Light mauve	*A Am ar B Ce cr*	Fig. 13
Light rose	*A Am Ar b ce Cr*	Fig. 14
Salmon	*A Am Ar b Ce cr*	Fig. 15
Pale rose purple	*A Am Ar B ce cr*	Fig. 16
White	*a* -- -- - -- --	Fig. 17

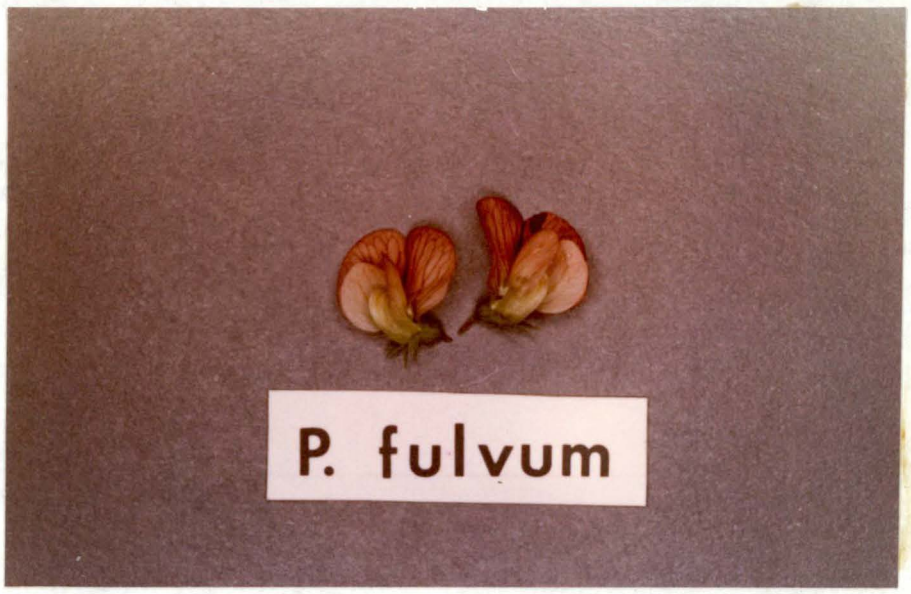


Fig. 4 Ochraceus flowers

Cm Cit

Fig. 5 Wild-type purple flowers

A Am Ar B Ce Cr

Fig. 6 Pinkish-white flowers
A am Ar B Ce Cr

these labels are inverted
AMB

Fig. 7 Violet flowers
A Am ar B Ce Cr

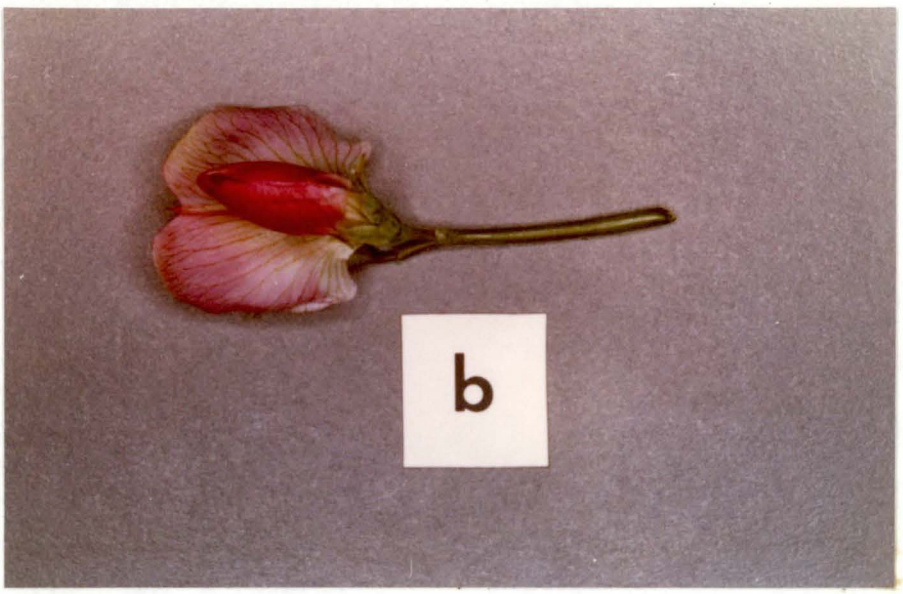


Fig. 8 Pink flowers

A Am Ar b Ce Cr

Fig. 9 Cerise flowers

A Am Ar B ce Cr

Fig. 10 Crimson flowers

A Am Ar B Ce cr

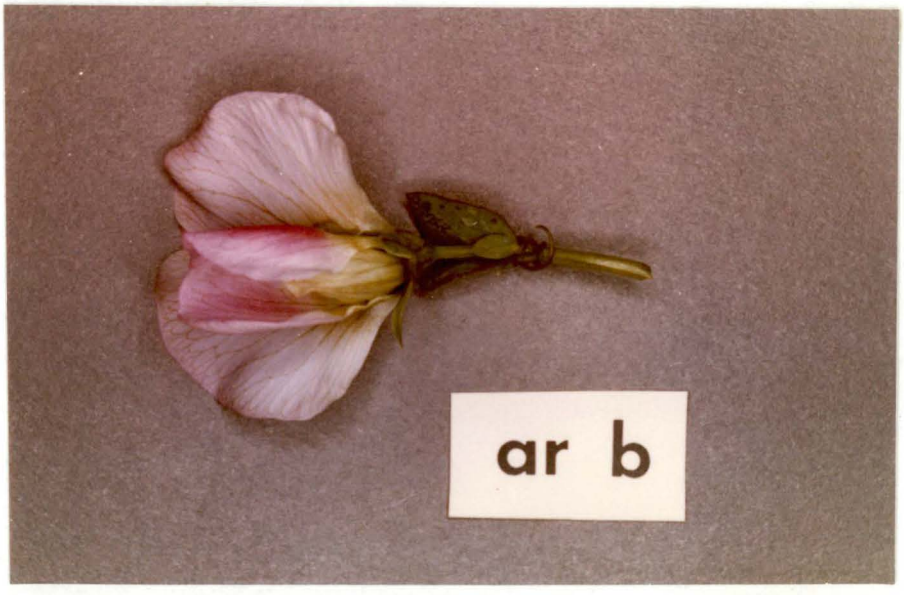


Fig. 11 Pale pink flowers

A Am ar b Ce Cr

Fig. 12 Pale lilac flowers

A Am ar B ce Cr

Fig. 13 Light mauve flowers

A Am ar B Ce cr

Fig. 14 Light rose flowers

A Am Ar b ce Cr

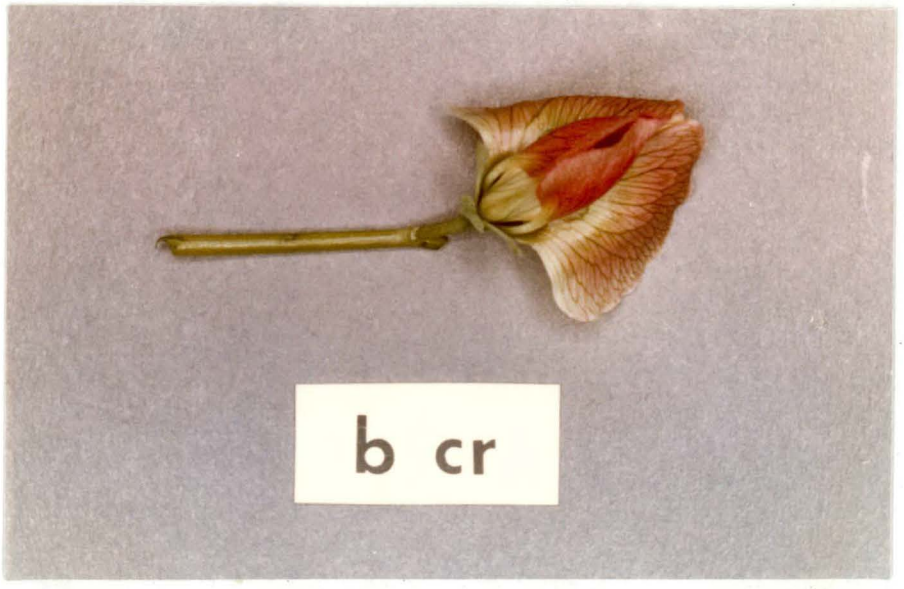


Fig. 15 Salmon flowers

A Am Ar b Ce cr

Fig. 16 Pale rose purple flowers
A Am Ar B Ce cr

Fig. 17 White flowers
a

Axil Colour

Mendel (1865) noted that the dominant *A* gene simultaneously conferred colour in the flowers, the seed coat and leaf axils of *Pisum*. Other loci are known which are more specific in their action; for example, *D* is necessary for axil colour and the complementary genes, *Pur* and *Pu*, allow the development of anthocyanin in pods.

The ground factor for axil colour, *D*, was demonstrated by Tschermak (1912), but was later shown to have three alleles : (Kappert, 1923; Wellensiek, 1925; Tedin and Tedin, 1926; Lamprecht, 1944).

D^{W} : Double ring of colour at the base of stipules in the leaf axils

D^{co} : Single ring of colour

D^{ma} : Spot of colour

d : Without coloured area at the base of the stipules in the leaf axils

The two new flower colour genotypes described by de Haan (1930) differed from the *Pisum* genotypes previously available in that they did not possess corresponding flower and axil colour. He found that the factor for crimson flowers should be taken as operating only in floral tissue, since *cr* genotypes possessed crimson flowers and purple axils; *b cr*, genotypes had salmon flowers and rose axils, and light mauve flowered plants, of genotype *ar cr* possessed violet axils. The leaf axil colour of his pinkish-white flowered plants, (*am*) was dull rose, and de Haan described the *am* gene as having a different effect on the flowers and leaf axils.

If this description of *am* genotypes is borne in mind, then the "white flowered, coloured axils" line used by Wellensiek (1947) is seen to be recessive *am*, since such plants do have white flowers (sometimes tinged with pink at the wing edges) and dull rose axils. These white flowers are

not acyanic in the sense associated with recessivity at the *A* locus, whereby anthocyanin is absent from all parts of the plant.

It would appear that neither Wellensiek nor Lamprecht fully appreciated the pleiotropy of *Am;* Wellensiek did not distinguish the colour of the leaf axils in his publication, using only coloured *(D)* or uncoloured *(d)*, while Lamprecht (1954) stated that de Haan's pinkish-white *(am)* line was characterized by purple leaf axils. de Haan made quite clear that the dull rose leaf axil colour of *am* genotypes was readily distinguishable from the other axil colours, namely purple, violet and rose.

de Haan tabulated flower and leaf axil colour, and although seven combinations of the loci *A, Ar Am, B* and *Cr* were not known then, by analogyy with the data already available, he inferred that different recessive combinations with *am*, (for example, *am b, am ar)*, would be distinguishable with the aid of the leaf axil colours. He had obtained a progeny segregating for both *am* and *b*, both of which he knew to affect axil colour, but he did not score this character; he scored only flower colour, and in the tabulation mentioned above, de Haan lists the genotype, *am b*, as unknown.

While de Haan described recessive *b* genotypes of *Pisum* as possessing rose axils, and recessive *am* genotypes as having dull rose axils, he took care to point out that these two colours were readily distinguished. However, since rose coloured flowers are now described as pink (see page 17) the leaf axil colour for such plants will subsequently be termed red, which better describes the colour. The tabulation of flower and leaf axil colours given below was compiled from observation of the genotypes and uses the nomenclature adopted earlier.

GENOTYPE	FLOWER COLOUR	AXIL COLOUR
A Am Ar B Ce Cr	Purple	Purple
A am Ar B Ce Cr	Pinkish-white	Dull rose
A Am ar B Ce Cr	Violet	Violet
A Am Ar b Ce Cr	Pink	Red
A Am Ar B ce Cr	Cerise	Purple
A Am Ar B Ce cr	Crimson	Purple
A am Ar b Ce Cr	Pinkish-white	Red
A Am ar b Ce Cr	Pale pink	Light purple
A Am ar B ce Cr	Pale lilac	Violet
A Am ar B Ce cr	Light mauve	Violet
A Am Ar b ce Cr	Light rose	Red
A Am Ar b Ce cr	Salmon	Red
A Am Ar B ce cr	Pale rose purple	Purple

Pod Colour

Anthocyanin may also be developed in pods of *Pisum*, when the flower colour genes are operating on an appropriate background genotype. The development of pod anthocyanins is controlled by a pair of complementary genes, *Pur* and *Pu*. Lamprecht (1953) distinguished three degrees of pod colour related to multiple alleles at the *Pur* locus:

Pur : Full anthocyanin colouration of pods

pur^a)
pur^b) : Progressively less colouration

pur : Without anthocyanin on the pods.

Ph. de Vilmorin (1912) obtained red pods from a crossing of a violet podded form with a yellow podded variety, but he did not give the results of segregation in F_2. J. de Vilmorin (1921) makes mention of coloured pods, but in relation to "ghost marbling". It is presumed he was dealing with one or other of Lamprecht's polymeric genes, pur^a or pur^b. Vilmorin attributes the red colour of pods to the expression of violet colour on a yellow *(gp)* background. Wellensiek (1925) reported several crosses using

plants having purple flowers and coloured pods. He pointed out that no plants having coloured pods possessed white flowers, and that total absence of violet colouration gave green pods, while total absence of red colour gave yellow pods.

Testa colour

Anthocyanin may also be found colouring the seed coat of *Pisum*, the expression of colour again being dependent on the particular genotype with reference to flower colour loci, dominance at the *A* locus being necessary for development of anthocyanin. The pattern of anthocyanin pigmentation of the testa is under the control of a number of loci, e.g. *F*, *U*; but these will not be discussed further since no biochemical studies were performed on this material during the course of the present investigation.

Biochemistry

Literature on the biochemistry of flavonoid pigmentation in *Pisum* is sparse. Bach (1919) appears to have been the first worker to examine the pigments of *Pisum* by chemical methods. He compared the various grades of anthocyanin in several flowers. The quantity of pigment appeared to be about the same in homozygous and heterozygous forms. He found the difference between purple and pink flowers was not exclusively in quantity : factor *B* did not intensify the activity of *A* and *Ar*, but originated a new pigment.

Govorov (1928) suggested that the peculiar dirtyish-pink or creamy hue of the standard of *Pisum sativum* L. subspecies *asiaticum* was determined by the presence, besides anthocyanin, of a special pigment belonging

to the flavone group. However, since no full translation of the Russian publication was available, it was not possible to determine on what chemical evidence, if any, the suggestion was based.

Dodds and Harborne (1964) isolated and identified the anthocyanins in the flowers of a series of known genotypes of *Pisum*, with the following results. Wild type, purple flowers had a mixture of the 3-rhamnoside-5-glucosides of malvidin, petunidin and delphinidin. Cerise had the same pigments, but only one sixth as much in the wing petals and one third as much in the standards. Crimson flowers were found to contain four delphinidin glycosides; two of these were delphinidin 3-glucoside and delphinidin 3,5-diglucoside; the other two were thought to be the related sophorosides. Pink flowered forms had the 3-rhamnoside-5-glucosides of peonidin, cyanidin and pelargonidin, as well as traces of other cyanidin-glucosides. L826, of genotype *ar b*, supplied to Dodds and Harborne by Blixt, (Weibullsholm, Sweden) possessed deep red pods, the main pigment of which was cyanidin 3-sambubioside-5-glucoside occurring with traces of cyanidin 3-sophoroside-5-glucoside.

Harborne (1936) found white flowered forms of *Pisum* to be unusual in lacking both anthocyanins and flavonols. Two trisaccharides derived from sophorose are present in the leaves of *Pisum* in all genotypes irrespective of their genetic constitution with regard to *A*. One, a glucosylsophorose, occurs as the trisaccharide unit of the flavonols of the garden pea, whereas the other, a galactosylsophorose, is found as a leaf constituent of the field pea.

1:6 Summary

The biochemistry and genetics of flower colour variation has been investigated over a relatively long period. Independent studies using radioactive materials has provided information regarding the biosynthesis of flavonoids, but very little work has been done on the enzymology of the later stages of this pathway. The contribution of flavonoids to plant colouration has now been reasonably well documented, and it is evident that there are many different interactions responsible for the array of such colours.

In the genus *Pisum* six major loci have been described as affecting flower colour, and twelve different flower colour genotypes, including white, have been documented. However, with the exception of the work of Dodds and Harborne, (1964), no attempt has been made to define the way in which the flower colour genes affect the chemistry of the flavonoids. From their investigation of a limited number of genotypes, Dodds and Harborne suggested that the genes *b* and *cr* have qualitative chemical effects while the *ce* gene has only a quantitative effect on *Pisum* anthocyanin.

It was suggested (Fedotov, 1936) that the *Ar* gene controls the acidity of the cell sap, since anthocyanins are natural indicators and, on examination, genotypes containing the dominant gene *Ar* have an intensification of red tints when compared to genotypes containing the recessive gene *ar*.

For *am* genotypes of *Pisum*, some chemistry has been reported by de Haan, (1930) and Wellensiek (1947), in reference to the reaction of flowers of this genotype with concentrated HCl. However, due to their conflicting results, the position was contradictory.

The terms of reference for the current investigation were firstly, the determination of a chemical profile for each of the available colour mutants, and secondly, the genesis of other colour mutants by a crossing programme where this appeared necessary for the elucidation of gene interaction in flavonoid pigmentation. Finally, by establishing the manner in which the loci *A*, *Am*, *Ar*, *B*, *Ce* and *Cr*, affect the chemistry of the constituent flavonoids of *Pisum*, it should be possible to append these loci to the proposed biosynthetic pathway of the flavonoids.

MATERIALS AND METHODS

2:1 Plant materials

Seeds from genetically pure lines of *Pisum* were used throughout the investigation. The seeds were obtained from Dr. S. Blixt, Plant Breeding Institute, Weibullsholm, Landskrona, Sweden, and from Dr. I.C. Murfet, Botany Department, University of Tasmania, Hobart. A full list of genotypes available is given in Table 1, with seeds of Swedish origin prefixed S, and those of Tasmanian origin prefixed L. Among the lines maintained at Weibullsholm are many of the original lines described in the literature, and this includes material from de Haan, Wellensiek, the Tedins and Lamprecht.

Plants were grown in a glasshouse, in containers having 2.5 kg of a 1:1 vermiculite-dolerite chip (13 mm mesh) medium. Plants were watered daily, and a modified Hoagland's nutrient solution applied weekly. In some instances, plants were field grown in soil previously treated with an application of N.P.K. fertiliser. Flowers produced under the latter conditions were treated separately from flowers of glasshouse-grown plants.

Generally, for field grown plants, flower colour was deeper, and seed yield was improved, when compared with plants grown in the glasshouse. However, as the control of pests was more difficult in the field, field-grown plants were used to provide material for isolation of chemical constituents, and for the bulking of seed. All genetic crosses were performed in the glasshouse.

2:2 Genetic crosses

Lines to be used in genetic crosses were planted, 2 seeds/ container, and grown in a heated glasshouse, in an effective day-length of not less

TABLE 1

Line numbers of genotypes used in this investigation.

GENOTYPE	LINE NUMBERS
(a) Flowers & Axils	
A Am Ar B Ce Cr	S180, S577, S936, S1017, S1293, S1402, S1516. L2, L41, L51, L60, L63.
A am Ar B Ce Cr	S239, S369, S1088, S1089, S1166, S1451.
A Am ar B Ce Cr	S25, S1391.
A Am Ar b Ce Cr	S592, S1167, S1185, S1238, S1324, S1508, S1515, S1520.
A Am Ar B ce Cr	S1227, S1458.
A Am Ar B Ce cr	S1221, S1328, S1366.
A am Ar b Ce Cr	S1467, S1512.
A am Ar B ce Cr	From C17.
A Am ar b Ce Cr	S21, S857, S1319, S1357.
A Am ar B ce Cr	From C27.
A Am ar B Ce cr	S1325, S1326.
A Am Ar b ce Cr	S1511.
A Am Ar b Ce cr	S1510, L12.
A Am Ar B ce cr	S1228, S1539.
a - - - - -	S102, S206, S680, S1143, S1241, S1525, L1, L8, L14, L15, L22, L23, L24, L58, L59.

TABLE 1 (cont.)

Line numbers of genotypes used in this investigation.

GENOTYPE	LINE NUMBERS
(b) Pods	
A Am Ar B Ce Cr Pur Pu	S577, S1017, L41, L56
A Am ar B Ce Cr Pur Pu	From C3.
A Am Ar b Ce Cr Pur Pu	From C133.
A Am Ar B ce Cr Pur Pu	From C33.
A Am Ar B Ce cr Pur Pu	From C2.
A Am Ar b ce Cr Pur Pu	From C33.
A Am ar b Ce Cr Pur Pu	S826.
(c) Other	
Pisum fulvum	S1256.
Lathyrus odoratus	Var. Chloe (Arthur Yates).

than 18 hours. Female parents were emasculated by slitting the keel petals with a scalpel blade before anthesis, and removing the immature anthers with fine-pointed forceps. Pollen was collected on a scalpel blade from mature anthers of pollen parents, and then transferred to the stigma of the emasculated female parent.

F_1 seed was collected when pods were dry, and planted under the conditions previously described. In most instances, F_1 plants were allowed to self-fertilize, to produce an F_2 generation. The F_2 was again planted out, and each plant's phenotype recorded. Chemical analyses were carried out where applicable, and included two-dimensional paper chromatography of anthocyanins and flavones (e.g. C2, C3), one-dimensional paper chromatography of anthocyanidins (e.g. C2, C3) and one-dimensional paper chromatography of flavones (e.g. C1, C29, C26), all on Whatman No. 1MM chromatography paper.

2:3 Paper Chromatography

Conditions used for paper chromatography were as described by Harborne, (1958b), and Whatman No. 1 MM and No. 3 MM chromatography papers were used throughout.

The loaded papers were developed by descending chromatography in large Shandon chromatography tanks, dried in a fume cupboard, and inspected with the aid of a Chromatovue paper viewer, having both long and short wave ultra-violet light. Solvent mixtures used for chromatography were :

BAW	(n-butanol:glacial acetic acid:water, 5:1:4 by volume, upper phase)
5%HOAc	(glacial acetic acid:water, 5:95 by volume)
BuHCl	(n-butanol:2N HCl, 1:1 by volume, upper phase)
WAH	(water:glacial acetic acid:conc. HCL, 82:15:3 by volume)
Forestal	(glacial acetic acid:conc. HCl:water, 30:3:10 by volume)
Formic	(formic acid:conc HCl:water, 5:2:3 by volume)
BEW	(n-butanol:95% ethanol:water, 4:1:22 by volume)
1% HCl	(conc. HCl: water , 3:97 by volume)

Eluting solvents for removal of flavonoids from the paper included

MAW	(methanol:glacial acetic acid:water, 85:10:5 by volume)
MAW/1% HCl	(MAW:conc. HCl, 99:1 by volume)

2:4 Extraction, Separation and Purification of Flavonoids

The general method of extraction, separation and purification of the flavonoids from *Pisum* material was that published by Harborne in 1958b. Preliminary separation of extracts was carried out on No. 3 MM chromatography paper in BAW solvent. As a general rule, 5% HOAc was the second solvent used in the separation. As circumstances required, other solvents were used, including (especially in the case of pod material in the initial stages of the investigation) 3% HCl. Also in the case of pod material, it was found necessary to elute anthocyanins from the paper using MAW/1% HCl, in order to obtain clean elutions.

At a later stage of the investigation, pod material was extracted, and extracts separated by the procedure of Jarman and Crowden, (1972): Fresh plant material was extracted with methanol containing a minimum quantity of HCl. The filtered extract was concentrated to a small volume in a rotary evaporator, water was added and evaporation continued until all traces of methanol were removed. The remaining aqueous solution was further diluted

with large quantities of water (at least 5 times the volume of original methanol extract), filtered to remove water-insoluble materials, and then passed through a column containing the weak carboxylic acid ion-exchange resin, Bio-Rex 70H$^+$ (Bio Rad Laboratories). The anthocyanin mixture was then eluted from the column with MAW (MeOH:HOAc:Water, 17:1:2) and concentrated to dryness by rotary evaporation. This preliminary treatment, while causing no apparent breakdown of the pigments, provided a mixture of anthocyanins free from bulky, viscous contaminants, and greatly facilitated the subsequent fractionation procedures.

Purification of individual anthocyanins was achieved by preparative scale paper chromatogrpahy on Whatman 3 MM paper, using BAW and 5% HOAc in succession.

Flavone contaminants could be removed from partially purified anthocyanins by passing them in a weakly acidic, aqueous solution through short (3cm x 0.5cm) polyamide-celite column. The anthocyanin was collected directly onto a column of the ion-exchange resin Zeo-Karb 226H$^+$ (Permutit Co.), from where it was eluted with MAW as a pure anthocyanin. Paper chromatographic monitoring of the process showed that this treatment caused no alteration of the anthocyanin structure.

Bands resulting from these processes were chromatographed until no further separation of bands was achieved.

2:5 Identification of the Anthocyanidins and Sugars of Anthocyanins

Eluates of the purified pigments were evaporated to dryness in vacuo.

The residues were redissolved in MEOH, 1 ml of 2N HCl added, and the anthocyanin subjected to a partial hydrolysis in a boiling water bath, aliquots being streaked on Whatman 3MM chromatography paper at 0 minutes (no heating applied), 6 minutes after initial heating and 12 minutes after heating. These papers were chromatrographed in BAW, to allow detection of intermediate hydrolysis products. The remaining solution was then subjected to a complete hydrolysis by boiling for a further 50 minutes. The hydrolysate was extracted with n-amyl alcohol to remove aglycones, and the aglycone extract (upper layer) evaporated to dryness. Aglycones were identified by co-chromatography with authentic anthocyanidins in forestal and formic solvents.

The aqueous residues of the complete hydrolysis (lower layer) were neutralized by several washings with 10% (v/v) solution of di-n-octyl-methylamine in chloroform. The neutralized aqueous extracts were concentrated *in vacuo*. Sugars, present as a result of the hydrolysis, were identified by co-chromatography with authentic sugars, using BBPW (n-butanol:benzene:pyridine:water; 5:1:3:3 by volume) and BAW solvents. The resultant papers were sprayed with a solution of aniline hydrogen phthalate (aniline:n-butanol:ether:water; 9.15:490:490:20 mls, to which is added 16 gm phthalic acid), and placed for 5 minutes in 100^{o}C oven. This treatment produced visible spots for comparison of sugars.

The spectra of purified pigments were recorded on a Unicam SP800 spectrophotometer, and interpreted after Harborne, 1958a.

2:6 Identification of Flavones

Eluates of the purified pigments were evaporated to dryness *in vacuo*. Residues were dissolved in 10 mls ethanol, and the spectrum in the ultra violet and visible range recorded on a Unicam SP800 spectrophotometer. To one portion of ethanolic solution was added 1 drop 20% $AlCl_3$ solution, and to another portion, 1 drop NaOH. Spectra of these solutions were also recorded.

Solid sodium acetate was then added to 5 mls of the ethanolic solution of the flavone, the mixture shaken, allowed to settle and the spectrum recorded. Solid boric acid was added to one half of the ethanolic/NaoAC solution, which was shaken and allowed to stand for 20 minutes before the spectrum was recorded. These spectra were interpreted after the manner of Jurd (1956, 1962) amd Mabry et al. (1970).

The remainder of the ethanolic solution was hydrolysed with 1 ml 2N HCl and the aglycone extracted with ethyl acetate, and co-chromatographed against reference aglycones in BAW. Sugars from the hydrolysis were obtained and identified as detailed previously.

2:7 Paper Chromatographic Survey of *Pisum* Material

Flowers of the various lines of *Pisum* were collected and stored at $2^{o}C$ in sealed plastic bags in a refrigerator until sufficient material was available for analysis. Pod material, both pigmented and non-pigmented, was analysed as fresh material, since this procedure gave sufficient material. Pigmented axils were cut from the plants after the fruiting stage, but before the plants had dried off, and axils were also treated as fresh material.

Wing and standard petals were treated separately in this survey. Keel petals were not investigated, since none of the *Pisum* lines available contained anthocyanin in the keel petals.

Anthocyanins

Plant material was crushed in a pestle and mortar in a solution of methanol: 1% HCl. The resultant solution was evaporated to dryness on a watch glass in a fume cupboard. The solid mixture resulting was moistened with 1 drop of MeOH/1%HCl and a spot applied to a sheet of 1 MM chromatography paper. The paper was chromatogrammed in BAW, dried, and then in 5% HOAc at 90^{o} to the original solvent front.

The resultant chemical profile for the organ of the particular genotype of *Pisum* was recorded on the paper, noting the presence and Rf values of each spot in visible light, and recording the colour of spots under long wave ultra violet light.

Other Flavonoids

For the extraction of flavonoids other than anthocyanins, (e.g. flavone, flavonol) plant material was covered with a solution of ethanol: water, 70:30 by volume, (70% EtOH) in a test tube. The test tube and contents were warmed in a water bath (about 60^{o}C), for about one hour.

After standing, the 70% ethanolic solution was evaporated to dryness on a watch glass. In this case, the resulting solid mixture was moistened with 70% EtOH, a spot applied to 1 MM chromatography paper, and the procedure then followed that given for anthocyanins. Papers containing these non-anthocyanin flavonoids were viewed under long wave UV light, and the colours of resultant spots recorded before and after treatment with ammonia fumes.

Weakly pigmented genotypes

Petals of weakly pigmented flowers contained insufficient pigment for analysis as described above, and therefore were hydrolysed with 2N HCl, and the resultant aglycones were extracted from the hydrolysate into n-amyl alcohol. The aglycones were evaporated to dryness in a fume cupboard, and co-chromatographed with reference aglycones in forestal and formic acids. If no anthocyanidins were apparent after hydrolysis, the hydrolysate was extracted with ethyl acetate to obtain any other phenols present. This extract was then chromatographed in forestal, formic and BAW solvents.

2:8 Investigation of the Sequence of Anthocyanin Accumulation during Floral Ontogeny

Flowers of pure lines of *Pisum* and *Lathyrus* were collected at various stages of floral development, and sorted into seven developmental stages, from the youngest, colourless buds to fading, senescent flowers, as shown in fig. 18. A pooled sample of 10 or more flowers, representing each developmental stage, was crushed in a pestle and mortar and then exhaustively extracted with a known volume of MeOH/1%HCl. The optical density of the resultant extracts was recorded on an Hitachi 101 spectrophotometer, at 530 nm (*b* genotypes) or 540 nm (*B* genotypes and *Lathyrus*), and this reading was used to determine the anthocyanin concentration per flower. Wing and standard petals were treated separately.

The extracts, after drying in a fume cupboard, were applied as 5 cm streaks to Whatman No 1 MM chromatography paper. Papers were developped overnight in BAW. Centre strips, 3 cm wide, were cut from dried chromatograms thus eliminating blurred edges of resultant bands, and the relative

1 Petals just emerged from the calyx. No anthocyanin colouration.

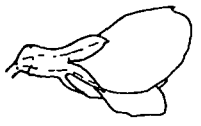
2 Anthocyanin colouration in the tips of the wing petals. No anthocyanin in standard petals.

3 Anthocyanin pigmentation general throughout the wing petals, but none in standard petals.

4 Anthocyanin pigmentation more concentrated in the wing petals, and traces of anthocyanin evident in the standard petals.

5 Anthocyanin general and more concentrated than in stage 4 for both wing and standard petals.

6 Fully open, mature flowers.



7 Senescent flowers.

Fig. 18

Stages in the floral ontogeny of *Pisum*.

density of each band in the strip was determined using an EEL densitometer.

In this manner, an absorbance profile, showing the relative proportions of anthocyanins present in the flowers was constructed, for each of the seven developmental stages investigated. These data were then used to determine concentration of anthocyanins in single flowers.

Flower development in *Pisum* is rapid, with progression through the seven stages occurring within 48 hours in warm weather. Selection and sorting of flowers into stages is quite accurate, and there is no overlap. Within each stage, however, individual flowers will show some variability with respect to pigment concentration. In order to assess the level of variability using this technique, a statistical analysis was carried out using six replicates of purple wing petals of *Pisum*. Results presented in Table 2 indicate the reliability of this method. Quantitatively the appearance of delphinidin 3-rhamnoside-5-glucoside at stage 4 of development was distinct. Total quantitative variation decreased with increasing age of flowers.

TABLE 2

Mean percentage composition ± S.E. of flower colour in *Pisum*.

Genotype *A Am B Ce Cr* (Purple flowers). Wing petals.

	Stage of Floral Development					
	2	3	4	5	6	7
Mv3R				1.4±0.4	1.6±0.3	2.0±0.4
Mv3R,5G	61.2±9.4	46.2±6.3	33.0±3.9	19.0±0.5	15.5±0.6	14.5±0.6
Pt3R,5G	38.8±9.4	53.8±6.3	47.2±2.6	43.9±1.0	44.2±0.8	44.2±0.8
Dp3R,5G			19.7±2.0	28.6±0.9	33.2±0.9	29.7±0.9
Dp3GX,5G) Dp3GG,5G)				7.1±0.7	6.3±0.9	9.5±0.9

PISUM GENETICS

The six flower colour loci, *A, Am, Ar, B, Ce* and *Cr*, are capable of producing sixty four different pure lines, of which half would be recessive *a* and possess white flowers. Some of the combinations with dominant *A*, that is those genotypes having coloured flowers, were neither available nor described in the literature. Also, in many cases it was not known how the flower colour loci interact with the three pod colour genes, *Pur, Pu* and *Gp*, or how they may influence the colour of the leaf axils. Accordingly, several crosses were undertaken to obtain some of the missing genotypes so that their phenotypes could be determined, and to identify the chemical constitution of their flavonoid pigments.

The genetic crosses undertaken are presented in three sections, the first one dealing with new genotypes with respect to the flower colour loci. In the second section, crosses relating to the effect of the loci *ar, b, ce, cr, gp, Pur* and *Pu* on pod colour are described, while in the final section dealing with genetics, a new locus, *Cgf*, is described and its linkage relations investigated.

To avoid repetition in the text, the flavonoid chemistry associated with the new phenotypes produced in the crossing programme will not be discussed until later. The pure lines and segregates are labelled only by their phenotype; that is, flower, axil and pod colours are described according to the nomenclature adopted earlier. The colours ascribed to the phenotypes are given distinctive names only when they are visually distinct, irrespective of their chemical constitution.

In the X^2 analysis in this section of the thesis the levels of statistical significance are represented in the following manner:

$^{+}P<0.05$ $^{++}P<0.01$ and $^{+++}P<0.001$

Any X^2 value not bearing an asterisk indicates a non-significant value.

3:1 New Genotypes with respect to the Flower Colour Loci

Three crosses concerning the production of new genotypes with respect to the flower colour loci are collected in this section. In Cross 17, the segregating loci are *am* and *ce*, in order that the double recessive segregate, *am ce* could be isolated. It was hoped that an inspection of the flavonoid chemistry of this segregate would give some indication of the relative time of action of the genes *am* and *ce*, in the biosynthetic pathway to the flavonoids.

The flower colour genes *ar* and *ce* are reasonably well known in *Pisum*, the single recessive genotype *A Am ar B Ce Cr* having violet flowers and axils and the genotype *A Am Ar B ce Cr* having cerise flowers and purple axils. However, the double recessive *A Am ar B ce Cr* has received little attention, and this combination was obtained in C 27.

C 22 was undertaken in response to conflicting evidence available in the literature with regard to chemical phenotyping of the genotypes *A am* and *a Am*, (see page 15), both of which have white coloured flowers, but the former genotype, *A am*, has dull rose coloured axils, while *a Am* does not possess anthocyanin in the axils.

CROSS 17

PARENTS	S1088	x	S1227
	Pinkish-white flowers		Cerise flowers
	am Ce		*Am ce*

F_1 Purple flowers

Am am Ce ce

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers	*Am Ce*	62
Cerise flowers	*Am ce*	16
Pinkish-white flowers	*am Ce)* *am ce)*	28
		106 Total

Single locus segregations :

Am locus $_{3:1}$ X_1^2 = 0.12

Ce locus $_{3:1}$ X_1^2 = 0.84

Marker gene :

Le locus $_{3:1}$ X_1^2 = 0.00

Since only three phenotypic classes were obtained in the F_2, it can be deduced that *am* is epistatic to *Ce*. Chemical analyses must therefore be confined to the pinkish-white phenotypic class as a whole, bearing in mind that it containes two genotypic classes with respect to the *Ce* locus.

CROSS 27

PARENTS	S25	x	S1227
	Violet flowers		Cerise flowers
	ar Ce		*Ar ce*

F_1 Purple flowers

Ar ar Ce ce

F_2 PHENOTYPE	GENOTYPE		OBSERVED NOS.
Purple flowers	*Ar*	*Ce*	91
Cerise flowers	*Ar*	*ce*	30
Violet flowers	*Ar*	*Ce*	25
Pale lilac flowers	*ar*	*ce*	8
			154 Total

Single locus segregations :

Ar locus 3:1 $X_1^2 = 1.05$

Ce locus 3:1 $X_1^2 = 0.01$

Joint segregation $X_1^2 = 0.03$

The phenotypic class, pale lilac flowers, constitutes a new flower colour of *Pisum*. The full genotype with respect to the flower colour loci for this phenotype being *A Am ar B ce Cr*.

Plants of this genotype, when grown in a glasshouse, have both wing and standard petals with traces of lilac colouration, mostly confined to the margins, as illustrated in Fig. 12. This colour corresponds to reference plate 15B4 of the Methuen Handbook of Colour. Pale lilac plants possess violet coloured axils, not visibly different to the axil colour of violet flowered plants.

One F_3 family, C27a/1/6, which was field sown during July, 1973, (late Southern Hemisphere winter) proved to have nearly white standard petals and lilac (Methuen 15B4) coloured wing petals. Other F_3 families grown at the same time in a glasshouse were coloured as in Fig. 12.

Cross 27 is illustrated in Fig. 19.

Fig. 19. An illustration of the flower colours involved in the production of the new flower colour, pale lilac.

CROSS 22

All available white flowered (a) lines were surveyed for the production of a red colouration on exposure of petals to concentrated HCl. (Wellensiek, 1947, see page 15). Only one such line, L8, did not give a positive reaction, and the cross below was undertaken to investigate the genetic control of this character.

PARENTS L8 x L22

No reaction with conc. HCl Red colouration with conc. HCl

F_1 Red colouration with conc. HCl

F_2 PHENOTYPE	Observed Nos.	
Red colour with conc. HCl	149	
No reaction with conc. HCl	10	
	159	Total

Single factor segregations :

3:1	X_1^2	=	29.69^{+++}
9:7	X_1^2	=	97.43^{+++}
15:1	X_1^2	=	0.09

The genetic analysis would suggest that this character is under the control of duplicate genes, such that the only genotype to give no reaction with concentrated HCl is the double recessive. However, due to insufficient time, no F_3 families could be inspected, and it would be premature to speculate as to the genetic and biosynthetic implications of this data.

3:2 Effects of the Genes *ar, b, ce, cr, gp, Pur* and *Pu* on Pod and Axil Colour in *Pisum.*

Pod colour in *Pisum* is under the control of the *Gp* locus, plants having the dominant allele bearing green pods, while homozygous recessives have yellow pods. The complementary loci, *Pur* and *Pu*, control the additional colouration of pods by anthocyanin, and subsequently the term "coloured pods" refers to plants bearing anthocyanin-coloured pods, being of genotype *Pur-Pu-*.

Older literature, (e.g. de Vilmorin, 1912; Wellensiek, 1925) refers to violet and red pods, both of which are borne on purple-flowered plants; the genotype of violet podded plants being *B Gp Pur Pu*, while red-podded plants have the genotype *B gp Pur Pu*; that is, the expression of the colour controlled by the loci, *Pur* and *Pu*, is dependent on the background genotype with respect to *Gp* locus.

Information on the effects of flower colour loci on pod colour and anthocyanins is minimal. Fedotov (1935) suggested that pods of genotype *Ar Pur Pu* have more red tints than do pods of genotype *ar Pur Pu*, and Lamprecht (1961) suggested that the flower colour loci of *Pisum* also have their effects in pod tissue. However, little material was available for confirmation of these suggestions.

Crosses relevant in this context are collected below. In this section, phenotypic classes without anthocyanin colouring of the pods bear an asterisk, and contain three genotypic classes with respect to the complementary loci for pod colour, namely *Pur-pupu, pur pur Pu-* and *pur pur pu pu.*

During the crossing programme it became evident that the pod colour loci, *Pur* and *Pu*, have pleiotropic effects on plant colouration, in that plants bearing coloured pods also possess specific plant colour-

ation by anthocyanin, namely, along the veins of the sepals and along the veins of the leaves, particularly the stipules.

CROSS 133

To confirm the effect of the *B* locus on pod anthocyanin colouration and to show the interaction of the expression of *B* and *Gp* loci in pods.

PARENTS L13 x L41

Pink flowers, yellow pods — Purple flowers, coloured pods

b gp pur pu — *B Gp Pur Pu*

F_1 Purple flowers, coloured pods

Bb Gpgp Purpur Pupu

F_2 Phenotype	Genotype	Observed Nos.
Purple flowers coloured pods	*B Gp Pur Pu*	45
Purple flowers, coloured pods	*B gp Pur Pu*	10
*Purple flowers, green pods	*B Gp* — —	30
*Purple flowers, yellow pods	*B gp* — —	6
Pink flowers, coloured pods	*b Gp Pur Pu*	10
Pink flowers, coloured pods	*b gp Pur Pu*	6
*Pink flowers, green pods	*b Gp* — —	14
*Pink flowers, yellow pods	*b gp* — —	7
		128 Total

$X^2_7 = 10.18$

Single factor secregations

B $\text{locus}_{3:1}$: $X^2_1 = 1.04$

Gp $\text{locus}_{3:1}$: $X^2_1 = 0.37$

Pur and *Pu* loci $_{9:7}$: $X^2_1 = 0.93$

L13 was a poor pollen parent, but proved adequate as a female parent. L42 produced viable pollen, and was useful for the cross in both directions. Poor pollen production was general in subsequent generations of the cross, with the result that it was not possible to obtain large progenies.

Numbers in the F_2 were small, due to limits on labour, time and glasshouse space. However, the X^2_7 value obtained is acceptable as an 81:27:63:21:27:9:21:7 ratio.

Plants having purple flowers and coloured pods belong to two genotypic classes which were phenotypically distinct. Purple flowered plants with *Gp* background possessed purple coloured pods (Fig. 20) while purple flowered plants with *gp* background possessed red coloured pods (Fig. 21).

Pink flowered plants of genotype *b Pur Pu* all possessed red coloured pods, but again there were two distinct phenotypes correlated with the background genotype with respect to the *Gp* locus; pink flowered plants with *gp* background possessed pods as illustrated in Fig. 22, while on *gp* background, the colour was as shown in Fig. 23.

There was very little difference in the red colour of the pods borne on plants of genotype *B gp Pur Pu* and *b Gp Pur Pu*, but these plants were readily genotyped using flower, axil and background pod colour.

Since there were now three distinct genotypes bearing red coloured pods, only one of which had previously been recorded in the literature, viz. purple flowers, red pods of genotype *B gp Pur Pu* , a genotypically descriptive terminology was adopted, as shown in tabular form below :

GENOTYPE	PHENOTYPE	TERMINOLOGY
B Gp Pur Pu	Fig. 20	Purple-Gp pods
B gp Pur Pu	Fig. 21	Purple-gp pods
b Gp Pur Pu	Fig. 22	Red-Gp pods
b gp Pur Pu	Fig. 23	Red-gp pods

It can be seen from this tabulation that the new terminology relates the colour of the pod to the genotype with respect to the *B* and *Gp* loci. Any plant having coloured pods and bearing the dominant *B* allele is described as having purple pods, while recessive *b* plants have red pods. As already described in the literature, the superposition of purple

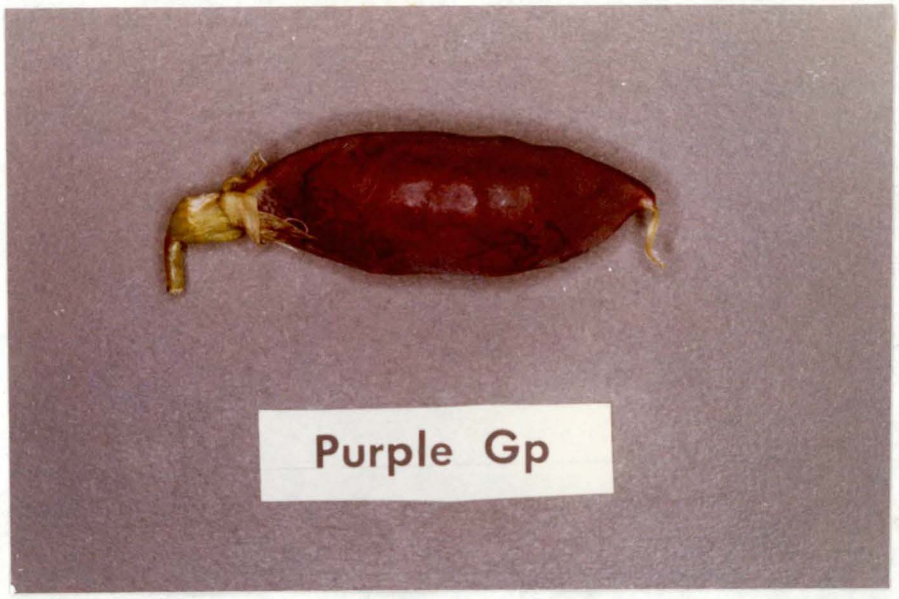


Fig. 20. Purple-Gp pods, borne on plants of genotype *A Am Ar B Ce Cr Pur Pu Gp*

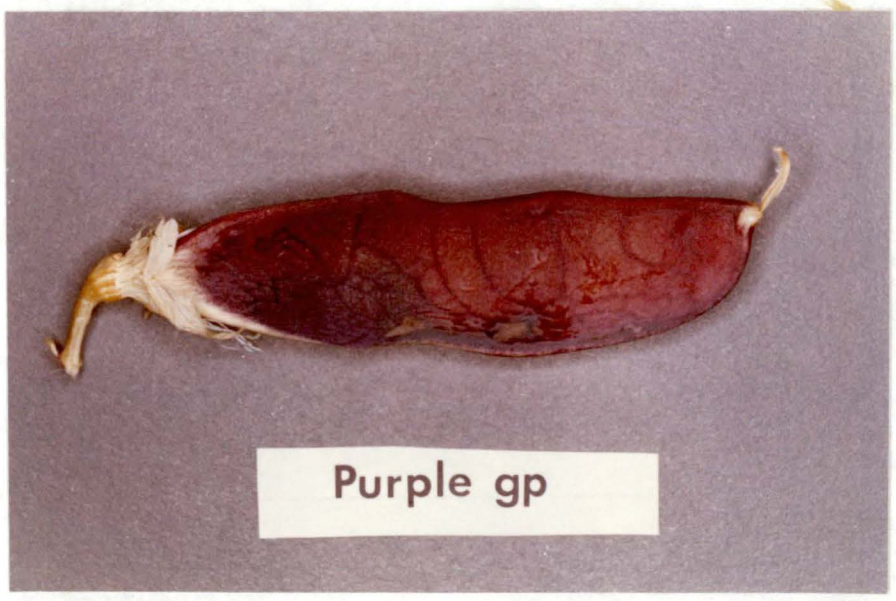


Fig. 21. Purple-gp pods, borne on plants of genotype *A Am Ar B Ce Cr Pur Pu gp*

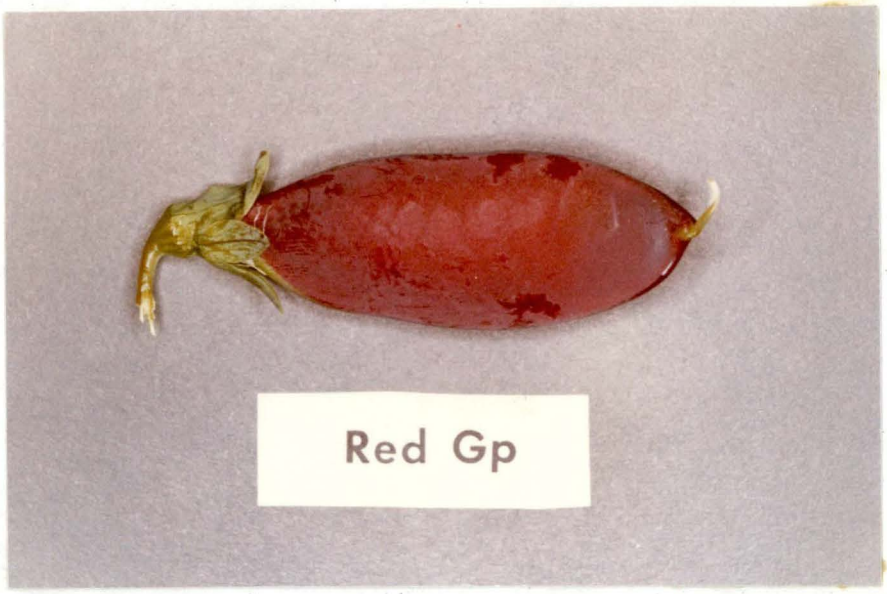


Fig. 22 Red-Gp pods, borne on plants of genotype
A Am Ar b Ce Cr Pur Pu Gp

Fig. 23 Red-gp pods, borne on plants of genotype
A Am Ar b Ce Cr Pur Pu gp

anthocyanin on a yellow background pod colour (controlled by recessive *gp*) in fact produces a red colour, but since the flower and axil colours of such plants (*B gp Pur Pu*) are evident before the coloured pods are produced, the description of such pods as "purple-gp" should not prove confusing. Therefore the *B* locus is seen to have a phenotypic effect in both floral and pod tissue, *B* genotypes bearing purple flowers and purple pods, while recessive *b* types possess pink flowers and red coloured pods.

It should be noted here that the colour of the leaf axils was found to be the same as the pod colour, and the *B* locus is operative in this tissue as well. The expression of pod colour, controlled by the *B* locus, is dependent on the background genotype with respect to the *Gp* locus, and this effect has been included in a new terminology of pod colour. The background genotype with respect to *Gp* does not affect the expression of axil colour; there are only two colours for leaf axils; purple and red.

CROSS 2

To investigate the effect of the *cr* gene on pod colour.

PARENTS	S1017	x	S1366
	Purple flowers, coloured pods		Crimson flowers, green pods
	Cr Pur Pu		*cr pur pu*

F_1 Purple flowers, coloured pods

Cr cr Pur pur Pu pu

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers, coloured pods	*Cr Pur Pu*	13
*Purple flowers, green pods	*Cr* — —	21
Crimson flowers, coloured pods	*cr Pur Pu*	12
*Crimson flowers, green pods	*cr* — —	11
		57 Total

Single factor segregations :

Cr locus $_{3:1}$: X_1^2 = 7.66**

Pur and *Pu* loci $_{9:7}$: X_1^2 = 3.49

The segregation at the *Cr* locus was significant at the 0.01 level, due to a deficiency of purple flowered plants. Some misclassification could have occurred, as it has been mentioned previously that *cr* has its effect only in floral tissue, axil colour being purple for crimson flowered plants (de Haan, 1930). Thus all phenotyping must be done on the flowers. It should be noted that flower colour in all F_2 progenies is variable due, presumably, to changes in the background genotypes generated in the crosses.

All coloured pods in the F_2, whether borne on purple or crimson flowered plants, were of the same colour, namely that shown in Fog. 20 as purple-Gp pods. This result confirms that the *cr* gene has its effect only in floral tissue, both pod and axil colour being unaffected by recessivity at the locus.

CROSS 3

To investigate the action of the *Ar* locus on pod colour.

PARENTS S1017 x S1391

Purple flowers, coloured pods — Violet flowers green pods

Ar Pur Pu — *ar* - -

F_1 Purple flowers, coloured pods

Ar ar Pur Pu

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers, coloured pods	*Ar Pur Pu*	56
*Purple flowers, green pods	*Ar* - -	22
Violet flowers, coloured pods	*ar Pur Pu*	23
*Violet flowers, green pods	*ar* - -	6
		107 Total

Single locus segregations

Ar locus 3:1	:	X_1^2 =	0.25
Pur and *Pu* loci 9:7		X_1^2 =	13.86^{+++}
3:1		X_1^2 =	0.08

All F_1 plants possessed purple flowers, but not all possessed coloured pods. Although S1017 is a pure line, some green pods are produced on selfing. Lamprecht (1953) noted that purple pod may mutate to green pod by as much as 40%. F_2 plants were grown only from seed of F_1 plants with coloured pods.

For the complementary loci for pod colouration by anthocyanin, *Pur* and *Pu*, two different segregations are given in the genetic analysis. The segregation as a 9:7 ratio for complementary genes was significant at the 0.001 level, while the single factor segregation was acceptable only at the 0.70 level. This would suggest that, contrary to previous information, the parent line, S1391, is not homozygous recessive for the two pod colour

genes, *Pur* and *Pu*. S1391 must bear the dominant alleles at one of these loci, but which one cannot be determined from this data.

For very young pods in F_2, pods borne on violet flowered plants were rather bluer in colour than young pods borne on purple flowered plants. However this phenotypic difference was apparently lost as pods matured, and all coloured pods were referable to Fig. 20, namely purple-Gp pods. Therefore, the effect of the *ar* gene on pod colour is only transitory, and mature plants of genotype *ar Pur Pu* bear purple pods, but have violet flowers and axils, as previously described (de Haan, 1930). Fedotov (1935) had suggested that coloured pods borne on violet flowered plants were bluer than those of purple flowered plants. This was observed for young pods, but was not found to be the case for mature pods borne on violet flowered plants.

CROSS 33

To investigate the action of the *Ce* locus on pod & axil colour

PARENTS C133b/4/10/4 x S1458

Pink flowers, coloured pods — Cerise flowers, yellow pods

b Ce gp Pur Pu — *B ce gp pur pu*

F_1 Purple flowers, coloured pods

B Ce ce Pur pur Pu pu

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers, coloured pods, purple axils	*B Ce Pur Pu*	42
*Purple flowers, yellow pods, purple axils	*B Ce* --- --	33
Cerise flowers, coloured pods, purple axils	*B ce Pur Pu*	15
*Cerise flowers, yellow pods, purple axils	*B ce* --- --	12
Pink flowers, coloured pods, red axils	*b Ce Pur Pu*	21
*Pink flowers, yellow pods, red axils	*b Ce* --- --	7
White flowers, coloured pods, red axils	*b ce Pur Pu*	10
*White flowers, yellow pods, red axils	*b ce* --- --	5
		145 Total

Single locus segregations :

B locus $_{3:1}$ X_1^2 = 1.68

Ce locus $_{3:1}$ X_1^2 = 1.22

Pur and *pu* loci $_{9:7}$ = 1.16

F_1 plants all possessed coloured pods, but the colour was not very deep in intensity.

However, in the F_2, this trend did not continue; pod anthocyanin was generally distributed and of deep intensity.

No disturbed segregations were evident, although because of difficulties with time and space, the numbers of F_2 plants were small. It should be noted that plants of genotype *b ce*, although bearing dominant *A*, have white flowers, but this combination of recessives does not affect the quantity of anthocyanin in the pods.

From the phenotypes listed above, it can be seen that *ce* does not affect the axil colour; in this cross, axil colour is determined solely by the genotype with respect to the *B* gene, being purple in the presence of the dominant allele, and red for *b* types. While *ce* is expressed in floral tissue, as can be seen above, the pod colour of appropriate genotypes is related to the axil colour, not the flower colour. Thus plants of genotype *B ce Pur Pu* have cerise flowers, purple pods and purple axils, while *b ce Pur Pu* types have white flowers, red pods and red axils. The two pod colours mentioned are referable to Fig. 21 and Fig. 23 respectively, since this cross is segregating on *gp* background.

The *ce* gene thus appears to affect the flower colour in a quantitative fashion, but has no effect on either pod colouration by anthocyanin, nor on axil colour.

In summarizing the results obtained for the effects of the flower colour loci in vegetative tissue, and their interaction with the pod colour loci *Gp*, *Pur* and *Pu*, only the *B* locus was found to have a phenotypic effect in all three tissues under investigation, namely the flowers, leaf axils and pods. The loci *Ce* and *Cr* have their phenotypic effect confined to floral tissues, and the genotypes *Ce Pur Pu* (cerise flowers) and *cr Pur Pu* (crimson flowers) both have purple axils and purple pods. The effect of the *Ar* gene is different again, in that plants of genotype *ar Pur Pu* have violet flowers and leaf axils, and immature pods are violet coloured, but when the pods have reached maturity, they are purple coloured.

3:3 A New Gene, *Cgf*, for the Production of Flavones in the Standard Petals of *Pisum*.

During the initial screening of all available genotypes of *Pisum* for their flavonoid constituents, flavone compounds were observed in the standard petals of all but two cyanic lines, S1516 and S1539, and were not observed as constituents of acyanic (*a*) genotypes.

A crossing programme was undertaken to investigate the genetic control of the biosynthesis of flavones, and once a Mendelian gene had been established, to investigate the linkage relations of the gene. Flavones are present in standard petals as C-glycosides, and the new locus was designated *Cgf*.

Other crosses included here were designed to produce new combinations of *Cgf* with other flower colour genes, which genotypes could then be included in studies on the biosynthesis of flavonoids in *Pisum*.

CROSS 1

To investigate the genetic control of flavone biosynthesis in standard petals of *Pisum*.

PARENTS S1458 x S1539

Cerise flowers yellow pods, flavone White flowers green pods, no flavone

ce Cr gp Cgf *ce cr Gp cgf*

F_1 Cerise flowers, green pods, flavone

cece Cr cr Gp gp Cgf cgf

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Cerise, green, flavone	*ce Cr Gp Cgf*	54
Cerise, green, no flavone	*ce Cr Gp cgf*	1
Cerise, yellow, flavone	*ce Cr gp Cgf*	35
Cerise, yellow no flavone	*ce Cr gp cgf*	1
White, green flavone	*ce cr Gp Cgf*	18
White, green, no flavone	*ce cr Gp cgf*	35
White, yellow, flavone	*ce cr gp Cgf*	0
White, yellow, no flavone	*ce cr gp cgf*	0
		144 Total

Single locus segregations :

Cr locus $_{3:1}$ $X_1^2 = 10.7^{+++}$

Gp locus $_{3:1}$ $X_1^2 = 0.0$

Cgf locus $_{3:1}$ $X_1^2 = 0.1$

Linkage X^2 from 2x2 contingency table (Mather, 1968)

Cr-Gp : $X_1^2 = 37.95^{+++}$

Cr-Cgf : $X_1^2 = 71.40^{+++}$

Gp-Cgf : $X_1^2 = 13.20^{+++}$

Estimates of recombination values (by method of Stevens, 1939)

Gp-Cgf : R.C.V. = 0.054 ± 0.08

Cr-Cgf : R.C.V. = 0.089 ± 0.03

Cr-Gp : Could not be estimated from data.

Phenotype of F_1 plants was different from cerise flower colour illustrated in Fig. 9, in that wing petals were a pale pink and standards were white. It was presumed that the expression of cerise flower colour had been modified by background genotype.

From the genetic analysis of the F_2 data, it can be seen that the *Gp* and *Cgf* loci give acceptable monohybrid ratios. The *Cr* locus shows a disturbed segregation, which, together with the observation of flower colour in the F_1, suggests misclassification of plants of genotype *ce cr* on some backgrounds.

The linkage X^2 values suggest that all three genes were linked in *Pisum* group V, and estimates of the recombination fraction were calculated, where possible, by the product ratio method (Stevens, 1939). Other attempts to obtain linkage data for *Cgf* are presented in Cross 29.

The results of this cross provide evidence for a new gene in *Pisum* designated *Cgf* , which, when present as a dominant allele, determines flavone C-glycoside production in the standard petals.

CROSS 29

To confirm the linkage relations of the *Cgf* locus in *Pisum* group V, as indicated in the results of Cross 1.

PARENTS C1a/1/13 x L2

Cerise flowers, yellow pods, no flavone Purple flowers, green pods, flavone

ce gp cgf *Ce Gp Cgf*

F_1 Purple flowers, green pods, flavone x C1a/1/13

Ce ce Gp gp Cgf cgf *ce ce gp gp cgf cgf*

TEST $CROSS_1$

PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple, green, flavone	*Ce Gp Cgf*	26
Purple, green no flavone	*Ce Gp cgf*	17
Purple, yellow, flavone	*Ce gp Cgf*	12
Purple, yellow, no flavone	*Ce gp cgf*	4
Cerise, green, flavone	*ce Gp Cgf*	12
Cerise, green, no flavone	*ce Gp cgf*	7
Cerise, yellow, flavone	*ce gp Cgf*	23
Cerise, yellow, no flavone	*ce gp cgf*	20
		121 Total

Single locus segregations :

Ce $locus_{1:1}$ $X_1^2 = 0.07$

Gp $locus_{1:1}$ $X_1^2 = 0.07$

Cgf $locus_{1:1}$ $X_1^2 = 5.17^{+}$

Joint segregations :

Ce - Gp : $X_1^2 = 21.5^{+++}$ R.C.V. = 28.9%

Ce - Cgf : $X_1^2 = 0.08$

Gp - Cgf : $X_1^2 = 0.05$

It may be noted there is a slight deficiency of recessive *cgf* in this cross. The disturbed segregation of *Cgf* was found to be contributed mainly from T C 29a, i.e. that part of the test-cross having C1a/1/13, of genotype *ce gp cgf*, as female parent. However, the other two loci segregating in this part of the cross each possessed $X_1^2 = 0.00$, and therefore the disturbance of the segregation of *Cgf* may be attributed

to chance.

As can be seen from the genetic analysis of F_2 data, this cross provides no evidence for linkage of *Cgf* with marker genes from Group V of *Pisum*. The reason for the discrepancy between the results from cross 1 and cross 29 is unknown. With conflicting evidence, the position of *Cgf* in the *Pisum* genome is uncertain.

CROSS 6

To confirm the interaction of the *A* and *Cgf* loci in flowers of *Pisum*, it having been noted that no white flowered (*a*) lines possessed flavones in the standard petals.

PARENTS	L 60	x	S 206
	Purple flowers, flavone		White flowers, no flavone
	A Cgf		*a Cgf*
F_1	Purple flowers, flavone		
	A a Cgf Cgf		

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers, flavone	*A Cgf*	116
Purple flowers, no flavone		
White flowers, no flavone	*a Cgf*	47
		163 Total

Single locus segregations :

A locus $_{3:1}$ $X_1^2 = 1.13$

The white flowered parent line, S206, was confirmed as being homozygous dominant at the *Cgf* locus, by the fact that no purple flowered plants which did not possess standard petal flavone were observed in the F_2.

The absolute linkage of coloured flowers and possession of flavone in standard petals, would suggest that *a* is epistatic to *Cgf*, i.e. the *A* gene has its effect before *Cgf* on the biosynthetic route to flavonoid in *Pisum*.

The recessive allele, *a*, is also epistatic to the other flower colour genes, *Am*, *Ar*, *B*, *Ce* and *Cr*, so the *A* gene is essential for both anthocyanin and flavone pigmentation in *Pisum* flowers.

CROSS 26

To confirm the genotype of S1516 with respect to the flavone locus, *Cgf*, and to obtain the genotype *A am Ar B Ce Cr cgf*.

PARENTS S1088 x S1516

Pinkish-white flowers, flavone Purple flowers, no flavone

am Cgf *Am cgf*

F_1 Purple flowers, flavone

Am am Cgf cgf

F_2 PHENOTYPE	GENOTYPE	OBSERVED NOS.
Purple flowers, flavone	*Am Cgf*	63
Purple flowers, no flavone	*Am cgf*	20
Pinkish-white flowers, flavone	*am Cgf*	21
Pinkish-white flowers, no flavone	*am cgf*	5
		109 Total

Single locus segregations :

Am locus 3:1 X_1^2 = 0.08

Cgf locus 3:1 X_1^2 = 0.26

Joint segregation X_1^2 = 0.23

Marker genes :

D locus 3:1 X_1^2 = 0.01

Le locus 3:1 X_1^2 = 0,05

The segregation of *Cgf* is acceptable as a monohybrid ratio, and confirms the genotype of the parent line, S1516, with respect to this locus. The use of this line has allowed the segregate *am cgf* to be obtained in a reasonably simple manner, since S1516 has wild-type purple flowers, while the other line recessive for *Cgf*, S1539, which was used in the other crosses in this section, is a double recessive with respect to flower colour, that is *ce cr*.

There was no obvious phenotypic difference between the genotypic classes *am Cgf* and *am cgf*, and these could be distinguished only with the

aid of chemical screening.

The recessive *am* in *Pisum* acts more specifically than the recessive *a* to block colour in the flowers, and it is noteworthy that *am* is not epistatic to *Cgf* even though the latter gene operates in floral tissue. The purpose, therefore, of obtaining the segregate *am cgf* was to provide material for a chemical investigation of the intermediates of flavonoid biosynthesis, in that, in the presence of dominant *A*, with no accumulation of either anthocyanin or flavone, a common precursor to these flavonoids may be accumulated.

3:4 Summary

The crosses described in this chapter have provided some new genotypes with respect to flower, leaf axil, and pod colour, which, on further selection, were available for inclusion in the investigation of the flavonoid chemistry of *Pisum*, to be described in subsequent chapters of this thesis. Information gained through the genetical studies has suggested which of the flower colour loci are operative in specific tissues. The results obtained in the crosses also point to the mode of operation of the flower colour loci in the biosynthesis of the flavonoid constituents, by indicating instances of epistasis, and complementary genes, which gives an indication of the relative time sequence for gene action.

A new gene, *Cgf*, for the control of flavone production in the standard petals was described and its linkage relations investigated. Further crosses were performed to gain suitable combinations of this gene with the previously described flower colour loci, in order that the flavones could be included in the chemical studies.

CHEMICAL SURVEY OF THE FLAVONOIDS IN *PISUM*

By means of a two-dimensional paper chromatographic survey of the lines of *Pisum*, tabulated in Table I, it was established that all lines of a particular genotype contained the same complement of pigments. For the identification and isolation of these pigments, extracts from a single line of each genotype were used. Anthocyanins were isolated from the following :

GENOTYPE	FLOWER COLOUR	LINE	TISSUE
A Am Ar B Ce Cr	Purple	L 60	Wing petals
A Am Ar b Ce Cr	Pink	S 592	Wing petals
A Am Ar B Ce cr	Crimson	S 1366	Wing petals
A Am Ar b Ce cr	Salmon	L 12	Wing petals
A Am Ar B Ce Cr Pur Pu	Purple	S 1017	Pods
Flavones were isolated from extracts of			
A am Ar B Ce Cr	Pinkish-white	S 369	Standard petals

The chromatographic survey showed that different genotypes may possess the same pigments. Thus isolations were carried out using the most heavily pigmented genotype of a particular pigment group. The pigment complement of other genotypes within the group was then found by comparison of specimen chromatograms.

4:1 White-flowered *Pisum*

No flavonoids could be detected in white flowers of *Pisum*, of genotype *a*--. Thus this species is similar to *Antirrhinum* (Geissman & Harborne, 1955) and *Petunia* (Mosig, 1960) in that there are varieties totally lacking in floral flavonoids. It appears that gene *A* of *Pisum* is therefore necessary for production of both anthocyanin and flavone in floral tissue. Leaf flavonols are present in all genotypes of *Pisum*, irrespective of

genotypical constitution with respect to *A*, and are evidently under the control of some other genetic system not yet identified.

4:2 Identification of new anthocyanins

During the course of this survey and the subsequent analysis of *Pisum* pigments, several new anthocyanins, not previously reported as plant constituents, were found. These anthocyanins were four glycosides of delphinidin; -- delphinidin 3-sophoroside-5-glucoside, delphinidin 3-sambubioside-5-glucoside, delphinidin 3-(p-coumaroyl-lathyroside)-5-glucoside, delphinidin 3-lathyroside-5-glucoside; and two glycosides of cyanidin; cyanidin 3-(p-coumaroyl-lathyroside)-5-glucoside and cyanidin 3-lathyroside-5-glucoside.

The structure of the disaccharides substituted at postition 3 of these anthocyanins was not determined unambiguously. In part, their structure was assumed by analogy with material of *Lathyrus odoratus*. However, the intermediates from partial hydrolyses had identical chromatographic behaviour to the relevant reference samples. The information concerning the 3-lathyroside-5-glucosides, and their acylated derivatives was obtained from partial and complete hydrolyses, two-dimensional chromatograms of intermediates of the hydrolyses, and from spectral data.

Delphinidin 3GX,5G and delphinidin 3GG,5G were first isolated from purple pod tissue of S1017 *Pisum* and were subsequently observed as constituents of the floral anthocyanins in particular genotypes, as listed in Table 3. These two delphinidin anthocyanins, together with their cyanidin analogues, are the major constituents of purple pods, but occur only as minor constituents in flowers.

Delphinidin and cyanidin glycosides of the type 3GalX,5G and the

acylated derivative 3-(p-coumaroyl)-GalX,5G were first observed on two-dimensional paper chromatograms prepared from semi-mature purple pods of L56 *Pisum*. They were later isolated from this source, and were also observed as constituents of some members of the F_2 progeny in cross 3 (see page 55). Cyanidin 3(p-coumaroyl)-GalX,5G and cyanidin 3GalX,5G were also observed in the pure line, S826, of genotype *b*.

As shown in Table 1, most pod genotypes are represented by only a single line, with the exception of purple pods; therefore no assessment of the quantitative and qualitative variation of pigment composition could be obtained. As mentioned previously in discussing the origin of new *Pisum* anthocyanins, purple pods did differ in pigment composition with respect to anthocyanins of the type 3GalX,5G and its acylated derivatives. However, at this time, the presence of these anthocyanins has not been definitely associated with any particular genotype.

4:3 Anthocyanins identified in flowers of various genotypes of *Pisum*.

These are listed in Table 3. Flowers of genotypes marked with an asterisk contained very little, if any, anthocyanin in relation to flowers of other genotypes, and therefore anthocyanins could not be idenitified in these cases. Such flowers were hydrolysed, and aglycones identified, as was also the case for pigmented axils.

Several *Pisum* lines were available for purple and pink-flowered genotypes, and five different purple-flowered lines were investigated for qualitative and quantitative variation of anthocyanin composition (see page 96). The anthocyanins listed in Table 3 as minor anthocyanins, are those most likely to vary between different lines of the same genotype.

From Table 3, it is clear that different flower colours may possess

TABLE 3

Flavonoid pigments identified in *Pisum*

GENOTYPE	MAJOR ANTHOCYANINS	MINOR ANTHOCYANINS
1. FLOWERS		
A Am Ar B Ce Cr	Delphinidin, petunidin and malvidin	Delphinidin 3-sambubioside-5-glucoside
A am Ar B Ce Cr	3-rhamnoside-5-glucosides	3-sophoroside-5-glucoside
A Am ar B Ce Cr	Malvidin 3-rhamnoside.	
A Am Ar B ce Cr		
A Am Ar b Ce Cr	Pelargonidin, cyanidin and peonidin	Cyanidin 3-sophoroside-5-glucoside 3-sambubioside-5-glucoside
A Am ar b Ce Cr	3-rhamnoside-5-glucosides	3-glucoside-5-glucoside
A Am Ar b ce Cr	Cyanidin 3-rhamnoside.	3-glucoside.
A Am Ar B Ce cr	Delphinidin 3-sambubioside-5-glucoside 3-sophoroside-5-glucoside	Delphinidin 3-sophoroside 3-glucoside
A Am ar B Ce cr^{+}	3-sambubioside	
A Am Ar B ce cr	3-glucoside-5-glucoside	
A Am Ar b Ce cr	Cyanidin 3-sambubioside-5-glucoside 3-sophoroside-5-glucoside 3-sambubioside 3-glucoside-5-glucoside	Cyanidin 3-sophoroside 3-glucoside

TABLE 3 (cont'd)

GENOTYPE	MAJOR ANTHOCYANINS	MINOR ANTHOCYANINS
2. PODS		
A Am Ar B Ce Cr Pur Pu *A Am ar B Ce Cr Pur Pu* *A Am Ar B ce Cr Pur Pu* *A Am Ar B Ce cr Pur Pu*	Delphinidin and cyanidin 3-sambubioside-5-glucoside 3-sophoroside-5-glucoside 3-(p-coumaroyl-lathyroside)-5-glucoside 3-sambubioside 3-sophoroside	Delphinidin and cyanidin 3-lathyroside-5-glucoside 3-glucoside-5-glucoside
A Am Ar b Ce Cr Pur Pu *A Am ar b Ce Cr Pur Pu* *A Am Ar b ce Cr Pur Pu*	Cyanidin 3-sambubioside-5-glucoside 3-sophoroside-5-glucoside 3-(p-coumaroyl-lathyroside)-5-glucoside 3-sambubioside 3-sophoroside	Cyanidin 3-lathyroside-5-glucoside 3-glucoside-5-glucoside 3-glucoside

TABLE 3 (cont'd)

GENOTYPE	FLAVONES / FLAVONOLS
1. FLOWERS	
All cyanic genotypes	Luteolin 6-C-glucoside (iso-orientin) 8-C-glucoside (orientin) Apigenin 6-C-glucoside (iso-vitexin) 8-C-glucoside (vitexin) 6-C-glycoside (sugar unidentified)
2. LEAVES	
All genotypes	Kaempferol and quercetin 3-sophorotriosides, as such, and acylated with p-coumaric acid.

TABLE 3 (cont'd)

GENOTYPE	ANTHOCYANIDINS
1. FLOWERS	
*A am Ar B Ce Cr	Delphinidin, petunidin and malvidin
*A am Ar b Ce Cr	Cyanidin and peonidin
2. AXILS	
A Am Ar B Ce Cr A am Ar B Ce Cr A Am ar B Ce Cr A Am Ar B ce Cr A Am Ar B Ce cr	Delphinidin and cyanidin
A Am Ar b Ce Cr A Am Ar b Ce cr A am Ar b Ce Cr	Cyanidin and peonidin
A Am Ar B ce Cr (S1227)	Delphinidin, cyanidin, malvidin and peonidin
A Am ar B Ce cr (S1325)	Delphinidin, cyanidin and malvidin

the same anthocyanin complement, e.g. purple flowers and violet flowers possess delphinidin, petunidin and malvidin 3-rhamnoside-5-glucosides and malvidin 3-rhamnoside as major pigments, together with other delphinidin glycosides as minor pigments. It appears that the flower colour loci, *Am*, *Ar* and *Ce*, have only quantitative effects, and do not affect the basic chemistry of the anthocyanins. The *B* gene is seen to be responsible for the 5'-hydroxylation of anthocyanins, such that *B*-genotypes possess the tri-substituted anthocyanins, delphinidin, petunidin and malvidin, while *b*-genotypes possess only di-or mono-substituted anthocyanins, cyanidin and peonidin. The *Cr* gene controls 3'- and 5'- methylation of anthocyanins, together with the pleiotropic effect of determining rhamnose as the sugar in the 3-O-glycoside, since *Cr*-genotypes contain both methylated and rhamnosylated anthocyanins, while *cr*-genotypes contain only non-methylated, non-rhamnosylated pigments.

4:4 Pod and axil colour in *Pisum*

Results in Table 3, obtained from the chromatographic survey of *Pisum* material, indicated there were both red and purple pods, which were chemically as well as visually distinct. The coloured pods first examined in our laboratory contained both delphinidin and cyanidin anthocyanins, while the pods examined by Dodds and Harborne (1964) had contained only cyanidin glycosides. This suggested the *B* gene, responsible for 5'-hydroxylation of anthocyanins, operated in pod tissue, as well as in flowers. Material to test this hypothesis was generated in Cross 133, and the results obtained, (see page 51), confirmed that *B* is operative in vegetative as well as floral tissue. Plants of genotype *B Pur Pu* all possessed purple flowers, purple pods and purple leaf axils, pigmented

with a mixture of delphinidin and cyanidin anthocyanins, while plants of genotype *b Pur Pu* all possessed pink flowers, red axils and red pods, pigmented with only cyanidin anthocyanins.

It was further noted in the survey and in Cross 133 that although methylated anthocyanins occurred in purple flowers, they were not present in related vegetative tissue, i.e. in purple pods or purple axils. However, for pink flowered plants, of genotype *b Pur Pu*, cyanidin and peonidin anthocyanins were present in both floral and vegetative tissues; the major constituents of floral tissue were 3-rhamnoside-5-glucosides of cyanidin and peonidin, while these glycosides were replaced in vegetative tissue by non-rhamnosylated pigments, of the type 3-sambubioside-5-glucoside and 3-sophoroside-5-glucoside. It was noted earlier that anthocyanin methylation and rhamnosylation were under the control of the *Cr* gene.

Results obtained in Cross 2 (page 54) confirmed that the effect of the *Cr* gene is confined to floral tissue. Plants of genotype *Cr Pur Pu* possessed purple flowers (methylated, rhamnosylated anthocyanins), purple pods (non-methylated, non-rhamnosylated anthocyanins) and purple axils (non-methylated, non-rhamnosylated anthocyanins), while crimson flowered plants (*cr Pur Pu*) possessed non-rhamnosylated delphinidin anthocyanins in the flowers, and non-rhamnosylated derivatives of delphinidin and cyanidin in pods and axils. Phenotypically, plants of genotypes *cr Pur Pu* have crimson flowers, purple axils and purple pods, and chemical differences are evident only between crimson and purple flowers.

Coloured pods generated in Cross 3 (page 55) were chemically investigated, since it appeared that pod colour was related to axil colour rather than flower colour, and it was known that the *Ar* locus does have an affect on axils. For very young pods (of length less than 3 cm), a phenotypic difference was observed between pods borne on violet-flowered plants and

on purple-flowered plants, the former possessing more blue tints than the latter. Chemically, the only correlation between colour and chemistry was that purple pods contained larger numbers of glycosides than did violet pods of comparable maturity. However, as pods matured, the phenotypic differences were lost, as were the chemical differences, such that all coloured pods in F_2 of Cross 3 appeared purple and contained the pigments listed for purple pods in Table 3. Hydrolysis of pods during maturation detected no differences in the delphinidin-cyanidin aglycone ratio.

Plants of genotype *ar b Pur Pu* possessed pale pink flowers, pale violet axils and deep red pods, and the following chemical constituents:

ORGAN	COLOUR	ANTHOCYANINS
Flowers	Pale pink	Cyanidin, peonidin and pelargonidin 3-rhamnoside-5-glucosides
axils	Pale violet	Cyanidin and peonidin non-rhamnosylated anthocyanins
Pods	Deep red	Cyanidin non-rhamnosylated anthocyanins

Again it can be seen that despite recessivity at *Ar*, neither pod colour nor anthocyanin composition is affected, and, as was found with genotype *ar Pur Pu*, a visually distinct axil colour cannot readily be correlated with any chemical difference.

Cross 33 (page 57) allowed the effect of the *Ce* locus on pod and axil colour and chemistry to be investigated. The chromotographic survey of *Pisum* material had suggested that the *Ce* locus has only quantitative effects on floral anthocyanins, and it was noted that the two lines recessive *ce*, S1227 *B ce Gp* and S1458 *B ce gp* , possessed purple axils, while lines of genotype *b ce Gp* e.g. S1511, possessed red axils. Results obtained in Cross 33 are similar to the results in Cross 2; where the segregating locus was known not to affect axil colour, pod colour was the same as axil colour.

In Cross 33 all plants were homozygous recessive *gp* and *B*-type plants possessed purple axils and purple-gp pods, while plants having red axils, due to recessive *b*, possessed red-gp pods, irrespective of the genotypical constitution with respect to *Ce*. Thus the chemical effect of *ce* is confined to floral tissue, and *ce* does not have its effect in vegetative tissues.

Again due to difficulties associated with time and space, a thorough chemical investigation of the F_2 of cross 33 was not possible. However, the analysis performed did make clear that purple-gp pods contained both delphinidin and cyanidin anthocyanins while red-gp pods contained only cyanidin anthocyanins. The chemistry of the axils tissue could not be investigated, because it was necessary to obtain all F_3 seed.

The relationships between flower colour, axil colour and pod colour, as described above, have been tabulated and presented in Table 4 (a and b).

From the above discussion of pod and axil colour, the rationale behind the terminology of pod colour given on page 52 becomes clear. The expression of the *B* gene, (which has its effect in floral, axil and pod tissue), is dependent on the background genotype with respect to *Gp* in the determination of pod colour.

GENOTYPE	COLOUR	ANTHOCYANIDINS	TERMINOLOGY
B Pur Pu Gp	Purple	Dp, Cy	Purple-Gp
B Pur Pu gp	Red	Dp, Cy	Purple-gp
b Pur Pu Gp	Red	Cy	Red-Gp
b Pur Pu gp	Red	Cy	Red-gp

Although *B gp* genotypes possess coloured pods which look red, due to the superposition of anthocyanin on a yellow background colour, they contain anthocyanins which are identical to those of purple coloured pods borne on *B Gp* genotypes, where the anthocyanin is superimposed on a green

background. Those genotypes which are recessive *b* also possess red coloured pods, containing only cyanidin anthocyanins, and are thus chemically distinct from the red coloured pods of *B gp* plants. The terminology previously adopted therefore relates to the chemistry of the pod anthocyanins, as well as the flower colour of the plants, both of which are under the control of the *B* gene, and does not directly relate to the colour of the pod.

4:5 Axil chemistry

Results obtained on hydrolysis of axil tissues are also presented in Table 3. Generally, axils of B-genotypes contained a mixture of delphinidin and cyanidin with no methylated aglycones, while *b*-genotypes contained cyanidin and peonidin. Gene *B*, which for floral and pod tissue was found to control 5'-hydroxylation of anthocyanins is also operative in axil tissue.

Two lines gave exceptional results : S 1227, genotype *A Am Ar B ce Cr*, possessed four aglycones in axil tissue - delphinidin, cyanidin, peonidin and malvidin; S1325, genotype *A Am ar B Ce cr*, contained delphinidin, cyanidin and malvidin. These were the only *B*-genotypes to possess methylated aglycones in axil tissue.

For *Pisum*, gene *B* controls 5'-hydroxylation of the anthocyanins in flowers, axils and pods. However, only in flowers of *B*-types is the cyanidin of *b*-types completely replaced by delphindin, axil and pod tissues containing both delphinidin and cyanidin.

The *Cr* locus of *Pisum* apparently controls methylation of anthocyanins in floral tissue only, as production of methylated glycosides in axils is not related to the genetic constitution with respect to *Cr*. Recessivity for *Cr*, while not permitting production of methylated anthocyanins in the

TABLE 4 a

Flower colour tabulated against axil colour, plant genotype being shown in the body of the table.

Flower colour / Axil colour	Purple	Pink	Crimson	Cerise	Violet	Pale lilac	Salmon	Pale pink	Light mauve	White
Purple	Wild type		*cr*	*ce*						*ce cr*
Red		*b*					*b cr*			*b ce*
Violet					*ar*	*ar ce*		*ar b*	*ar cr*	
Dull rose										*am*
Green										*a*

TABLE 4 b

Axil colour tabulated against pod colour, flower colour given in the body of the Table.

Pod colour \ Axil colour	Purple	Red	Violet
Purple	Wild type Cerise Crimson		Violet
Red		Pink White	Pale pink

flowers, does not necessarily prevent methylation of anthocyanins in axils. Methylated anthocyanidins were not found in pod tissues of *Pisum*, although most lines investigated were homozygous dominant at the *Cr* locus.

4:6 Other pigments

Of all cyanic lines of *Pisum* available, only two, S 1516 and S 1539, did not possess flavone pigments in the flowers. Five constituents, all C-glycosides, were isolated and four were fully identified (Table 3). The fifth flavone, a minor constituent, is reported as an apigenin 6-C-glycoside, though its sugar moiety remains unidentified.

The existence in *Pisum* of C-glycosyl flavones was first suspected by the difficulty of acid hydrolysis for the pigments isolated by the method given on pp.4 & 5. All flavone-O-glycosides are hydrolysed by heating for 4 hours in 2N HCl-ethanol (1:1), whereas C-glycosyl derivatives are largely unaffected (Harborne, 1965). C-glycosyl-flavones are spectrally identical in the U.V. range to the parent flavones from which they are derived. They differ from O-glycosides in that they undergo spectral shifts with all four of the usual reagents, since all of their phenolic hydroxyl groups are free.

Therefore, while the nature of the parent flavone could be ascertained for the minor constituent of standard petal flavones by means of its U.V. spectrum, because of the small amount of material available, its sugar substituent was not identified.

The major leaf flavonoids are the same as those reported in the cultivated "Alaska" pea, i.e. quercetin and kaempferol 3-triglucosides, occurring as such and also acylated with p-coumaric acid. The sugar is a sophorose derivative, probably sophorotriose (Harborne, 1963) and thus

the leaf flavonols are structurally related to some of the anthocyanins, particularly those of vegetative tissues.

The complement of C-glycosyl-flavone in standard petals was invariant, and all flavone-containing lines investigated contained the same pigments. The distribution of flavones differed from that of the anthocyanins, the former being found only in standard petals, while anthocyanins were found in both wings and standards. No flavonoid pigmentation was found in the keel petals of *Pisum*. The survey of chromatograms of flower petal extracts suggested that flavones were present in highest concentration in those genotypes possessing little anthocyanin, i.e. in genotypes recessive for *Ce* and *Am*.

Flavones in standard petals of *Pisum* are under the control of a newly described locus, *Cgf*, being present in those plants possessing the dominant allele.

4:7 Pinkish-white flowered *Pisum*

The original lines of de Haan and Wellensiek, with respect to *Am* and *Aw*, have been preserved by the Plant Breeding Institute, Weibullsholm, Sweden, and both were available for the present investigation. As previously mentioned, while de Haan and Wellensiek gave similar phenotype to the lines with regard to flower colour, they reported different chemical results with respect to the chemical phenotyping of genotypes *A am* and *a Am*. Our results have not aided the interpretation, for a red colour is produced when flowers of both genotypes are exposed to concentrated HCl. However, the chemical product obtained is not the same in both instances.

Genotype *A am* has pinkish-white flowers, which, at least under glasshouse conditions, shows faint anthocyanin colouration at the margins of

wing petals. This colour is intensified on exposure of wings to concentrated HCl, and if the wings are boiled in 2N HCl for 30 mins., the red product is extractable into amyl alcohol, and can then be seen to be anthocyanidin.

For acyanic flowers of *Pisum*, of genotype *a Am* or *a am*, again a red colour is produced in petals on exposure to concentrated acid. When petals of *a*-genotypes are boiled in 2N HCl, the red product could not be extracted into amyl alcohol, which suggests that the red colouration is not anthocyanidin. The nature of the red colouration produced by acid in acyanic *Pisum* remains unidentified. However, non-coloured pigments which give a red colour with concentrated acid in the cold have been previously reported as natural plant products (Werckmeister, 1955).

4:8 *Pisum fulvum*

Genetical investigation of *Pisum fulvum* were undertaken by Lamprecht (1961), and although in crosses with *Pisum arvense* a complicated segregation for flower colour was produced in F_2, no chemistry of these segregates was undertaken.

Our investigation of flowers of *Pisum fulvum* showed the presence of two anthocyanins in floral tissue, delphinidin 3-glucoside and petunidin 3-glucoside. These anthocyanins were present in both wing and standard petals. The complement of flavones, previously recorded for standard petals of other *Pisum* lines, were present in *P. fulvum* also. The flavones were found in the same relative quantities as found for other *Pisum* flowers, and therefore could be eliminated as being responsible for the ochraceus colour of the flowers, which was the only qualitative character found to separate *P. fulvum* and *P. arvense* (Lamprecht, 1961).

Flowers of *Pisum fulvum* are self-coloured, in that wing and standard petals are the same (see Fig. 4). Since flavone pigments are present only in standard petals, and there is no difference in colour between wings and standards, again flavones are seen not to be responsible for the different flower colour of this species.

Carotenoid pigments were obtained in some quantity from petals of *Pisum fulvum*, but these were not separately identified. Co-occurrence of anthocyanin and lipid-soluble carotenoids has been shown previously to produce orange coloured flowers, e.g. in the genus *Nemesia*(Hess, 1969), and, by analogy, it could be suggested that the ochraceus flower colour of *Pisum fulvum* is due to the presence of these two classes of compounds.

Further credence is given to this hypothesis, by reference to genetical results obtained in crosses of *P. fulvum* with *P. arvense*. Fedotov (1935) whose results were discussed by Lamprecht (1961), suggested that two genes concerned with flower colour, *Cv* and *Cm*, were segregating in his crosses. *Cm* was found not to be dependent on *A* for its effect. Lamprecht (1961) suggested two genes for flower colour, *Cit* and *Cm*, are responsible for the ochraceus flower colour, but he gives no indication as to whether these loci are dependent on the *A* gene for their expression. It would appear that flower colour of *P. fulvum* is the result of the presence of both anthocyanin and carotenoid pigments, and that both these classes of pigments are under separate genetic control, a situation previously recorded in *Nemesia* (Hess, 1969).

Crosses between *P. fulvum* and white flowered *Pisum*, as reported by Fedotov and Lamprecht, should be repeated, and the resulting segregates examined chemically, to determine unambiguously the genetic control of flower pigments in ochraceus flowers. Also, since *P. fulvum* possesses rather simple anthocyanins, relative to other genotypes examined, some

further evidence concerning the genetic control of glycosylation of anthocyanins in floral tissue of *Pisum* could be obtained.

SEQUENCE OF ANTHOCYANIN ACCUMULATION DURING FLORAL ONTOGENY IN *PISUM* GENOTYPES

Developmental studies of anthocyanin accumulation in relation to accumulation of other flavonoids have indicated that although the various classes of flavonoids may branch from a common biosynthetic pathway, (a suggestion first put forward by Lawrence & Scott-Moncrieff in 1935), the sequence of their formation and accumulation in plant tissues may be quite independent. Reznik (1961) showed that in flowers of *Primula obconica*, the time of initiation and rate of synthesis of kaempferol and malvidin glycosides are quite unrelated. Grisebach and Bopp (1959) studied the incorporation and subsequent disappearance of ^{14}C-labelling in quercetin and cyanidin in buckwheat, to see if there was any evidence of interconversion. They found that the label was incorporated into both substances at the same time and disappeared also at the same rate. Labelled rutin (quercetin 3-glucoside) was not converted to cyanidin and they could find no evidence of interconversion.

Generally, as tissues mature, there is a progression from less complex to more complex molecular structures; studies using a variety of plant species have indicated that B-ring hydroxylation and methylation, and 3- and 5-glycosylation are all terminal reactions in flavonoid biosynthesis (Barber, 1962a & b; Hess, 1964; Patschke & Grisebach, 1965). In *Pisum*, these reactions are controlled by three Mendelian genes --*A* , basic for anthocyanin pigmentation; *B*, 5'-hydroxylation and *Cr*, 3'- and 5'-methylation and also the pleiotropic effect of determining rhamnose as the sugar in the 3-O-glycoside (Statham, Crowden and Harborne, 1972).

5:1 Qualitative Loci: Quantitative Changes in Anthocyanin.

The *Pisum* genotypes *B Cr*, *b Cr*, *B cr* and *b cr* which bear purple, pink crimson and salmon coloured flowers respectively, are treated together

since these genotypes vary with respect to the *B* and *Cr* loci, both of wnich have been shown to affect the chemical structure of anthocyanins. Commercial *Lathyrus odoratus* var. Chloe has purple coloured flowers and was included in the investigation of the sequence of anthocyanin accumulation, since it has been reported to possess similar anthocyanins in floral tissues to *Pisum*, namely the 3-rhamnoside-5-glucosides of delphinidin, petunidin and malvidin, as well as malvidin 3-rhamnoside. (Harborne, 1960b; 1963a).

The varying complement of anthocyanins in developing flowers of *Pisum* (*B Cr*, *b Cr*, *B cr* and *b cr* genotypes) and of *Lathyrus odoratus* var. Chloe is given in Tables 5-8 respectively,

Anthocyanidins

In those *Pisum* genotypes which permit methylation of anthocyanins (*Cr* types), the more complex (methylated) anthocyanidins appeared first, and non-methylated pigments were evident only at later stages of floral development. However, by stage 6 (mature flowers), there was a predominance of part- and non-methylated over fully methylated pigments, for wing petals of these genotypes.

For standard petals, on the other hand, anthocyanins did not appear until about stage 4 of development, and thereafter the sequence of accumulation reflected the progression seen in earlier developmental stages (i.e. 2 to 4) of wing petals, such that non-rhamnosylated glycosides of delphinidin were not evident in standard petals of purple flowers of *Pisum* or *Lathyrus*.

Lathyrus flowers are much larger than those of *Pisum*, and contain more anthocyanin per flower. Also pigment is accumulated in the standard petals of *Lathyrus* earlier than is the case for *Pisum*. However, the

TABLE 5

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*. Genotype *A Am Ar B Ce Cr* (purple flowers).

	Stage of Floral Development					
	2	3	4	5	6	7
a. Wing Petals						
Total Anthocyanin (μ moles)	7	28	40	112	142	80
Mv3R	-	-	-	2	2	2
Mv3R,5G	4	13	13	21	22	12
Pt3R,5G	3	15	19	49	62	35
Dp3R,5G	-	-	8	32	47	24
Dp3GX,5G) Dp3GG,5G)	-	-	-	8	9	7
Methylated pigments	7	28	32	82	86	49
non-methylated pigments	-	-	8	40	56	31
rhamnosylated pigments	7	28	40	104	133	73
non-rhamnosylated pigments	-	-	-	8	9	7
b. Standard Petals						
Total Anthocyanin (μ moles)	-	-	7	24	47	29
Mv3R	-	-	-	-	6	-
Mv3R,5G	-	-	4	12	12	8
Pt3R,5G	-	-	3	12	21	14
Dp3R,5G	-	-	-	-	8	7
Methylated pigments	-	-	7	24	39	22
non-methylated pigments	-	-	-	-	8	7

TABLE 6

Quantitative changes in anthocyanins during floral ontogeny of *Pisum*. Genotype *A Am Ar b Ce Cr* (pink flowers).

	Stages of floral development					
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μ moles)	3	7	14	21	37	24
Cy3R	-	-	-	4	2	2
Pg3R,5G	-	-	2	4	5	3
Pn3R,5G	2	3	5	6	10	5
Cy3R,5G	1	4	7	7	11	7
Cy3G$_1$,5G	-	-	-	-	5	4
Cy3GX,5G) Cy3GG,5G)	-	-	-	-	4	3
Methylated pigments	2	3	5	6	10	5
non-methylated pigments	1	4	9	15	27	19
rhamnosylated pigments	3	7	14	21	28	17
non-rhamnosylated pigments	-	-	-	-	9	7

TABLE 7

Quantitative changes in anthocyanins during floral ontogeny of *Pisum*

	Stage of floral development					
	2	3	4	5	6	7
a. Genotype *A Am Ar B Ce Cr* (Crimson flowers)						
Total anthocyanins (μ moles)	5	11	26	60	62	34
Dp3GX,5G) Dp3GG,5G)	5	8	19	45	51	31
Dp3GX) Dp3G,5G)	-	3	7	15	11	3
b. Genotype (Salmon flowers)						
Total anthocyanin (μ moles)	4	10	17	26	37	23
Cy3GX,5G) Cy3GG,5G)	4	10	13	16	24	15
Cy3GX) Cy3G,5G)	-	-	4	10	13	8

TABLE 8

Quantitative changes in anthocyanins during floral ontogeny of *Lathyrus odoratus* var. Chloe. (Purple flowers)

	Stage of floral development					
	2	3	4	5	6	7
a. Wing Petals						
Total anthocyanin (μ moles)	9	44	144	474	1140	778
Mv3R	-	-	-	-	23	23
Mv3R,5G	9	37	80	161	365	234
Pt3R,5G	-	7	45	185	399	249
Dp3R,5G	-	-	19	114	274	202
Dp3GX,5G) Dp3GG,5G)	-	-	-	14	79	70
Methylated pigments	9	44	125	346	787	506
non-methylated pigments	-	-	19	128	353	272
rhamnosylated pigments	9	44	144	460	1061	708
non-rhamnosylated pigments	-	-	-	14	79	70
b. Standard Petals						
Total anthocyanin (μ moles)	14	53	117	382	842	485
Mv3R	-	-	-	15	59	-
Mv3R,5G	10	29	68	126	252	151
Pt3R,5G	4	19	47	138	303	199
Dp3R,5G	-	5	6	103	228	136
Methylated pigments	14	48	111	279	614	349
non-methylated pigments	-	5	6	103	228	136

pattern of pigment variation and the sequence of accumulation during floral development was the same as found for *Pisum*.

Glycosides

In genotypes recessive for *Cr*, i.e. crimson and salmon flowered *Pisum*, all constituent glycosides are derivatives of a single anthocyanidin, being delphinidin and cyanidin respectively.

In *Lathyrus*, and in *Cr*-type *Pisum*, as floral ontogeny progressed complex 3-O-glycosides appeared, in which rhamnose was replaced by sambubiose and sophorose. These latter glycosides typify the pigments found in flowers of *cr* types of *Pisum* and in coloured pods, where neither methylation nor rhamnosylation occurs. Both wing and standard petals of *cr*-type *Pisum* show the appearance of pigments with complex 3-O-glycosides in the earliest bud stages. Glycosylation at position 5 of *Pisum* anthocyanins involves only glucose. Pigments without 5-O-glycosyl substitution occur at late stages of floral development in all genotypes, and may result from partial degradation of the anthocyanins.

For flowers of *Cr*-type *Pisum*, these observations could result from a disproportionate activity of the *Cr* gene relative to the varying rate of synthesis of the flavylium nucleus during successive stages of floral development. Thus, early in floral development, anthocyanin synthesis is low, and the enzymic activity controlled by *Cr* is apparently able to effect methylation of all anthocyanin precursor. At later stages of development, the *Cr* gene product is apparently not supplied at the rate of production of the flavylium nucleus, and non-methylated anthocyanins are accumulated. This theory is supported by the temporal difference in anthocyanin production in wing and standard petals of *Pisum*. In the standards, total anthocyanin content is less than in the wings at all

stages of development, and while some non-methylated pigments are accumulated in standards, their proportion is less than in the case of wing petals. Alternatively, it could be proposed that the *Cr* gene is "switched off" at an early stage in the senescence biochemistry of *Pisum* flowers, so that partially and non-methylated anthocyanins are accumulated.

Reference has been made previously to the pleiotropic effect of *Cr*. While the two biochemical functions ascribed to this gene, (B-ring methylation and 3-O-rhamnosylation) are inseparable by genetic analysis, a temporal difference in these functions is evident from analysis of the sequence in which anthocyanins are accumulated during floral development. Thus, anthocyanidin methylation may be reduced while 3-rhamnosylation can still proceed, so that, for example, delphindin 3-rhamnoside-5-glucoside is accumulated. At a later stage of floral development, non-rhamnosylated anthocyanins are accumulated. These observations would suggest that *Cr* is, in fact either two tightly linked loci, or alternatively, some product of *Cr* is necessary for both anthocyanidin and for biosynthesis of 3-rhamnosyl-glycoside, and that the latter reaction has priority over methylation when the gene product of *Cr* is limited in amount.

In *cr*-types of *Pisum*, where no methylation nor rhamnosylation occurs, the sequence of glycoside accumulation showed glycosides of the type 3-sambubioside-5-glucoside and 3-sophoroside-5-glucoside to be accumulated during the first stages of floral ontogeny, with the simpler 3-glucosides and 3-glucoside-5-glucosides being evident only in later stages. Since no specific function has been ascribed, as yet, to anthocyanins, it may be that they are a means of storing excess sugars for later metabolism.

5:2 Purple-flowered Lines of *Pisum*

The two-dimensional chromatographic survey of the anthocyanins present in the various *Pisum* genotypes suggested that, in terms of anthocyanins present, there were no major differences between different lines of the same genotype, where several lines of one genotype with respect to flower colour were available, notably purple- and pink-flowered *Pisum*. While the survey showed no major qualitative differences between lines of the same genotype, within the purple-flowered lines there were obvious quantitative differences, with, e.g. L60 and S1293 being more heavily pigmented in wing petals than some other genotypes, e.g. L51, L63, S1516. Also, there were some lines in which the difference in the colour of the wing and standard petals was marked, e.g. S1017, S577. The lines S936 and S1402 were unsuitable for further chemical analysis because of the small size of the flowers when compared with other lines.

Five purple-flowered lines of *Pisum*, of diverse origin were subjected to an analysis of the sequence of anthocyanin accumulation during floral development. The origin of these lines is given below:

L60	Tasmania, Cross (L53 x L2).
L51	Australia, Commercial "Vinco".
S180	Hungarian wild type.
S1017	Weibullsholm, Sweden Cross 424.
S1293	Jordan Valley.

The results of the analyses are presented in Tables 5 and 9 to 12 respectively. An analysis of variance for these results, together with results obtained using L 60 (Table 5) is given in Appendix A.

It would appear that S1293 is rather different from all other purple-flowered lines investigated, in that, initially, the pattern of anthocyanin accumulation in S 1293 is opposite to that found in other genotypes

of *Pisum* and in *Lathyrus*. For S1293, (Fig. 24) the first anthocyanins accumulated are the 3-rhamnoside-5-glucosides of delphinidin and petunidin, while for other purple flowered, violet flowered, cerise flowered and pale lilac flowered lines, the first anthocyanins accumulated are the corresponding glycosides of malvidin and petunidin.

It should be noted that the sequence of anthocyanin accumulation in this line S 1293 of *Pisum* is similar to that reported for other genera, namely *Primula*, *Petunia* and *Impatiens balsamina* (Hagen, 1966).

Another point of interest is the fact that for S 1017, stage 2 flower buds contained only a single anthocyanin, malvidin 3-rhamnoside-5-glucoside. For other purple flowered lines, there were usually two anthocyanins apparent at this stage, malvidin 3-rhamnoside-5-glucoside and petunidin 3-rhamnoside-5-glucoside, and in each case, the former was present in greater concentration than the latter. It was inferred from this observation that the malvidin glycoside was the first anthocyanin to be accumulated, but that the time of initiation of synthesis of malvidin and petunidin was not greatly different, with the result that when those flower buds first seen to possess anthocyanin were examined chemically, both anthocyanins were present. Results obtained using S 1017 would appear to support this inference.

5:3 Quantitative loci

Violet, cerise and pale lilac flower colours of *Pisum* result from the action of the quantitative loci, *Ar* and *Ce*, which, while they have an effect on the colour of the flower, do not affect the chemistry of the constituent anthocyanins (Table 3; Statham et al., 1972). It had been suggested (Fedotov, 1935) that the *Ar* locus controls acidity of cell sap,

Fig. 24 S1293

Purple flowered *Pisum*, ex Jordan Valley.

TABLE 9

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*

Genotype *A Am Ar B Ce Cr* (Purple flowers).

L 51. Wing Petals.

	Stage of Floral Development					
	2	3	4	5	6	7
Total anthocyanin (μ moles)	9	17	26	62	273	91
Mv3R	-	-	-	-	18	-
Mv3R,5G	6	11	12	21	61	26
Pt3R,5G	3	6	10	26	93	43
Dp3R,5G	-	-	4	15	80	22
Dp3GG,5G) Dp3GX,5G)	-	-	-	-	21	-

TABLE 10

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*.
Genotype *A Am Ar B Ce Cr* (Purple flowers).
S 180 Wing Petals.

	Stage of Floral Development					
	2	3	4	5	6	7
Total Anthocyanin (μ moles)	10	47	142	358	498	409
Mv3R	-	-	-	12	10	-
Mv3R,5G	7	13	26	55	76	60
Pt3R,5G	3	34	66	146	207	241
Dp3R,5G	-	-	47	132	181	108
Dp3GG,5G) Dp3GX,5G)	-	-	3	13	24	-

TABLE 11

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*.

Genotype *A Am Ar B Ce Cr* (Purple flowers)

S 1017 Wing Petals.

	Stage of Floral Development					
	2	3	4	5	6	7
Total anthocyanin (μ moles)	4	26	75	163	213	126
Mv3R	-	-	4	3	4	-
Mv3R,5G	4	9	12	36	39	39
Pt3R,5G	-	17	27	57	77	51
Dp3R,5G	-	-	32	59	87	36
Dp3GG,5G) Dp3GX,5G)	-	-	-	8	6	-

TABLE 12

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*.

Genotype *A Am An B Ce Cr*(Purple flowers)

S 1293 Wing Petals

	Stage of Floral Development					
	2	3	4	5	6	7
Total anthocyanin (μ moles)	5	45	115	323	426	287
Mv3R	-	-	-	24	23	14
Mv3R,5G	-	7	24	45	67	42
Pt3R,5G	2	20	41	109	139	105
Dp3R,5G	3	18	41	110	140	100
Dp3GG,5G) Dp3GX,5G)	-	-	9	35	57	26

those genotypes possessing the dominant allele being noticeably more red in colour than genotypes possessing only recessive alleles. However, no quantitative data were presented to support the suggestion. Quantitative data relating to cerise flowered *Pisum* has been presented by Dodds and Harborne (1964), who found that cerise wing petals contained one-third as much anthocyanin as purple wing petals, while cerise standard petals possessed only one-sixth the amount of anthocyanin found in standard petals of purple flowers.

pH on the cell sap of wing petals of purple (L 60) and of violet (S 25) flowers of *Pisum* was determined by the method of Nozzolillo (1970), and the results, presented in Table 13, suggest that pH could not account for the observed difference in flower colour between purple and violet flowers.

Sequence data relating to the genotypes *ar Ce*, *Ar ce* and *ar ce* of *Pisum* are shown in Tables 14 to 16 respectively. Comparison of Tables 5 and 15, relating to purple and cerise flowers, show that while cerise flowers contain markedly less anthocyanin than purple, there is also a distributional difference, in that cerise wing petals contain three times more of the methylated anthocyanins, malvidin 3-rhamnoside, malvidin 3-rhamnoside-5-glucoside and petunidin 3-rhamnoside-5- glucoside, than the non-methylated delphinidin 3-rhamnoside-5-glucoside. It is also noted that the non-rhamnosylated anthocyanins found as minor pigments in purple flowers, delphinidin 3-sambubioside-5-glucoside and delphinidin 3-sophoroside-5-glucoside, are not accumulated in cerise flowers of *Pisum*. No non-methylated anthocyanins are evident in standard petals of cerise flowers, where the total anthocyanin is one-fifth that found in wing petals at stage 6 of developments.

Violet flowers of *Pisum* contain all the pigments present in purple flowers, and no significant distributional differences could be found

using this technique. (Analysis of variance, page iv). However, it was noted there was a significant colour difference, probably due to the fact that delphinidin 3-rhamnoside-5-glucoside is accumulated earlier in violet than in purple flowers. Data relating to standard petals of violet flowers reflect that for purple flowers, in that no non-rhamnosylated anthocyanins are accumulated.

Data presented in Table 16 for pale lilac flowers of *Pisum* are not directly comparable with those presented for purple, violet and cerise flowers. As noted earlier, plants of this genotype, *ar ce*, when grown in a glasshouse possessed lilac colouration in both wings and standards, the pigment being most concentrated at the margins of the petals. Insufficient plants of this genotype were available for analysis from the F_2, and therefore F_3 families were raised, one being field grown (July, 1973). Flowers used for sequence analysis were obtained from field sown plants, and under these conditions, anthocyanin colouration was better developed in wing petals.

Inspection of Table 16 shows that under field conditions, pale lilac wing petals contain as much anthocyanin as glasshouse grown purple and violet wing petals but the distribution of pigments is as seen for cerise wings, in that no non-rhamnosylated pigments are accumulated. Also, while methylated pigments are in abundance, there is relatively less malvidin than found for cerise. Pale lilac standards differ from cerise, in that delphinidin 3-rhamnoside-5-glucoside is accumulated in the former, but not in cerise, where standards contain only malvidin 3-rhamnoside-5-glucoside and petunidin 3-rhamnoside-5-glucoside.

In Table 17 sequence data relating to S 1458 (cerise flowered *Pisum*) which were field grown, July, 1973, are presented. It had been noted previously that this particular line possessed flowers of a deeper colour

TABLE 13

pH values for purple and violet petals of *Pisum*.

	Purple flowers	Violet flowers
Wings	5.83	5.67
	5.62	5.76
	5.76	5.58
	5.77	5.63
	5.67	5.72
	5.65	5.72
	$\bar{x}$ = 5.72	$\bar{x}$ = 5.68
Standards	5.76	5.83
	5.74	5.69
	5.62	5.74
	5.75	5.72
	5.73	5.67
	5.73	5.65
	$\bar{x}$ = 5.72	$\bar{x}$ = 5.70

TABLE 14

Quantitative changes in anthocyanin during floral ontogeny in *Pisum*. Genotype *A Am ar B Ce Cr* (Violet flowers).

	Stage of Floral Development					
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μ moles)	7	25	79	159	227	87
Mv3R	-	-	2	4	3	1
Mv3R,5G	4	8	21	38	49	24
Pt3R,5G	3	15	32	59	75	32
Dp3R,5G	-	2	24	51	86	24
Dp3GG,5G) Dp3GX,5G)	-	-	-	7	14	6
Methylated pigments	7	23	55	101	127	57
non-methylated pigments	-	2	24	58	100	30
rhamnosylated pigments	7-	25	79	152	213	81
non-rhamnosylated pigments	-	-	-	7	14	6
Standard Petals						
Total anthocyanin (μ moles)	-	-	7	17	32	23
Mv3R			1	1	2	2
Mv3R,5G			2	5	6	2
Pt3R,5G			3	7	14	12
Dp3R,5G			1	4	10	7
Methylated pigments			6	13	22	16
non-methylated pigments			1	4	10	7

TABLE 15

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*.

Genotype *A Am Ar B Ce Cr* (Cerise flowers)

Glasshouse grown.

	Stage of Floral Development					
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μ moles)	-	8	14	27	68	19
Mv3R	-	-	-	-	3	-
Mv3R,5G	-	5	5	11	25	8
Pt3R,5G	-	3	7	13	26	8
Dp3R,5G	-	-	2	3	14	3
Methylated pigments	-	8	12	24	54	16
non-methylated pigments	-	-	2	3	14	3
Standard Petals						
Total anthocyanin (μ moles				6	13	9
Mv3R				-	-	-
Mv3R,5G				5	9	6
Pt3R,5G				1	4	3
Dp3R,5G				-	-	-

TABLE 16

Quantitative changes in anthocyanin during floral ontogeny in *Pisum*.

Genotype *A Am ar B ce Cr* (Pale lilac flowers).
Field grown.

	Stage of Floral Development					
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μ moles)	-	60	109	155	310	248
Mv3R						
Mv3R,5G		24	34	41	89	66
Pt3R,5G		30	55	84	150	127
Dp3R,5G		6	20	30	71	55
Methylated pigments		54	89	125	239	193
non-methylated pigments		6	20	30	71	55
Standard Petals						
Total anthocyanin (μ moles)			8	29	95	51
Mv3R,5G			3	6	21	15
Pt3R,5G			5	18	56	25
Dp3R,5G			-	5	18	11
methylated pigments			8	24	77	40
non-methylated pigments			-	5	18	11

TABLE 17

Quantitative changes in anthocyanins during floral ontogeny in *Pisum*.

Genotype *A Am Ar B Ce Cr* (cerise flowers)

Field grown.

	Stage of Floral Development				
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μ moles)		51	153	326	692	393
Mv3R,5G		-	14	28	74	43
Pt3R,5G		27	61	126	282	156
Dp3R,5G		24	78	172	336	194
Methylated pigments		27	75	154	356	199
non-methylated pigments		24	78	172	336	194
Standard Petals						
Total anthocyanin (μ moles)			8	28	130	35
Mv3R,5G			6	23	111	28
Pt3R,5G			2	5	19	7
Dp3R,5G			-	-	-	-

Fig. 25.

From left to right: Cerise flowers glasshouse grown, cerise flowers field grown, and purple flowers glasshouse grown.

when field sown than when glasshouse grown (see Fig. 25), but another cerise-flowered line, S 1227, did not. The chemical analysis of constituent anthocyanins for glasshouse- and field-sown S1458 shows significant differences, on statistical analysis. (Appendix B). Generally, in such field grown plants, wing petals possess more delphinidin 3-rhamnoside-5-glucoside than when glasshouse grown. Again no delphinidin 3-rhamnoside-5-glucoside is accumulated in standard petals.

Inspection of Fig. 25 shows the colour of field grown S1458 (*ce*) to be more purple than cerise, and this difference in colour is reflected in the chemistry of such flowers. The pattern of accumulated anthocyanins in wing petals of field grown S1458 approaches that of both field- and glasshouse-grown wild-type purple flowers, as represented by L60. It appears there is an inefficient or incomplete methylation of anthocyanins, so that non-methylated anthocyanins are accumulated.

Since this observation was not noted for both lines of genotype *ce ce*, it must be presumed that the effect is due to some difference in the background genotype, rather than being specific to the *Ce* locus.

5:4 Modification of the Expression of the Flower Colour Loci

It has already been noted that S 1458 (cerise flowered) and pale lilac flowered *Pisum* have a modified expression of flower colour, depending on the environments in which they are grown, these environments being :

1. Glasshouse grown.
2. Field grown.

Similar effects have been noted in other lines carrying recessive alleles of *Ce*, notably S 1228, S 1539 (*ce cr*), and S 1511 (*b ce*).

Wellensiek (1951) with whom the genotype *ce cr* originated, described the flower colour as "light", while Lamprecht (1957) and Blixt (1962) describe the flower colour of this genotype as palleopurpureus or pale rose purple, but these latter authors also suggest that often the flowers are irregularly coloured greenish-white on a cream background.

Environmental effects

S 1228 is the original line developed by Wellensiek, while S 1539 originated in Weibullsholm Cross 1365. Both these lines, when grown in a glasshouse, possessed no anthocyanin colouration of the flowers, being greenish-white, and having coloured axils. In September, 1970, seeds of both these lines were field sown for the purpose of bulking seed. These plants flowered in early December, 1970, and it was noticed that both lines possessed traces of pale reddish coloured anthocyanin in both wing and standard petals.

The two lines were again field grown in the winter of 1973, and anthocyanin coloured was evident in flowers. This sowing was able to be regularly inspected, and it was noted that flowers produced 5 to 6 nodes after the onset of flowering were more deeply coloured than those first produced. At early stages of flowering, anthocyanin colouration was confined to veinal areas and petal margins, while later produced flowers possessed more general and more concentrated anthocyanin. Flowers of this latter type were used to typify the flower colour for this genotype, as shown in Fig. 16.

Another genotype which possessed similar modification of flower colour was S 1511 (*b ce*), which had been selected from Weibullsholm Cross 1283 (parents unknown). This genotype had been glasshouse-grown on several occasions, but has been field grown only once, in conjunction with S 1228 and S 1539, during winter, 1973. Again, development of floral

anthocyanin was more evident in flowers produced several nodes after the onset of flowering. The flower colour for genotype *b ce* is described as light rose (see Fig. 14). A search of the literature was unsuccessful in finding a description of this genotype; Lamprecht (1957) lists the genotype as unknown.

Anthocyanin colouration of S 1539 could not be induced by external modification of temperature regimes, in the following manner :

1. Glasshouse grown
2. 8 hours daylight in glasshouse;
   16 hours darkness in 3° cold room.
3. 8 hours artificial light in 3° cold room;
   16 hours darkness in 3° cold room.

In fact, the physiological modification of the expression of flower colour on some genetic backgrounds remains undetermined, and could profit from further work, using strictly controlled conditions of temperature, light, growing medium, and nutrient supply.

A further complication of this issue is provided by the following observations; During winter, 1973, several *Pisum* lines were field sown as listed below :

Genotype	Line Number
A Am Ar B Ce Cr	L 56, S 577
A am Ar B Ce Cr	S 369
A Am Ar b ce Cr	S 1511
A Am Ar B ce Cr	S 1227, S 1458
A Am Ar B ce cr	S 1228, S 1539

Two-dimensional chromatograms of wing and standard petals were prepared for each of above lines, since to that date, no information regarding the anthocyanins of S 1511 and S 1539 was available. As noted previously, S 1539 does not possess flavones in standard petals, while all other lines listed above do possess these pigments.

Chromatograms of wing and standard petals extracts of S 1539 showed the presence of yellow fluorescent spots not previously detected on *Pisum* petal chromatograms. These same spots were later recorded for S 1228, when field grown, although this line also possessed flavone pigments in standard petals. No other of the field grown lines listed above showed the presence of the yellow spots on petal chromatograms.

These yellow spots possessed identical chromatographic behaviour to yellow spots present on chromatograms of *Pisum* leaf extracts, grown either in the glasshouse or the field. Hydrolysis of ethanolic extract of flowers of S 1539 and S 1228, and subsequent co-chromatography of aglycones produces, showed the presence of the flavonols, kaempferol and quercetin.

It should be noted in connection with the modification of expression of the genotype with respect to flower colour that, in general, the recessive alleles of *Ce* are involved. However, other factors are also indicated, since, while quantitative evidence has been presented for S 1458, the other line available as homozygous recessive for *Ce*, S 1227, (Wellensiek's original line) did not show such large differences in colour when glasshouse and field grown. Unfortunately, due to pressures of time and space, this line was not investigated under the different conditions as fully as was S 1458. Visual examination of chromatograms of both lines S 1227 and S 1458, when glasshouse grown, suggested no great difference in anthocyanin complement, while for field grown samples, distributional differences in anthocyanins were noted, as given below :

S 1227 : Mv 3R,5G > Pt 3R,5G > Dp 3R,5G ; plus Mv 3R, Pt 3R + Dp 3R

S 1458 : Dp 3R,5G > Pt 3R,5G > Mv 3R,5G ; plus Mv 3R only.

The pattern of anthocyanin observed for field grown S 1227 does not differ from that of glasshouse-grown S 1227, and is typical of cerise flowered *Pisum*, in that non-methylated anthocyanins are present in less

concentration than are the methylated anthocyanins. However, when S 1458, also having cerise flowers is field grown, the flower colour would be described as purple, and it can be seen that in this instance, the non-methylated anthocyanin, delphinidin 3-rhamnoside-5-glucoside, is present in the greatest concentration, while the methylated anthocyanins petunidin and malvidin 3-rhamnoside-5-glucosides, are present in lesser concentrations. This situation more nearly parallels the pattern observed for purple flowered than for cerise flowered *Pisum*.

These flower colours may be compared by an inspection of fig. 25, where purple flowers, glasshouse grown cerise flowers and field grown cerise flowers of S 1458 are shown.

Background genetic effects

The effect of background modifiers on the expression of flower colour in *Pisum* is noted here, but no specific information is available. In Cross 1 (page 61), the F_1 flower colour was different from that expected, which was thought to be due to differences in background modifiers. Another cross, Cross 5, (L60 x S 1539) was carried out, but had to be abandoned in F_2 because optimum conditions for the expression of flower colour on diverse genetic backgrounds recombined in the cross could not be determined, with the result that most flowers were of a light colour. Since *Ce* was segregating in this cross, and this gene has only a quantitative effect on flower colour, there was no way of distinguishing chemically between purple and cerise phenotype, or crimson and "pale rose purple" flower colours.

5:5 Summary

The results presented in this section of the thesis have given some indication of the mode of gene action in the production of flower colour in *Pisum*. The genes responsible for flower colour may be divided into two categories, those which affect the chemical structure of the anthocyanins, and those which affect the quantity of anthocyanin in floral tissues. For the latter genes, it would appear that they may have their phenotypic effect by modifying the chemical effects of one of the qualitative loci, *Cr*. Further evidence bearing on this hypothesis could be obtained by an investigation of the enzymes involved in anthocyanin synthesis in floral tissues, but there are serious difficulties associated with such methods. Some of these difficulties are the small size of *Pisum* flowers and the observed modification of the expression of the quantitative loci in *Pisum* by the genetic background and environmental conditions.

RADIOISOTOPE STUDIES

As phenylalanine and acetate are known to be precursors of the B- and A-rings, respectively, of the flavonoids, it was hoped that by supplying radiocarbon isotopes in the form of U-^{14}C phenylalanine and 1-^{14}C-acetate and 2-^{14}C-acetate to flowering plants of *Pisum*, some further evidence on the sequence in which flavonoids are accumulated could be gained. The situation in *Pisum* is not simple, in that wing petals generally do not possess flavonoids other than anthocyanin, but flavones accompany anthocyanin in standard petals. In standard petals, if precursor could be incorporated into the biosynthetic pathway to flavonoid, it would be possible to examine the radio-activity of

a. flavones : dihydroxylated versus monohydroxylated, i.e. activity of luteolin derivatives versus activity of apigenin derivatives.

b. anthocyanin : malvidin versus petunidin versus delphinidin.

c. flavones versus anthocyanin.

Flavones were not included in the sequence studies reported earlier, but relevant literature suggests that flavones and flavonols are generally accumulated in tissues earlier than anthocyanins. Pecket (1966) found that in *Lathyrus odoratus* petals, flavonol glycoside was present in the earliest flower bud stage examined, which was defined as possessing no anthocyanins. For standard petals of *Lathyrus* there was very little variation of flavonol concentration with time, while for wing petals, a roughly similar quantity to that in standard petals is present in the earliest bud stage, but a considerable increase in quantity, as measured by optical density at 360 nm, soon takes place, so that maximum absorption for flavonol occurred at the time anthocyanin began to accumulate in wing petals. Flavonol content then decreased as wing petals matured. Reznik (1961) found kaempferol glycoside to be present during all stages of floral development in *Primula obconica*, while the first

anthocyanin, malvidin 3-glycoside, was present only in stages 4 and 5. The time for full development of flowers in *Primula* was given as 27 days.

The method of administering radioactive chemicals to *Pisum* plants was modified from Macovschi (1958) and Hess (1963). U-tubes, having a reservoir on one side, were constructed from Pasteur pipettes, and attached to plants with strips of adhesive tape. An ungreased cotton thread, pulled from commercial cheese cloth, was inserted into the U-tube, one end being sewn into a plant at a flowering node. (See Fig. 26). The hole made in the plant where the needle was withdrawn was covered with lanolin. A radioactive solution, of activity 2×10^6 c.p.m., was introduced into the tube, via a micro Pasteur pipette.

U-tubes were attached to the plant just prior to onset of anthocyanin accumulation in wing petals and entry of solution into the plant occurred within 12 hours of application. No provision was made to cover the thread between tube and plant, as normally there was no more than 2 mm thread uncovered, and with uptake occurring over a short period, losses by evaporation would be minimal.

Flowers were collected from the node of insertion of radioisotope, and from subsequent nodes, each node being treated separately. Individual flowers were insufficient for analyses; thus flowers from several treated plants were bulked for each node. Petals were crushed, and extracted with MeOH/1% HCl and a two-dimensional chromatograms of the extract prepared as described earlier. Individual spots were cut from chromatograms, and radioactivity of spots recorded. Autoradiographs of labelled plants indicated the movement of label upwards from the point of insertion, and that the label was well distributed through the plant. The label was found not to be confined to one side of the plant, such that it would be expected that only alternate nodes would be labelled.

Fig. 26 Apparatus for the administration of radioisotpic solutions to *Pisum* plants.

Although radioisotopy experiments were repeated several times, and chromatographic spots were counted using both Geiger-Muller and scintillation counters, no measurable activity could be recorded. Since no satisfactory results could be obtained in these experiments, it is suggested that floral development in *Pisum* is rather too rapid for incorporation of recoverable quantities of radioisotope into the flavonoids. For *Pisum* grown in a glasshouse, flower development proceeds through all developmental stages within 48 hours at a maximum. Also, *Pisum* flowers are small, relative to genera reported in the literature to have been subjected to this method, i.e. *Lathyrus*, *Primula*, *Petunia*.

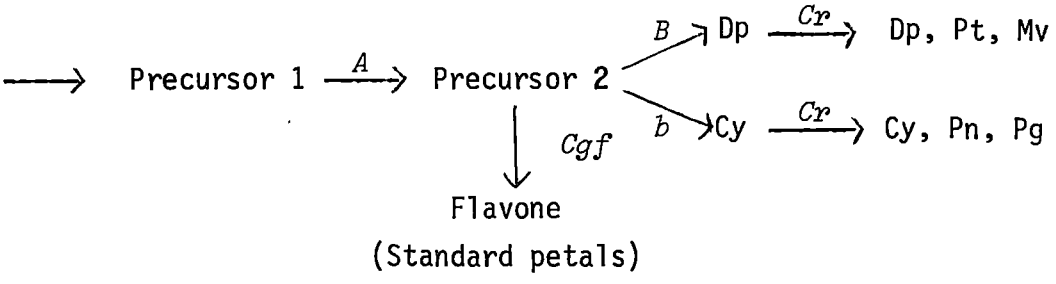
DISCUSSION

For the six major genes known to control flower colour in *Pisum*, the results of the chemical survey of the various genotypes have suggested that gene *A* is necessary for the general production of flavonoids in all plant organs; genes *B* and *Cr* modify the basic chemical structure of floral anthocyanins but are without effect on other classes of flavonoids, and the genes *Am*, *Ar*, and *Ce* only affect anthocyanins quantitatively.

A single Mendelian gene, not previously described, determines flavone production in the standard petals of *Pisum*, the pigments being accumulated in the presence of the dominant allele. Following the genetic analysis reported earlier, this new locus was designated *Cgf*, but to date no specific information on the linkage relationships of the locus has been established. The flower colour genes operate only on anthocyanins which suggests that the biosynthetic pathway to the flavones is branched off from that of anthocyanin, and the results gained from genetic crosses have shown that recessive *a* is epistatic to *Cgf*. In *Streptocarpus* (Lawrence and Sturgess, 1957) as in *Pisum*, the gene controlling 5'-hydroxylation does not affect floral flavone hydroxylation. However, studies in other genera have shown there to be similar hydroxylation patterns for different classes of flavonoids, mainly anthocyanin and flavonol; for example *Lathyrus*, (Harborne, 1960), *Antirrhinum* (Geissman et al, 1954), *Dianthus* (Geissman et al, 1956) and *Impatiens* (Clevenger, 1964).

The *B* gene of *Pisum* controls the 5'-hydroxylation of the B-ring of anthocyanins, and was found to have its effect in all tissues containing anthocyanin. No genotypes were investigated which contained only pelargonidin anthocyanins, and no separate locus for the 3'-hydroxylation comparable to *Sm* of *Lathyrus*, has been reported for *Pisum*. Pelargonidin anthocyanins were observed in flowers of *b Cr* genotypes, but not in flowers of *b cr* genotypes. No pelargonidin was observed in the vegetative tissues of *b* genotypes.

In *Pisum*, the *Cr* gene has two separate biochemical effects on anthocyanins in floral tissues; it permits methylation of the B-ring, and determines rhamnose as the glycosyl substituent at position 3. The anthocyanins of flowers of *cr* genotypes were neither methylated nor rhamnosylated. However, the effect of the *Cr* gene is not expressed in vegetative tissues, so that irrespective of genotype with respect to *Cr*, all pods and axils were pigmented with non-methylated, non-rhamnosylated anthocyanins. Methylated anthocyanins (but not rhamnosylated) were found in the axil tissue of just two lines during the survey, but their presence was not related to the genotype with respect to *Cr*. The operation of these loci in the biosynthesis of flavonoids of *Pisum* may be illustrated as shown here :



The sequence of accumulation of anthocyanins during floral ontogeny was investigated in eight different genotypes of *Pisum* and in one commercial variety of *Lathyrus*; for one *Pisum* genotype, *A B Cr* having purple flowers, 5 lines of diverse origin were examined. The results obtained showed that, in contrast to results obtained for other genera, the appearance of anthocyanins during floral development does not directly reflect the sequence of reactions in the proposed biosynthetic pathway. Reznik (1961), Hess (1963) and Hagan (1966) had found the sequence of anthocyanin accumulation during floral ontogeny to proceed from simple to more complex chemical structures, while for *Pisum* and *Lathyrus*, the most complex anthocyanins found for any genotype are the first observed.

However, this does not suggest that anthocyanin biosynthesis is different in these latter species, as presumably, glycosylation of anthocyanins is stepwise in *Pisum* as has been found in other genera such as *Streptocarpus* (Harborne, 1958) and *Solanum* (Harborne, 1962) and the results presented merely give evidence of the sequence of accumulation of anthocyanins.

One of the purple flowered lines of *Pisum*, S 1293, possessed an anthocyanin accumulation sequence which parallels that found for genera such as *Petunia* and *Primula*, and in this line the accumulation sequence reflected the proposed biosynthetic sequence. This particular line is derived from an accession from the Jordan Valley in Israel, and it would be of interest to investigate whether the sequence of anthocyanin accumulation in *Pisum* is geographically variable.

Although examination of purple pod tissues of *Pisum* would allow an investigation of the sequence of appearance of delphinidin and cyanidin, it was found that the method used for floral tissue was unsuitable, because of the ultimate complexity of the anthocyanin complement of pods. Also, the highly glycosylated (and acylated) anthocyanins found in *Pisum* pods are difficult to separate under normal circumstances, and the initial analytical prodecures normally result in mixtures of delphinidin and cyanidin analogues

Cerise, violet and pale lilac flowered genotypes of *Pisum* were included in the investigation of the sequence of anthocyanin accumulation during floral ontogeny. These genotypes possess basically the same anthocyanins as do wild type purple flowers, and the sequence study allowed differences in the proportions of the constituent anthocyanins to be investigated.

Cerise flowers, at all stages of floral development, possess less total anthocyanin than purple flowers of *Pisum*, and together with this difference in quantity, the proportions of the anthocyanins differ between purple and cerise wing petals, there being only 20% delphinidin in cerise petals compared to 40% delphinidin in purple wing petals. Glycosides which

do not contain rhamnose were not accumulated during the floral ontogeny of cerise flowers, but were found in the latter stages of development for purple flowers of *Pisum*. Thus the fully mature flowers of cerise coloured *Pisum* (*A B ce Cr*) are rather like non-mature flowers of purple flowered *Pisum* (*A B Ce Cr*). As discussed earlier, on page 94 , there appears to be some relationship between the total anthocyanin concentration, and the relative concentration of constituent anthocyanins; notably, the less total anthocyanin, the higher proportion of methylated anthocyanins.Also in petals containing low concentrations of anthocyanins, e.g. standard petals of purple flowered *Pisum*, and standard and wing petals of cerise flowers, no non-rhamnosylated pigments are accumulated. It is interesting to note that both these biochemical operations are under the control of the *Cr* gene in *Pisum* and it would appear that in the absence of the dominant *Ce* allele, the expression of *Cr* is modified. Thus, the *Ce* gene of *Pisum* has been classified as a quantitative locus, in that it does not directly control one step in the biosynthesis of anthocyanins.

In a qualitative survey of two dimensional paper chromatograms of petal extracts of several colour mutants of *Lathyrus odoratus* supplied by Arthur Yates and Co., some supportive evidence for this relationship between total anthocyanin content and the proportion of methylated anthocyanins was found. For *Lathyrus odoratus*, the colour mutants may be arranged into three series - one series based on pelargonidin; one on di-substituted anthocyanins and the third on tri-substituted anthocyanins. In this third series, the colours range from light blue through to deep reddish-purple. The deeper the flower colour, the greater the number of anthocyanins. The port-wine coloured variety possessed malvidin, petunidin and delphinidin glycosides, while the pale lilac coloured variety, possessed only the single anthocyanin Mv3R,5G.

Some evidence was presented to suggest that the expression of

recessive *ce* genes may be modified. No precise information is available, but the effect was particularly noticeable on some genetic backgrounds, presently not defined. When one cerise-flowered line (S 1458) was field grown, the total anthocyanin concentration of the petals was greatly increased, as was also the proportion of delphinidin anthocyanins. Thus the proportion of methylated anthocyanins was affected under these environmental conditions, but still no non-rhamnosylated anthocyanins were apparent.

For pale-lilac flowered *Pisum* (genotype *ar ce*), glasshouse grown plants accumulate very little anthocyanin in either wing or standard petals, and it was not possible to investigate the sequence of anthocyanin accumulation for such plants. However, when plants of this genotype are field grown, again the total anthocyanin of petals is increased, as was the case for one cerise-flowered genotype. The proportions of the anthocyanins present in mature petals of pale lilacs which were field grown were similar to those given for field grown cerise flowers, and again, while delphinidin anthocyanins represented 43% of the total anthocyanin of wing petals no non-rhamnosylated anthocyanins were accumulated.

No satisfactory explanation of the mode of action of the *ar* gene, responsible for violet flowers in *Pisum*, is yet available. Results presented earlier appear to negate the suggestion of Fedotov (1935) that this gene has its effect by controlling the acidity of the cell sap, since no significant differences in the pH of the cell sap of purple and violet petals was evident.

On visual examination, mature violet flowers of *Pisum* appear to be the same colour as senescent purple flowers. The analysis of the sequence of anthocyanin accumulation in violet wing petals partly supported the hypothesis that violet flowers may be prematurely aged (chemically) purple flowers, in that non-methylated anthocyanins make their appearance earlier

in the floral ontogeny of violet than in purple flowers. However, this trend was not evident at later stages of development, and at maturity, where the visual colour difference between violet and purple is most evident, the analysis of variance on the sequence analysis showed no significant differences in either the total anthocyanin content nor in the pigment composition of purple and violet wing petals.

Since there is really very little visual difference in the two colours, purple and violet, it is expected that if there is any chemical difference between these two genotypes which contain the same anthocyanin complement, such differences will be small. This investigation should be repeated on a larger scale, to identify the effect of the *ar* gene. Since the *ce* gene in *Pisum* which is also a quantitave locus, appears to have its effect by controlling the amount of anthocyanin synthesis and the concomitant effect of modifying the efficiency of the *Cr* gene, it does seem possible that the *ar* gene may operate in a similar fashion but in the opposite direction. Total anthocyanin synthesis in violet flowers may be slightly increased, with the result that the delphinidin anthocyanins, that is the non-methylated anthocyanins, are present in slightly greater proportion in violet than in purple flowers.

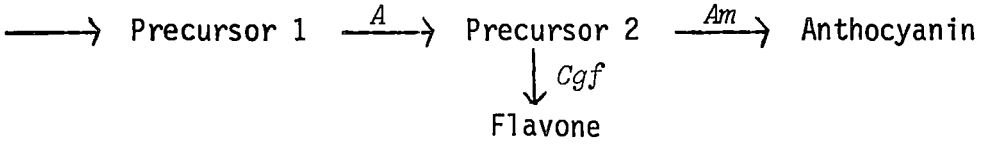
Some further reason for supporting this hypothesis is gained from comparing the anthocyanin complement of pink flowered (*b*) and pale pink flowered (*ar b*) *Pisum*. Pelargonidin 3-rhamnoside-5-glucoside is a minor constituent of pink wing petals, but in pale pink wing petals, this compound makes its appearance earlier in the floral ontogeny and forms a greater proportion of the total anthocyanin content of mature wing petals. As suggested for violet flowers, of genotype *ar*, the presence of the *ar* gene in pale pink flowered *Pisum*, appears to affect the relative proportions of non-methylated anthocyanins in the mature flower, and also these compounds are accumulated earlier in the floral ontogeny than is the case

for genotypes possessing the dominant *Ar* gene.

If the presumed mode of operation of the quantitative loci of *Pisum* is correct, it is not possible to show the genes *Ar* and *Ce* on the biosynthetic pathway, since these genes do not have a simple biochemical effect. They appear to have their effect by modifying the action of one of the qualitative loci, *Cr*, in a manner which is not yet clearly defined.

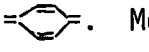
Pinkish-white flowered *Pisum* of genotype *am*, normally accumulate only minute quantities of anthocyanins in floral tissues, although, in the presence of *Cgf*, standard petal flavone is accumulated. The genotype *am b* also possesses pinkish-white flowers, and hydrolyses of floral and axil tissues showed the presence of dihydroxylated anthocyanidins, as found for pink flowers and axils, of genotype *b*. This evidence would suggest that the *am* gene of *Pisum* is an inhibitor of floral anthocyanin, and that the *B* gene has its chemical effect before the time of action of *am*.

At this point we may illustrate the biosynthesis of flavonoids in *Pisum* as shown below:



Material generated in Cross 26, where the genotype *am cgf* forms one-sixteenth of the F_2, may allow some elucidation of the suggested precursor 2, Information regarding the precursors to flavonoid biosynthesis at the point of cyclization is sparse. The results obtained in this investigation have provided some material which could be of use, but the material was generated too late for further investigations to be carried out.

In surveying a number of white flowered *Pisum*(of genotype *a*) for the reaction of petal tissue with concentrated HCl, it was found that such lines belonged to two classes - one class gave a red colouration of the tissue when dropped into concentrated HCl, while the other class, consist-

ing of only a single line, L8, did not give such colouration. In Cross 22, the genetic control of this chemical character of white-flowered *Pisum* was investigated, and a dihybrid segregation was indicated. No F_3 data were collected, but the production of a coloured product with conc. HCl would suggest some cyclic compound since, as discussed in Noller (1965) two types of chemical functions are usually present in highly coloured compounds: unsaturated groups, which have been called chromophores, and groups that intensify the colour, called auxochromes. Chemical functions assigned to the chromophores includes C=C and C=O and the quinonoid structure  . Most highly coloured substances which contain an aromatic ring can be written in such a way that they contain a quinonoid structure. The hydroxyl (-OH) group is listed as an important auxochrome, and with the development of the electronic theory of chemical structure, the chromophore-auxochrome theory of colour has been reinterpreted and extended.

Apparently, in *Pisum*, in the absence of the *A* gene, cyclization of precursors to form a flavonoid structure is not possible. The present evidence would suggest that in white-flowered plants, the intermediates in flavonoid synthesis are accumulated as different compounds, which react differently in the presence of concentrated acid.

The results obtained on the chemical investigation of the ochraceous flowered *Pisum fulvum* (fig. 4) suggested that this line is worthy of further attention, particularly in crosses made with white-flowered *Pisum*, the genetic results of which have been reported in the literature (Lamprecht, 1961a). While ochraceus flowers possess both anthocyanin and flavone, as found for other *Pisum* flower colours, they also contained quantities of carotenoid. By crossing *Pisum fulvum* to white flowered lines (*a*) it should be possible to obtain F_2 segregates possessing only carotenoid, as well as those possessing anthocyanin flavone and carotenoid.

Further genetic crossing using recessive *cgf* parents, would allow the production of segregates possessing just anthocyanin and carotenoid, and at that time, the relative contribution to *Pisum* flower colour of each of the classes of anthocyanin, flavone and carotenoid could be investigated. The ochraceus flowers contained only two anthocyanins, delphinidin and petunidin 3-glucosides, and are thus more simple, chemically, than other genotypes, and the crossing programme suggested above would also allow some investigation of the mode of glycosylation in *Pisum*.

It can be seen that the variation of flower colour in the genus *Pisum* is due to the action of the loci *A*, *Am*, *Ar*, *B*, *Ce* and *Cr*. The precise manner in which this variation of flower colour is achieved is due to the chemical action of the genes *A*, *B* and *Cr*, together with modifications of the actions of these by the loci *Am*, which to a great extent inhibits the production of floral anthocyanin, and by *Ce* and *Ar*, which, together with controlling the amount of anthocyanin accumulation, also affect the expression of the chemical action of *Cr*.

The newly described locus, *Cgf*, is responsible for the accumulation of flavone-C-glycosides in the standard petals of *Pisum* flowers, but these flavones do not co-pigment with floral anthocyanins to affect the flower colour.

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APPENDICES

APPENDIX A

Comparison of pigment composition during floral development for five purple-flowered lines of *Pisum*.

			(Dp3GX,5G (Dp3GG,5G	Dp3R,5G	Pt3R,5G	Mv3R,5G	MV3R
STAGE NUMBER	2	L51	0	0	3	6	0
		S1293	0	3	2	0	0
		S180	0	0	7	3	0
		S1017	0	0	0	4	0
		L60	0	0	2	7	0
	3	L51	0	0	6	11	0
		S1293	0	18	20	7	0
		S180	0	0	34	13	0
		S1017	0	0	17	9	0
		L60	0	0	18	14	0
	4	L51	0	4	10	12	0
		S1293	9	41	41	24	0
		S180	3	47	66	26	0
		S1017	0	32	27	12	4
		L60	8	55	68	25	0
	5	L51	0	15	26	21	0
		S1293	35	110	109	45	24
		S180	13	132	146	55	12
		S1017	8	59	57	36	3
		L60	14	61	81	38	8
	6	L51	21	80	93	61	18
		S1293	57	140	139	67	23
		S180	24	181	207	76	10
		S1017	6	87	77	39	4
		L60	15	76	102	51	11
	7	L51	0	22	43	26	0
		S1293	26	100	105	42	14
		S180	0	108	241	60	0
		S1017	0	36	51	39	0
		L60	9	62	82	34	0

APPENDIX A (cont'd)

Analysis of variance

	SS	d.f.	MS	F
Line	22,482	4	5,621	26 ***
Stage of development	79,619	5	15,924	73.6 ***
Pigment composition	76,678	4	19,170	88.6 ***
Line - stage	16,873	20	844	3.9 ***
Stage - pigment	41,334	20	2,067	9.6 ***
Line - pigment	21,829	16	1,364	6.3 ***
Error	17,314	80	216	
Total	276,128	149		

APPENDIX B

Variation of anthocyanidin composition during floral development in glasshouse- and field-grown plants of S1458 - cerise flowered *Pisum*.

	Glasshouse-grown			Field-grown		
Replicate	Dp	Pt	Mv	Dp	Pt	Mv
	14	26	28	336	282	74
	22	34	31	260	205	82
	14	29	29	296	264	88
	16	31	27	261	236	101

Analysis of variance

	SS	d.f.	MS	F
Treatment	198,744	1	198,744	478 **
Pigments	41,968	2	20,984	51 **
Replicate	1,515	3	506	1 ns
Treatment-pigment interaction	49,526	2	24,763	60 **
Error	6,239	15	416	
Total	297,992	23		

APPENDIX C

Comparison of pigment composition during floral development in purple-flowered (L60) and violet-flowered (S25) *Pisum*.

		Purple flowers					Violet flowers				
Stage	Rep.	(Dp3GX,5G (Dp3GG,5G	Dp3R,5G	Pt3R,5G	Mv3R,5G	Mv3R	(Dp3GX,5G (Dp3GG,5G	Dp3R,5G	Pt3R,5G	Mv3R,5G	Mv3R
2	1	0	0	1	5	0	0	0	10	8	0
	2	0	0	3	3	0	0	0	4	5	0
	3	0	0	2	7	0	0	0	2	4	0
	4	0	0	3	4	0	0	0	3	4	0
3	1	0	0	26	20	0	0	15	33	14	0
	2	0	0	31	20	0	0	13	14	7	0
	3	0	0	18	14	0	0	14	29	18	0
	4	0	0	30	28	0	0	2	15	8	0
4	1	3	33	49	21	0	0	30	72	23	0
	2	2	33	54	26	0	0	35	33	25	0
	3	8	55	68	25	0	0	37	64	27	0
	4	3	35	54	21	0	0	24	32	23	0
5	1	15	66	114	42	8	0	61	93	31	5
	2	10	41	62	30	7	0	56	61	49	8
	3	14	61	81	38	8	0	65	133	35	8
	4	11	67	90	43	8	0	57	60	38	4
6	1	25	116	198	69	18	12	93	118	30	6
	2	30	77	112	44	14	16	67	69	48	17
	3	15	76	102	51	11	10	68	130	41	14
	4	16	84	120	44	10	14	86	75	49	3
7	1	12	55	88	41	0	12	44	87	34	0
	2	13	44	68	41	0	13	45	60	48	0
	3	16	50	75	29	0	6	24	32	24	0
	4	9	62	82	34	0	4	76	89	33	0

Analysis of variance

	SS	d.f.	MS	F	
Colour	897	1	897	7.4	**
Stage of development	78,243	5	15,649	129	***
Pigment composition	105,592	4	26,398	218	***
Replicate	2,254	3	751	6	
Colour-stage	1,243	5	249	2.1	
Stage-pigment	43,190	20	2,160	17.9	***
Colour-pigment	484	4	121	1	
Colour-stage-pigment	1,700	20	85	1	
Error	24,069	199	121		
Total	255,416	239			

APPENDIX D

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BIOCHEMICAL GENETICS OF PIGMENTATION IN *PISUM SATIVUM*

CARMEL M. STATHAM and R. K. CROWDEN

Botany Department, University of Tasmania, Box 252C, Hobart, Tasmania 7001, Australia

and

J. B. HARBORNE

Phytochemical Unit, Botany Department, The University, London Road, Reading RG1 5AQ

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Abstract—The anthocyanins and flavones present in different colour forms of *Pisum sativum* have been identified and related to the genetic constitution of the plants. Two new anthocyanin pigments, delphinidin 3-sophoroside-5-glucoside, and delphinidin 3-sambubioside-5-glucoside have been isolated from purple pods and from flowers with the *cr cr* genotype. Chemical effects have been designated to the *B*-(hydroxylation) and *Cr*-(methylation) loci, whereas *Am*, *Ar* and *Ce* appear to have only quantitative effects. Variation of genetic expression in different tissues of the plant is demonstrated.

INTRODUCTION

INVESTIGATIONS into the inheritance of flower colour in *Pisum* began with the classical studies of Mendel,[1] who crossed purple-flowered with white-flowered plants. After the re-discovery of Mendel's work in 1901, the genetic difference which he observed was ascribed to the locus *A*. Subsequent genetical investigations have demonstrated the involvement of six major loci in the production of flower colour in peas: *A*: Mendel,[1] Tschermak;[2] *B*: Tschermak;[2] *Ar*: Tedin;[3] *Cr*: de Haan,[4] Fedotov;[5] *Am*: de Haan;[4] and *Ce*: Wellensiek.[6] Other gene systems are known which determine pigmentation elsewhere in the plant.[7,8] These include the loci *Gp*, *Pu* and *Pur*, which are concerned with pod colouration. Superimposed on these 'pigment production' genes, are the effects of other genes concerned with distribution and patterning of the pigments.

In a preliminary investigation using only a limited number of colour forms, Dodds and Harborne[9] identified a range of anthocyanins in flowers with genotypes differing at the *b* and *cr* loci, and were able to propose a chemical basis for the expression of these genes. The major anthocyanins present in red-coloured pods of plants with the genotype *A b Cr* were also identified. In this investigation we have extended the earlier work to cover a wider range of genotypes, and have also examined in some detail the pigmentation of organs other than flowers.

[1] G. MENDEL, *Verh. des Naturf. Vereines in Brunn*, **4**, 3 (1865).
[2] E. VON TSCHERMAK, *Zeits. Ind. Abst. Vererb.* **7**, 81 (1912).
[3] H. TEDIN, *Hereditas* **1**, 68 (1920).
[4] H. DE HAAN, *Genetica* **12**, 321 (1930).
[5] V. S. FEDOTOV, *Proc. USSR Congr. Genetics* **2**, 523 (1930).
[6] S. J. WELLENSIEK, *Genetica* **25**, 525 (1951).
[7] S. BLIXT, *Agri. Hort. Genet.* **20**, 95 (1962).
[8] H. LAMPRECHT, *Agri. Hort. Genet.* **21**, 19 (1963).
[9] K. S. DODDS and J. B. HARBORNE, *A. Rep. John Innes Inst.* **34** (1963).

RESULTS

Identification of Delphinidin 3-sophoroside-5-glucoside, and Delphinidin 3-sambubioside-5-glucoside

These two anthocyanins are new plant pigments. The glycosides were first identified as major constituents of purple pods of *Pisum*, and were later found to be minor constituents of crimson (*cr cr*) and purple (wild type) flowers. The related cyanidin glycosides had earlier been reported as constituents of red pods in *Pisum*.[9]

The structure of the disaccharides substituted at the 3 positions were not determined unambiguously. However, the intermediates from partial hydrolyses had identical chromatographic behaviour to reference samples of delphinidin 3-sophoroside and delphinidin 3-sambubioside. Delphinidin 3-sophoroside-5-glucoside and delphinidin 3-sambubioside-5-glucoside have very similar mobilities in most of the usual chromatographic solvents. However, they are adequately separated using 3% HCl. The relevant chromatographic data are summarized in Table 1.

TABLE 1. R_f DATA OF NEW DELPHINIDIN ANTHOCYANINS

Anthocyanin	Solvent			
	BAW	5% HOAc	WAH	3% HCl
Dp 3GG, 5G	0·12	0·60	0·68	0·63
Dp 3GX, 5G	0·12	0·60	0·62	0·57

Anthocyanins Identified in the Various Genotypes of Pisum

These are listed in Table 2. Flowers of genotypes marked with an asterisk contained very little anthocyanin in relation to flowers of other genotypes. From the results in the table, it is clear that the loci *Am*, *Ar* and *Ce* have quantitative effects only. They do not affect the chemistry of the anthocyanins.

Other Pigments

Of the thirty-two different cyanic lines of *Pisum* available for study, all but two, S1228 and S1539, contained flavones in the flowers. Five compounds, all *C*-glycosides, were isolated and four were identified (Table 2). The fifth, a very minor constituent, is reported as an apigenin 6-*C*-glycoside though its sugar moiety remains unidentified.

The complement of *C*-glycosyl-flavone was invariant, and all the lines investigated contained the same pigments. However, the distribution of flavones differed from that of the anthocyanins, the former being found only in the standard petals, while the anthocyanins were found in both wings and standards. No flavonoid pigmentation was observed in the keel petals of the flowers. The qualitative survey of the two-dimenisonal chromatograms of flower petal extracts showed that flavones were present in highest concentrations in those flowers possessing little anthocyanin, that is, in genotypes containing recessive *ce* and *am*. The major leaf flavonoids are the same as those reported in the cultivated 'Alaska' pea, i.e. quercetin and kaempferol 3-triglucosides occurring as such and also acylated with *p*-coumaric acid. The sugar is a sophorose derivative, probably sophorotriose,[10] and thus the leaf flavonols are structurally related to some of the minor flower anthocyanins.

[10] J. B. HARBORNE, *Experientia* **19**, 7 (1963).

TABLE 2. FLAVONOID PIGMENTS IDENTIFIED IN *Pisum sativum*

Genotype	Anthocyanins
1. *Flowers*	
A Am Ar B Ce Cr *A *am* Ar B Ce Cr	Delphinidin, petunidin and malvidin 3-rhamnoside-5-glucosides and malvidin 3-rhamnoside. Minor pigments
A Am *ar* B Ce Cr A Am Ar B *ce* Cr	Delphinidin and petunidin 3,5-diglucoside, delphinidin 3-sophoroside-5-glucoside, 3-sambubioside-5-glucoside, and 3-sophoroside
A Am Ar *b* Ce Cr *A *am* Ar *b* Ce Cr	Pelargonidin, cyanidin and peonidin 3-rhamnoside-5-glucoside. Minor pigments
*A *am ar b* Ce Cr A Am *ar b* Ce Cr A Am Ar *b ce* Cr	Cyanidin 3-rhamnoside, 3-glucoside, 3,5-diglucoside, and 3-sambubioside-5-glucoside.
A Am Ar B Ce *cr* A Am *ar* B Ce *cr*	Delphinidin 3-glucoside, 3,5-diglucoside, 3-sophoroside-5-glucoside, and 3-sambubioside-5-glucoside. Minor pigments
*A Am Ar B *ce cr*	Delphinidin 3-sophoroside and 3-sambubioside
A Am Ar *b* Ce *cr* *A Am *ar b* Ce *cr*	Cyanidin glycosides only
2. *Pods*	
A Am Ar B Ce Cr Pur Pu Gp	Delphinidin and cyanidin 3-sophoroside-5-glucoside and 3-sambubioside-5-glucoside
A Am Ar *b* Ce Cr Pur Pu Gp	Cyanidin 3-sophoroside-5-glucoside and 3-sambubioside-5-glucoside
3. *Axils*	
A Am Ar B Ce Cr A *am* Ar B Ce Cr A Am *ar* B Ce Cr A Am Ar B *ce* Cr (S1458) A Am Ar B Ce *cr*	Delphinidin and cyanidin glycosides
A Am Ar B *ce* Cr (S1227)	Delphinidin, malvidin, cyanidin and peonidin glycosides
A Am *ar* B Ce *cr*	Delphinidin, malvidin and cyanidin glycosides
A Am Ar *b* Ce Cr A Am Ar *b* Ce *cr* A *am* Ar *b* Ce Cr	Cyanidin and peonidin glycosides
	Flavones
1. *Flowers* All genotypes with flavones present	Luteolin 6-*C*-glucoside (Iso-orientin) and 8-*C*-glucoside (Orientin). Apigenin 6-*C*-glucoside (Iso-vitexin), 8-*C*-glucoside (Vitexin) and 6-*C*-glycoside (Sugar unidentified)
2. *Leaves* All genotypes	Kaempferol and quercetin 3-sophorotriosides, as such and also acylated with *p*-coumaric acid

DISCUSSION

This survey has shown that, of the six major genes known to control flower colour in *Pisum*, *A* is necessary for general flavonoid production in the plant, and for anthocyanin production in the flowers, axils and pods; *B* and *Cr* modify the structure of the anthocyanins, but appear to have no effect on other classes of flavonoids, while the three other genes, *Am*, *Ar* and *Ce* have only quantitative effects. No flavonoids could be detected in eight lines with white flowers (genotype *a a*). Thus, in *Pisum*, as in *Antirrhinum*[11] and probably *Petunia*,[12] there are varieties which are totally lacking in flavonoids. It appears, therefore, that gene *A* in *Pisum* is necessary for production of both anthocyanins and flavones in the flowers.

The *B* locus in *Pisum* apparently controls the 5'-hydroxylation of the B-ring of the anthocyanins. No separate locus for the 3'-hydroxylation of anthocyanins, comparable to the *Sm* locus of *Lathyrus*, has been reported for *Pisum*, and none of the genotypes available for our study contained only pelargonidin, as would be expected if such a locus had been preserved in cultivation.

Genetic control of hydroxylation of anthocyanins has usually been found to be tissue specific. For example, in *Impatiens balsamina*,[13] the flowers may have delphinidin, cyanidin or pelargonidin, but the sepals of most genotypes have only cyanidin. Even when the genetic control of hydroxylation affects pigment synthesis throughout the plant, the expression of the genes may vary from one tissue to another. Thus, in *Primula sinensis*,[14] *K* types have delphinidin in both leaf and petal, while *k k* types have mainly cyanidin in the leaf, but mainly pelargonidin in the petals. In *Pisum*, gene *B* controls 5'-hydroxylation of the anthocyanins in the flowers, axils and pods. However, only in the flowers of *B* genotypes is the cyanidin of *b b* genotypes completely replaced by delphinidin. Axils and pods contain both delphinidin and cyanidin glycosides.

The locus *Cr* controls the methylation of anthocyanins in the flowers. Production of methylated glycosides in the axils is not related to the genetic constitution with respect to the *Cr* locus. Recessivity for *Cr*, while not allowing production of methylated anthocyanins in the flowers, does not necessarily prevent methylation of anthocyanins in the axils. No methylated anthocyanin has been found in pod material, although all lines under investigation were homozygous dominant at the *Cr* locus.

Also, it appears that plants of the genotype *cr cr* do not possess the rhamnosyl transferases which must be present in plants of genotype *Cr*. All flowers of the latter type possessed not only methylated anthocyanins, but also glycosides of the type 3-rhamnoside, and 3-rhamnoside-5-glucoside. The observation that methylation and glycosylation are under simultaneous control by a single locus has been noted in some other plants, for example, *Solanum*[15] and *Petunia*.[16] In these two instances, the gene concerned is thought to be tightly linked to the gene controlling acylation of anthocyanins, and the three different biochemical effects are thought to be part of a compound locus. However, in both *Solanum* and *Petunia*, the glycosylation reaction involved is the addition of glucose to the 5-position of the *A*-ring, while in *Pisum*, the glycosylation effect related to *cr cr* appears to be the lack of an enzyme required for the addition of rhamnose to position 3. Again in *Solanum*, methylation is

[11] T. A. GEISSMAN and J. B. HARBORNE, *Arch. Biochem. Biophys.* **55**, 477 (1955).
[12] G. MOSIG, *Z. Vererbungsl.* **91**, 171 (1960).
[13] R. E. ALSTON and C. W. HAGEN, *Genetics* **43**, 35 (1958).
[14] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 259, Academic Press, New York (1967).
[15] J. B. HARBORNE, *Biochem. J.* **74**, 262 (1960).
[16] C. MEYER, *Z. Vererbungsl.* **95**, 171 (1964).

complete in the flowers, but incomplete in the tubers, which suggests that the degree of methylation exhibits activity gradients. A similar situation occurs in *Pisum*, where delphinidin, petunidin and malvidin occur in *B* flowers, and pelargonidin, cyanidin and peonidin in *b b* flowers, but methylated anthocyanins may or may not occur in the axils of all genotypes, and have not been found in the pods.

Each of the *Pisum* genotypes investigated contained a complex mixture of anthocyanins, a situation observed before in *Lathyrus*[17] and *Streptocarpus*.[18] In fact, in both *Pisum* and *Lathyrus*, the major pigments of the flowers are the 3-rhamnoside-5-glucosides, with the minor pigments being a complex mixture of many different glycosidic types. The differences in the flower colour between the two genera are due to the fact that no specific methylating factor has been observed in *Lathyrus*, while in *Pisum* no specific 3′-hydroxylating locus has been recorded.

A single Mendelian gene not previously described in peas appears to determine flavone production in the flowers of *Pisum*, the presence of the dominant allele resulting in pigment production. This is similar to the report for *Petunia*.[12] Experimental data describing this new locus will be published elsewhere.

In contrast with an earlier report,[19] Harborne[17] found that related anthocyanins and flavonols occur together in the flowers of all varieties of *Lathyrus* that he examined. Thus in *Lathyrus*, the loci *E* and *Sm* which control hydroxylation of anthocyanins, also determine the flavonol hydroxylation pattern. In *Pisum*, the genes affecting flower colour operate only on the anthocyanins. The pattern of flavone *C*-glycosides was the same in all coloured flowers, irrespective of the flower colour genes. These genes also were without effect on the flavonols found in the vegetative tissues.

EXPERIMENTAL

Plant materials. Seeds from genetically pure lines were used throughout. The seeds were obtained from the Plant Breeding Institution, Weibullsholm, Landskrona, Sweden (S lines), and Dr. I. C. Murfet, Botany Department, University of Tasmania, Hobart (L lines), Table 3. Seeds were grown in a glasshouse in containers having 1 : 1 vermiculite–dolerite chips (¼in. mesh) solid medium. Plants were watered daily, and normal Hoagland's nutrient solution applied weekly.

Analysis and identification of pigments. Flowers were collected when petals were fully expanded and had developed maximum pigmentation. Wings, standards and keels were examined separately. The pods were collected after they had grown to full length, but before the seeds were fully developed. Pigmented axils were cut from the plant on the completion of flowering, but before the plants had dried.

Fresh tissue was extracted using MeOH : 1% HCl for anthocyanins, and 70% EtOH for other flavonoids and phenolics. The extracts were examined by two-dimensional chromatography on Whatman No. 1 chromatography paper using BAW (*n*-BuOH–HOAc–H_2O, 4 : 1 : 5, v/v, upper phase), and 5% HOAc. When it had been established that all lines of a particular genotype contained the same complement of pigments, most of the isolation work was carried out using extracts from a single line of that genotype. Anthocyanins, were isolated from the following lines:

A Am Ar B Ce Cr	L60	Purple flowers	Wings
A Am Ar *b* Ce Cr	S592	Pink flowers	Wings
A Am Ar B Ce *cr*	S1366	Crimson flowers	Wings
A Am Ar B Ce Cr Pur Pu Gp	S1017	Purple pods	
A Am Ar *b* Ce Cr Pur Pu Gp		Red pods	
Flavones were extracted from A *am* Ar B Ce Cr	S369	Pinkish-white flowers	Standards.

Purification of pigments was carried out by band-loading extracts onto Whatman 3MM chromatography paper, and developing in BAW, 5% HOAc, and for pod material, 3% HCl. The pigments were identified by

[17] J. B. Harborne, *Nature, Lond.* **187**, 240 (1960).
[18] W. J. C. Lawrence and V. C. Sturgess, *Heredity* **11**, 303 (1957).
[19] G. H. Beale, G. M. Robinson, R. Robinson and R. Scott-Moncrieff, *J. Genet.* **37**, 375 (1939).

TABLE 3. *Pisum* VARIETIES USED FOR ANALYSIS AND IDENTIFICATION OF PIGMENTS

Genotype	Line No.	Colour
1. *Flowers*		
A Am Ar B Ce Cr	S577, S1017, S1402, S1516 L2, L41, L51, L60	Purple (Wild Type)
A *am* Ar B Ce Cr	S369, S1088, S1451	Pinkish-white
A *am* Ar *b* Ce Cr	S1512	
A *am ar b* Ce Cr	S1467	
A Am *ar* B Ce Cr	S25, S1391	Violet
A Am *ar b* Ce Cr	S1357	
A Am *ar* B Ce *cr*	S1325	
A Am *ar b* Ce *cr*	S1089	
A Am Ar *b* Ce Cr	S592, S1185, S1508, S1515, L13	Pink
A Am Ar *b ce* Cr	S1511	
A Am Ar *b* Ce *cr*	S1510, L12	
A Am Ar B *ce* Cr	S1227, S1458	Cerise
A Am Ar B *ce cr*	S1228, S1539	
A Am Ar B Ce *cr*	S1221, S1366	Crimson
aa———————	S102, S206, S680, S1143 S1241, S1525, L22, L23	White
2. *Pods*		
A Am Ar B Ce Cr Pur Pu Gp	S577, S1017, L41	Purple
A Am Ar *b* Ce Cr Pur Pu Gp	One line selected from the cross L41 × L13	Red

spectral and chromatographic analysis, comparison with authentic reference compounds, and by partial and complete hydrolysis, using the methods of Harborne[20,21] and Mabry *et al.*[22]

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[20] J. B. HARBORNE, *Biochem J.* **70**, 22 (1958).
[21] J. B. HARBORNE, *J. Chromatog.* **1**, 473 (1958).
[22] T. J. MABRY, K. R. MARKHAM and M. B. THOMAS, *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin (1970).

Key Word Index—Pisum sativum; Leguminosae; pea; flavonoids; anthocyanins; biochemical genetics.

ANTHOCYANIN BIOSYNTHESIS IN PISUM. SEQUENCE STUDIES IN PIGMENT PRODUCTION.

by,

Carmel M. Statham and R. K. Crowden
Botany Department, University of Tasmania, Box 252c, G.P.O., Hobart, Tasmania, Australia 7001.

Manuscript Received.....

Key Word Index. Anthocyanin; biosynthesis; Pisum; Lathyrus; sequence of accumulation; biochemical genetics; methylation; rhamnosylation.

Abstract The sequence of anthocyanin accumulation during flower development in four different flower-colour mutants of Pisum and in Lathyrus odoratus var. Chloe, shows a progression from methylated to non-methylated anthocyanidins, and the replacement of 3-O-rhamnoside by 3-O-sambubioside and 3-O-sophoroside. This behaviour is explained in terms of the activity of gene Cr.

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INTRODUCTION

Studies using a variety of plant species have indicated that B-ring hydroxylation and methylation, and 3- and 5-glycosylation are all terminal reactions in anthocyanin biosynthesis (Barber[1,2]; Hess[3]; Patschke and Grisebach[4]). In Pisum, these reactions are controlled by three Mendelian genes, - A, basic for anthocyanin pigmentation; - B, 5'-hydroxylation and Cr, 3'- and 5'-methylation. As well Cr has the pleiotropic effect of determining rhamnose as the sugar in the 3-O-glycoside (Statham et al.[5]).

Developmental studies of anthocyanin production in relation to other flavonoids, indicate that although the various pigments may branch from a common biosynthetic pathway, the sequence of their formation and accumulation in plant tissues may be quite independent. Generally, however, as tissues mature there is a progression from less complex to more complex molecular structures. Reznik[6] showed that white flowers of Primula obconica accumulated kaempferol 3-monoglucoside in the earliest bud stage, together with kaempferol 3-diglucoside in the second bud stage. In fully mature flowers, a third flavonol kaempferol 3-triglucoside, was also present. Reznik showed also that while malvidin 3-glucoside was the first anthocyanin to appear, mature flowers contained malvidin 3,5-diglucoside as well.

Similarly, Hagen[7] showed a progression from pelargonidin 3-glucoside through pelargonidin 3,5-diglucoside to acylated pelargonidin 3,5-diglucoside during successive stages of bud development in a red-flowered genotype of Impatiens balsamina.

Hess[8] using three genetically homozygous lines of Petunia hybrida, showed that anthocyanidins appeared in the sequence

cyanidin, delphinidin, peonidin, petunidin, malvidin, as floral development progressed. Thus in Petunia, the chemical structures of anthocyanidins present at different stages of flower development reflect the proposed biosynthetic sequence.

Also, Hess found that simple glycosides of anthocyanins appeared before more complex ones.

This present paper describes developmental variation with respect to anthocyanins in flowers of four genetically pure lines of Pisum, and in purple flowers of Lathyrus odoratus. Unlike Primula and Petunia, both Pisum and Lathyrus appear to accumulate anthocyanins in the reverse order to that expected from the biosynthetic pathway.

RESULTS

The varying complement of anthocyanins in developing flowers of Pisum (B Cr, b Cr, B cr and b cr genotypes), and in Lathyrus odoratus var Chloe, is shown in Tables 2 - 5 respectively.

Flower development in Pisum is rapid, the progression through the seven developmental stages occurring within about 48 hours in warm weather. The selection and sorting of flowers into seven stages is quite accurate and there is no overlap. Within each stage, however, individual flowers will show some variability with respect to pigment concentration. In order to assess the level of variability using this technique, a full analysis was carried out using six replicates of purple wing petals of Pisum. The mean percentage composition for each pigment at each stage of development was determined, and the results

presented in Table 1, indicate the reliability of this method. Quantitatively, the appearance of delphinidin 3-rhamnoside-5- glucoside at stage three was distinct. The total quantitative variation decreased with increasing age of the flower.

Anthocyanidins In those Pisum genotypes which permit methylation of the anthocyanins (Cr types), the more complex (methylated) anthocyanidins appeared first, and non-methylated pigments were evident only at later stages of floral development. However, by stage 6 (mature flowers), in wing petals there was a predominance of part and non-methylated over fully methylated pigments. In the standard petal on the other hand, anthocyanins did not appear until about stage 4, and the sequence of accumulation thereafter reflected the progression seen in earlier developmental stages (2 - 4) of wing petals. The total anthocyanin concentration was, at all times, less than in the wing petals of the same plant.

In Lathyrus the flowers are much larger than those of Pisum, and contain more anthocyanin per flower. Also, pigment is accumulated in the standard petals of Lathyrus earlier than is the case for Pisum. However, the pattern of pigment variation and the sequence of accumulation during floral development was the same as found for Pisum.

Glycosides In Lathyrus and in Cr type Pisum, as floral ontogeny progressed complex 3-O-glycosides appeared, in which rhamnose is replaced by sambubiose and sophorose. These latter glycosides typify the pigments found in flowers of cr type Pisum (and in coloured pods[5]), where neither methylation nor rhamnosylation occurs. Both wing and standard petals of cr type Pisum show the appearance of pigments with complex 3-O-glycosides in the earliest bud stages. Glycosylation at position 5 involves glucose only. Pigments without 5-O-glycoside substitution occur at late stages of floral development in all genotypes, and probably result from partial degradation of the anthocyanins.

DISCUSSION

Unlike both Primula obconia and Petunia hybrida, the appearance of anthocyanins in Pisum and Lathyrus does not directly reflect the sequence of reactions in the proposed biosynthetic pathway for anthocyanins. This situation could result from a disproportionate activity of the Cr gene relative to the varying rate of synthesis of the flavylium nucleus during successive stages of floral development. Thus, early in floral development, anthocyanin synthesis is low, and the enzymic activity controlled by the Cr gene is able to effect methylation of all anthocyanin nuclei. At later stages, the Cr gene product is apparently unable to keep pace with the rate of production of the flavylium nucleus, and non-methylated anthocyanins are accumulated. This notion is supported by the temporal difference in anthocyanin production in wing and standard petals. In the standards, total anthocyanin content is less than in the wings, and whereas some non-methylated pigments are accumulated, their proportion is less than in the case of the wings. Alternatively, it might be

supposed that the Cr gene is "switched off" at an early stage in the senescence biochemistry of the flowers, so that partially and non-methylated anthocyanins are allowed to accumulate.

Reference has been made previously to the pleiotropic effect of Cr^5. While the two biochemical functions ascribed to this gene (B-ring methylation and 3-rhamnosylation), are inseparable by genetic analysis, a temporal difference in these two functions is evident from these experiments. Thus, anthocyanidin methylation may be reduced, while 3-rhamnosylation can still proceed, so that for example, petunidin and delphinidin 3-rhamnoside-5-glucosides are accumulated. At a later stage of floral development, non-rhamnosylated anthocyanins are also accumulated. This observation would suggest that Cr is, in fact, two tightly linked loci. An alternative explanation would suppose that some product of Cr is necessary for both anthocyanidin methylation and for biosynthesis of the 3-rhamnosyl glycoside, and that the latter reaction has priority over methylation, when the Cr product is limited in amount.

EXPERIMENTAL

Plant Materials Seeds from genetically pure-breeding lines were used throughout. Seeds were obtained from the Plant Breeding Institute, Weibullsholm, Sweden (S lines), and from Dr. I.C. Murfet, Botany Department, University of Tasmania (L lines). Seeds were grown in a glasshouse in containers have a 1:1 vermiculite - dolerite chips (0.5 cm mesh) solid medium. Plants were watered daily and normal Hoagland's nutrient solution applied weekly.

The particular genotypes under investigation, and the anthocyanins contained in their flowers are given in Table 6.

The sequence of anthocyanin development in the commercial variety of Lathyrus odoratus var. Chloe, (Arthur Yates and Co., Pty. Ltd., Sydney, Australia) having purple flowers was also investigated. The pigment composition was identical with that for purple-flowered Pisum.

Pigment Analysis Flowers of pure lines of both Pisum and Lathyrus were collected at various stages of development, and sorted into seven developmental stages, from the youngest colourless buds to fading, senescent flowers, as shown in Fig. 1. A pooled sample of 10 or more flowers, representing each developmental stage, was crushed and exhaustively extracted with a known volume of MeOH/1% HCl. The optical density of these extracts was then recorded on an Hitachi 101 spectrophotometer, at 530 nm (b genotypes) or 540 nm (B genotypes and Lathyrus), and used to determine anthocyanin concentration / flower. Wing and standard petals were treated separately. Keel petals were not investigated, since none of the Pisum lines used contained anthocyanin in the keel.

The extracts, after concentration to a small volume, were applied as 5 cm streaks to Whatman No. 1 chromatography paper. The papers were developed overnight in BAW (Butanol: acetic acid:water, 4:1:5, upper phase). Centre strips 3 cm wide were cut from the chromatograms, thus eliminating the blurred edges of the resultant bands, and the relative density of each band in the strip determined using an EEL densitometer. In this manner, an absorbance profile showing the relative proportions of anthocyanins present in the flowers was constructed, for each of the seven developmental stages investigated. These data were then used to determine concentrations of anthocyanins in single flowers as shown in Tables 2 - 5.

Abbreviations Mv = malvidin, Pt = Petunidin, Dp = delphinidin, Pn = peonidin, Pg = Pelargonidin

3R = 3-rhamnoside, 3R,5G = 3-rhamoside-5-glucoside,

3GG = 3-sophoroside, 3GX = 3-sambubioside, 3G,5G = 3-glucoside-5-glucoside, 3GX,5G = 3-sambubioside-5-glucoside, 3GG,5G = 3-sophoroside-5-glucoside.

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TABLE 1

Mean percentage composition ± S.E. of flower colour in Pisum Genotype A Am B Ce Cr (Purple flowers). Wing petals

	Stage of Floral Development					
	2	3	4	5	6	7
Mv3R				1.4±0.4	1.6±0.3	2.0±0.4
Mv3R,5G	61.2±9.4	46.2±6.3	33.0±3.9	19.0±0.5	15.5±0.6	14.5±0.6
Pt3R,5G	38.3±9.4	53.8±6.3	47.2±2.6	43.9±1.0	44.2±0.8	44.2±0.8
Dp3R,5G			19.7±3.0	28.6±0.9	33.2±0.9	29.7±0.9
Dp3GX,5G) Dp3GG,5G)				7.1±0.7	6.3±0.9	9.5±0.9

TABLE 2

Quantitative changes in anthocyanins during floral ontogeny in Pisum. Genotype A Am Ar B Ce Cr (purple flowers).

	Stage of Floral Development					
	2	3	4	5	6	7
a. Wing Petals						
Total Anthocyanin (μmoles)	7	28	40	112	142	80
Mv3R				2	2	2
Mv3R,5G	4	13	13	21	22	12
Pt3R,5G	3	15	19	49	62	35
Dp3R,5G			8	32	47	24
Dp3GX,5G) Dp3GG,5G)				8	9	7
Σ Methylated pigments	7	28	32	82	86	49
Σ non-methylated pigments			8	40	56	31
Σ rhamnosylated pigments	7	28	40	104	133	73
Σ non-rhamnosylated pigments				8	9	7
b. Standard Petals						
Total Anthocyanin (μmoles)			7	24	47	29
Mv3R					6	
Mv3R,5G			4	12	12	8
Pt3R,5G			3	12	21	14
Dp3R,5G					8	7
Σ Methylated pigments			7	24	39	22
Σ non-methylated pigments					8	7

TABLE 3

Quantitative changes in anthocyanins during floral ontogeny of Pisum. Genotype A Am Ar b Ce Cr (pink flowers).

	Stages of floral development					
	2	3	4	5	6	7
Wing Petals						
Total anthocyanin (μmoles)	3	7	14	21	37	24
Cy3R				4	2	2
Pg3R,5G			2	4	5	3
Pn3R,5G	2	3	5	6	10	5
Cy3R,5G	1	4	7	7	11	7
Cy3G,5G					5	4
Cy3GX,5G) Cy3GG,5G)					4	3
Σ Methylated pigments	2	3	5	6	10	5
Σ non-methylated pigments	1	4	9	15	27	19
Σ rhamnosylated pigments	3	7	14	21	28	17
Σ non-rhamnosylated pigments					9	7

TABLE 4

Quantitative changes in anthocyanins during floral ontogeny of Pisum

	Stage of floral development					
	2	3	4	5	6	7
a. Genotype A Am Ar B Ce cr (Crimson flowers)						
Total anthocyanin (μ moles)	5	11	26	60	62	34
Dp3GX,5G) Dp3GG,5G)	5	8	19	45	51	31
Dp3GX) Dp3G,5G)		3	7	15	11	3
b. Genotype A Am Ar b Ce cr (Salmon flowers)						
Total anthocyanin (μ moles)	4	10	17	26	37	23
Cy3GX,5G) Cy3GG,5G)	4	10	13	16	24	15
Cy3GX) Cy3G,5G)			4	10	13	8

TABLE 5

Quantitative changes in anthocyanins during floral ontogeny of Lathyrus odoratus var. Chloe. (Purple flowers)

	Stage of floral development					
	2	3	4	5	6	7
a. Wing Petals						
Total anthocyanin (μ moles)	9	44	144	474	1140	778
Mv3R					23	23
Mv3R,5G	9	37	80	161	365	234
Pt3R,5G		7	45	185	399	249
Dp3R,5G			19	114	274	202
Dp3GX,5G) Dp3GG,5G)				14	79	70
Σ Methylated pigments	9	44	125	346	787	506
Σ non-methylated pigments			19	128	353	272
Σ rhamnosylated pigments	9	44	144	460	1061	708
Σ non-rhamnosylated pigments				14	79	70
b. Standard Petals						
Total anthocyanin (μ moles)	14	53	117	382	842	485
Mv3R				15	59	
Mv3R,5G	10	29	68	126	252	151
Pt3R,5G	4	19	47	138	303	199
Dp3R,5G		5	6	103	228	136
Σ Methylated pigments	14	48	111	279	614	349
Σ non-methylated pigments		5	6	103	228	136

TABLE 6

Anthocyanins in flowers of *Pisum* mutants.[5]

GENOTYPE	PHENOTYPE	ANTHOCYANINS
A Am Ar B Ce Cr	Purple flowers	Delphinidin, petunidin and malvidin 3-rhamnoside-5-glucoside Malividin 3-rhamnoside Delphinidin 3-sophoroside-5-glucoside Delphinidin 2-sambubioside-5-glucoside
A Am Ar b Ce Cr	Pink flowers	Pelargonidin, cyanidin and peonidin 3-rhamnoside-5-glucoside Cyanidin 3-rhamnoside Cyanidin 3-sophoroside-5-glucoside Cyanidin 3-sambubioside-5-glucoside
A Am Ar B Ce cr	Crimson flowers	Delphinidin 3-sophoroside-5-glucoside Delphinidin 3-sambubioside-5 glucoside Delphinidin 3-sambubioside Delphinidin 3-glucoside-5-glucoside
A Am Ar b Ce cr	Salmon flowers	Cyanidin 3-sophoroside-5-glucoside Cyanidin 3-sambubioside-5-glucoside Cyanidin 3-sambubioside Cyanidin 3-glucoside-5-glucoside

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FIG. 1.

Stages in Floral Ontogeny in *Pisum*.

Development Stage

1. Petals just emerged from the calyx. No anthocyanin colouration.

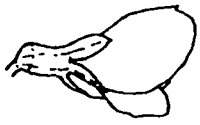
2. Anthocyanin colouration in the tips of wings only.

3. Anthocyanin colouration general throughout wing petals.

4. Anthocyanin colouration general throughout wing petals, and traces of anthocyanin in the standard petals.

5. Anthocyanin general in both wings and standards.

6. Fully open flowers.



7. Flowers senescent.

INHERITANCE OF FLAVONE PRODUCTION IN PISUM FLOWERS

Statham, Carmel M. and I. C. Murfet University of Tasmania, Hobart

While studying the biochemical-genetics of various pigmentation genes in Pisum, we became aware that some varieties possessed flavones in their flowers whereas others appeared to lack these substances which are related to the anthocyanin pigments but cause no visible difference in the color of the flower. A cross between line S1458* possessing flavone in the standard petal and line S1539* lacking flavone gave an F_1 with flavone, and an F_2 segregation of 107 plants with flavone and 37 without flavone. These numbers are in accord with a monohybrid segregation ($X^2_1 = 0.1$) and we propose that a dominant gene Cgf is responsible for the production of flavone in the standard petal. The flavones are present in the standards as glycosides, the glucose molecules being attached to the aglycone by C-C linkages. These pigments are therefore C-glycosyl flavones, hence the gene symbol Cgf. The basic anthocyanin gene A appears to be necessary for the expression of Cgf since flavones do not appear to be present in either varieties or segregates lacking A. This suggests that A operates earlier than Cgf in the biosynthetic pathway. The recessive a acts widely to prevent anthocyanin coloring in the flower, leaf axils, seed coats, etc. The recessive am acts more specifically to block color in the flower and it is noteworthy that am is not epistatic to Cgf even though the latter operates in the flower.

The joint segregation data in the first two rows of Table 1 come from the F_2 of cross S1458 x S1529. Segregation at the Cr locus is rather heavily disturbed but there is a definite indication that Cgf is close to Gp and Cr in linkage group 5. To check this linkage, a line pure breeding for ce, gp, and cgf was selected and crossed to line 2, Ce Gp Cgf. A testcross of this F_1 to the triple recessive produced the data shown as 29TC in Table 1. The testcross data show no evidence of linkage between Cgf and the group 5 markers. With conflicting evidence, we are therefore uncertain as to the position of Cgf. It may be noted there is a slight deficiency of recessive cgf in the testcross.

*Weibullsholm lines by courtesy of Dr. S. Blixt

Table 1. Segregation and assortment data for Cgf and markers Ce, Cr, and GP

Cross	Genes	Phase	Phenotype XY	Xy	xY	xy	Chi-squared Seg.X	Seg.Y	Joint	CrO %
1 F_2	Cr-Cgf	C	89	2	18	35	10.7***	0.1	71.4***	9 ± 3
1 F_2	Gp-Cgf	R	72	36	35	1	0.0	0.1	13.2***	5 ± 8
29 TC	Ce-Gp	C	43	16	19	43	0.1	0.1	21.5***	29
29 TC	Ce-Cgf	C	38	21	35	27	0.1	5.2*	0.8	46
29 TC	Gp-Cgf	C	38	24	35	24	0.1	5.2*	0.1	49

*$P < 0.005$; **$P < 0.01$; ***$P < 0.001$

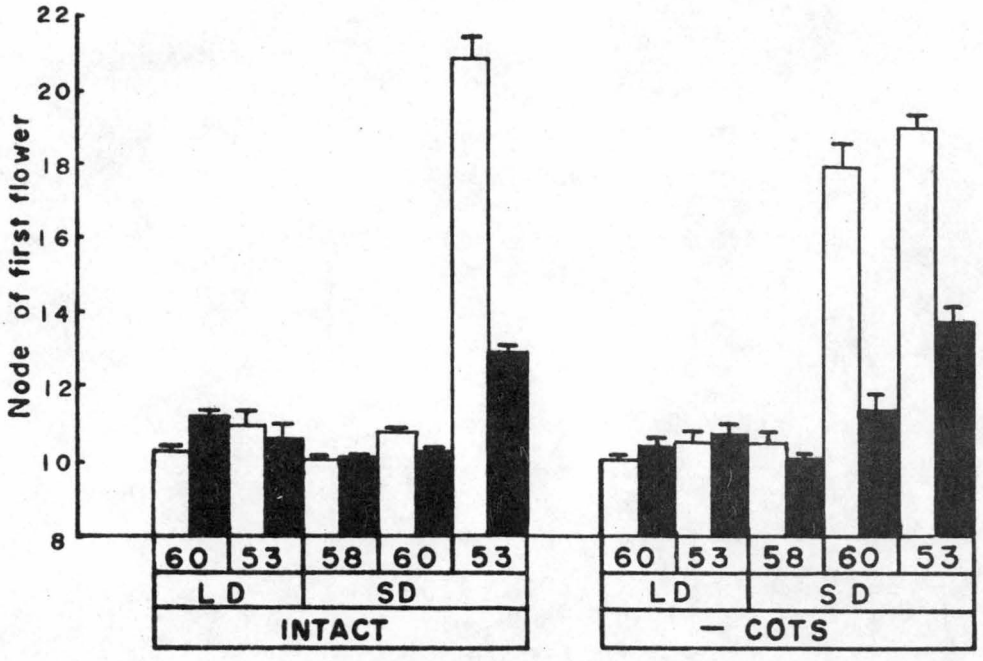


Figure 1. The mean node of first initiated flower and S.E. for lines 58 (lf e sn hr), 60 (lf E Sn hr) and 53 (lf e Sn hr). 15 plants/treatment. ■=vernalized for 6 weeks at 2-4°C; □=unvernalized; LD=continuous light from start of germination, SD=8 hr photoperiod; -COTS=cotyledons removed at 6 days for unvernalized plants and 6 weeks for vernalized plants (developmental stage approximately equivalent).

THE FLOWER COLOR PALE LILAC

Statham, Carmel M. and I. C. Murfet University of Tasmania, Hobart

The pigmentation genes ar and ce are reasonably well known, the single recessive A Am ar B Ce Cr having violet flowers and A Am Ar B ce Cr having cerise flowers. However, the double recessive A Am ar B ce Cr has received little attention. We obtained this combination in the F_2 of the cross S25* (ar) x S1227* (ce) and under glasshouse conditions we found the flowers to have a pale lilac coloration present in both the wings and standard, the pigmentation being strongest at the margins. This color corresponds to the reference plate 15B4 in the Methuen Handbook of Color. The observed F_2 numbers of 91 purple (Ar Ce), 30 cerise, 25 violet, and 8 pale lilac indicate normal segregation and free recombination. The pale lilac plants possessed violet-colored leaf axils which were not visibly different from those of violet plants. One pale lilac F_3 family was grown in the field during the winter. Under these conditions the standard petal appeared nearly white, whereas the lilac color of the wings was intensified relative to the glasshouse-grown plants.

*Weibullsholm lines by courtesy of Dr. S. Blixt