BIOCHEMICAL ASPECTS OF SEEDCOAT COLOUR INHERITANCE IN *PHASEOLUS VULGARIS* L.

W. J. FEENSTRA

BIOCHEMICAL ASPECTS OF SEEDCOAT COLOUR INHERITANCE IN *PHASEOLUS VULGARIS* L.

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE WND. RECTOR MAGNIFICUS
IR. W. F. EIJSVOOGEL,
HOOGLERAAR IN DE HYDRAULICA, DE BEVLOEIING,
DE WEG- EN WATERBOUWKUNDE EN DE
BOSBOUWARCHITECTUUR,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
OP VRIJDAG, 19 FEBRUARI 1960 TE 16 UUR

DOOR

WILLEM JAN FEENSTRA

H. VEENMAN EN ZONEN N.V. – WAGENINGEN – 1960

STELLINGEN

I

De aanwezigheid van het gen *V* in de zaadhuid van *Phaseolus vulgaris* L. heeft als gevolg de vervanging van één of twee waterstofatomen in de benzeenring van de precursors, waaruit de flavonoide verbindingen gevormd worden, door hydroxylgroepen.

H. LAMPRECHT, Hereditas **16**, 1932: 169–211.
Dit proefschrift.

II

Er bestaat een correlatie tussen het aantal substituenten in de benzeenring van de als gevolg van de aanwezigheid van de genen *C* of *C*^{r} in de zaadhuid van *Phaseolus vulgaris* L. gevormde flavonoide kleurstoffen en de structuur van het heterocyclische gedeelte van het molecule.

Dit proefschrift.

III

De glans van het zaad van *Phaseolus vulgaris* L., die een gevolg is van de aanwezigheid van het gen *Sh*, wordt veroorzaakt door het voorkomen van een geelbruine kleurstof in de parenchymatische laag die gelegen is onder de in elk zaadhuidtype aanwezige glasachtige bovenlaag, bestaande uit epidermis en kristalcellen-laag.

R. PRAKKEN, Genetica **16**, 1934: 177–294.
Dit proefschrift.

IV

De mate waarin men er in slaagt zogenaamde series van multiple allelen tot sterk gekoppelde genen terug te brengen hangt af van het aantal individuen per nakomelingschap dat men kan onderzoeken.

V

Tijdens de biosynthese van flavonolen en anthocyanidinen wordt de A-ring niet, zoals volgens MOEWUS het geval zou zijn, gevormd uit phloroglucinol, doch opgebouwd uit acetaateenheden.

F. MOEWUS, Ann. N.Y. Acad. Sci. **61**, 1955: 660–664.
H. Z. GRISEBACH, Z. Naturforsch. **12b**, 1957: 227–231.
J. E. WATKIN, E. W. UNDERHILL en A. C. NEISH, Can. J. Biochem. and Physiol. **35**, 1957: 229–237.

Diss. W. J. FEENSTRA
Wageningen, 1960

VI

Door LEETE c.s. is niet afdoende bewezen dat in de nicotinemoleculen, gevormd in de wortels van tabaksplanten na toediening van op de 2-plaats met C^{14} gemerkt ornithine, het C^{14}-isotoop ook op de 5-plaats in de pyrrolidine kern voorkomt.

E. LEETE en J. S. SIEGFRIED, J. Am. Chem. Soc. **79**, 1957: 4529–4531.

VII

Het uitblijven van bloei onder lange-dag omstandigheden na plantvernalisatie op jeugdige leeftijd bij *Cheiranthus allionii* Hort. kan verklaard worden door devernalisatie.

VIII

Op grond van entingsproeven kan hoogstens tot onderlinge vervangbaarheid, doch niet tot het identiek zijn van de bloeistimuli in verschillende plantensoorten geconcludeerd worden.

J. A. D. ZEEVAART, Meded. Landbouwhogeschool **58** (3), 1958: 1–88.

IX

In tegenstelling tot de mening van GEISSMAN is het waarschijnlijk dat flavonoide verbindingen wier absorptiespectrum geen maximum toont in het voor de mens zichtbare gebied, doch wel in het nabije ultraviolet, een bloembiologische betekenis hebben.

T. A. GEISSMAN, in "Modern Methods of Plant Analysis", vol. III. Ed. K. PAECH and M. V. TRACEY, Springer Verlag, Berlin, Göttingen, Heidelberg, XIII + 626 pp., 1955: 450–498.
K. DAUMER, Z. Vergleich. Phys. **41**, 1958: 49–110.

X

Zoals nu reeds in het „Veiligheidsbesluit ioniserende stralen" het geval is, dient ook in de wettelijke voorschriften regelende het omgaan met niet-radioactieve voor de gezondheid schadelijke chemicalien rekening te worden gehouden met de mogelijkheid van schade voor het nageslacht.

Veiligheidsbesluiten gebaseerd op de „Veiligheidswet 1934".

WOORD VOORAF

Gaarne wil ik op deze plaats allen danken die bijgedragen hebben tot mijn wetenschappelijke vorming en tot de voltooiing van dit proefschrift.

Hooggeleerde PRAKKEN, hooggeachte promotor, het was Uw initiatief dat tot het hier beschreven onderzoek leidde. Uw voortdurende belangstelling tijdens de uitvoering was voor mij een belangrijke steun; dat U mij daarbij een grote vrijheid liet tot ontplooiing van eigen ideeën, stemt mij tot dankbaarheid.

Hooggeleerde DEN HERTOG, hooggeachte promotor, als student mocht ik op Uw laboratorium werkzaam zijn en de prettige sfeer aldaar leren kennen. Dat U mede mijn promotor hebt willen zijn, terwijl het onderzoek voor dit proefschrift grotendeels buiten Uw laboratorium heeft plaats gevonden, is voor mij een reden tot dankbaarheid; Uw critische opmerkingen zijn voor mij van veel nut geweest.

Hooggeleerde WELLENSIEK, tijdens mijn studie heb ik Uw leiding en steun ondervonden; ik zal daaraan altijd met grote erkentelijkheid terugdenken.

Zeergeleerde MINDERHOUD, Uw colleges in bijenteelt waren voor mij vreugdevolle uren.

I wish to express my sincere thanks to Dr. BATE-SMITH, Low Temperature Research Station, Cambridge, England, for the opportunity of working during five weeks in his department, and to Dr. SWAIN for reading the manuscript and for correcting the English text.

Beste DUYVENDAK, PARLEVLIET en VAN DER VEEN, de prettige wijze waarop U mij steeds tegemoet trad, waardeer ik ten zeerste.

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Overdruk

581.169 : 581.48 : 535.6
581.19 : 581.169
635.652 : 581.169 : 535.6

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BIOCHEMICAL ASPECTS OF SEEDCOAT COLOUR INHERITANCE IN *PHASEOLUS VULGARIS* L.

(met een samenvatting in het Nederlands)

by

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CONTENTS

CHAPTER I

INTRODUCTION

The investigations which are reported in this paper form part of a programme to establish the relation between genes and pigment synthesis in the seedcoat of the French bean, *Phaseolus vulgaris*. The first combined genetical and chemical work on plants, leading to a better understanding of the action of the genes and giving some information about the biosynthesis of the compounds involved, was that by WHELDALE (65) in 1909 on the pigments in the flower of *Antirrhinum majus*. Up to the present, mainly the sap-soluble flower pigments have been investigated in higher plants. The majority of these substances, e.g. anthocyanidins (red, violet and blue pigments), flavones and flavonols (yellow pigments), belong to the class of flavonoid compounds. In plant all these substances occur mainly as glycosides.

The carbon skeleton of these pigments consists of two benzene rings, linked by a chain of three carbon atoms. This chain usually forms part of a third six membered ring with an oxygen atom and two carbon atoms of one of the benzene nuclei. In fig. 1 the structures of some typical compounds are shown. The numbering of the C-atoms is given in fig. 1a, the two C_6-rings being designated A and B.

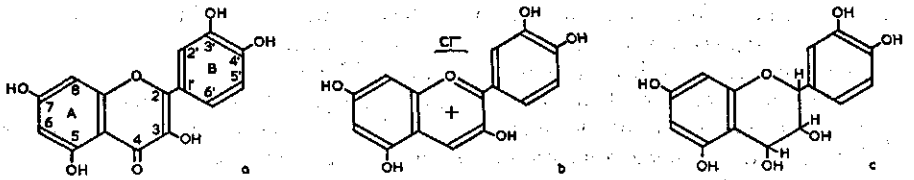


FIG. 1. Structures of some flavonoid compounds.
a: quercetin (a flavonol); *b:* cyanin (an anthocyanidin); *c:* leuco-cyanidin (a leuco-anthocyanidin).

Until 1948 attention was paid mainly to the anthocyanins (i.e. glycosides of the anthocyanidins). The establishment of the structure of many of these compounds by WILLSTÄTTER, KARRER and ROBINSON and their schools, and the development, by R. and G. M. ROBINSON (45, 46, 47, 48), of methods enabling their rapid identification had been highly stimulating. The investigations, carried out by BEALE, LAWRENCE, the ROBINSONS, SCOTT-MONCRIEFF and others, showed that many genetically determined changes in the colour of flowers were related to alterations in the structure of the anthocyanin pigments present. Examples of such changes were the substitution of one or two hydrogen atoms in the B-ring by hydroxyl groups, the methylation of existing hydroxyl groups, and the conversion of the 3-monoglycoside or 3-pentose-glycoside structures into 3,5-diglycosides. Reviews of this work have been given by SCOTT-MONCRIEFF (50, 51).

The application of paperchromatography (introduced by BATE-SMITH (2) in 1948) and spectrophotometry for the identification of the flavonoid pigments opened new perspectives. These techniques made possible not only a better analysis of the anthocyanins, but also the investigation of the nature of the yellow compounds, which previously had been neglected by the lack of any suitable method for their identification on a micro scale. The work on *Antirrhinum* by DAYTON (11), GEISSMAN and co-workers (16, 19, 25, 26), and SHERRATT (55), on *Dianthus* by GEISSMAN *et al.* (17), on *Cyclamen* by SEYFFERT (52), and on *Impatiens* by ALSTON and HAGEN (1) may be mentioned here as examples.

More recently stress has been laid on studies of the routes by which the various flavonoid pigments are synthesized, and this work has been adequately summarised by BOGORAD in 1958 (6).

Phaseolus vulgaris shows a very large variation in seedcoat colour. The genetical background of this variation has been studied extensively, but the nature of the pigments involved has remained rather obscure. PRAKKEN (42), in 1940, expressed the desirability that thorough chemical investigations should proceed along with the genetical studies. This combined chemical and genetical work is in progress now, and this paper reports the results of the first part of it.

In this investigation the relation between genes and pigment synthesis has been studied in an extra-floral plant part. As mentioned earlier relations of this kind until now have mostly been studied in flowers. One major difference between flowers and seeds lies in the fact that flowers are exposed to light, whereas the seed develops surrounded by other tissues of the fruit in darkness. In a number of cases where anthocyanin formation has been studied in organs directly exposed to the light, it has been found that light plays an important role

in the synthesis (6). The anthocyanin synthesis which occurs in the seedcoat of *Phaseolus vulgaris*, however, must be light independent.

The chemical-genetical studies on beans in the Laboratory of Genetics at Wageningen were started in 1954 with an investigation into the action of two gene pairs and one multiple allelic series of three alleles. All twelve possible different homozygous genotypes, in the form of pure lines, were examined.

The pigment mixtures, present in the seedcoats, were analysed qualitatively with use of paper chromatography and absorption spectrophotometry. Fifteen components, leuco-anthocyanidins, and glycosides of flavonols and anthocyanidins, were identified. With some other glycosides, however, only the aglycone was determined.

By spectrographic methods a number of genotypes were compared quantitatively with regard to their contents of anthocyanins, flavonol glycosides or leuco-anthocyanidins.

CHAPTER II

REVIEW OF PREVIOUS INVESTIGATIONS CONCERNING THE CHEMISTRY AND LOCATION OF BEAN SEEDCOAT PIGMENTS

With one exception the chemical work so far carried out on bean seedcoat pigments has not extended beyond the determination of the class to which the compounds belonged (e.g. anthocyanins, flavonol glycosides, etc.). The individual pigments were not identified.

SHAW and NORTON (54) stated that the pigments from red and purplish-red seedcoats which they investigated were anthocyanins, basing their conclusion on the solubility of the compounds in cold water, and the changes of the colour with acid and alkali. They could not identify the pigments from yellow, brown or black seeds.

SKALINSKA (56) concluded, from genetic evidence, that the pigments in various colour types must be related. She stated that black and violet seeds from her material contained anthocyanins, basing her identification on the reactions of alcoholic extracts of these colour types with solutions of neutral lead acetate and iron salts. She found that the yellowish and brown seeds contained substances which, when treated with nascent hydrogen in the presence of hydrochloric acid, yielded pigments with anthocyanin-like properties. The precursors were identified as "flavonols", but were presumably glycosides of these compounds. Yellow beans were found to contain "pseudobases" (i.e. leuco-anthocyanins or leuco-anthocyanidins) which yielded anthocyanidins when treated with hot hydrochloric acid. The browning of old beans was ascribed to oxidation of "pseudobases".

ROBINSON and ROBINSON (46), employing their methods to identify the pigments in the coloured parts of various plants, analysed the seedcoat of the purplish-red bean variety 'Canadian Wonder' and detected pelargonidin-monoglycoside.

Some authors have given data concerning the location of the pigments. According to KAJANUS (27) black, blue, dark green, greenish-brown and yellow colours are caused by pigments filling the lumina of the epidermic palisade cells (see fig. 2). Orange-brown and brownish-yellow pigments are situated in the palisade cell walls. Only the reddish-yellow "ground colour" is caused by

a yellow pigment in the underlying parenchymous layers of the seedcoat. TJEBBES and KOOIMAN (63) have described an analogous distribution of the pigments.

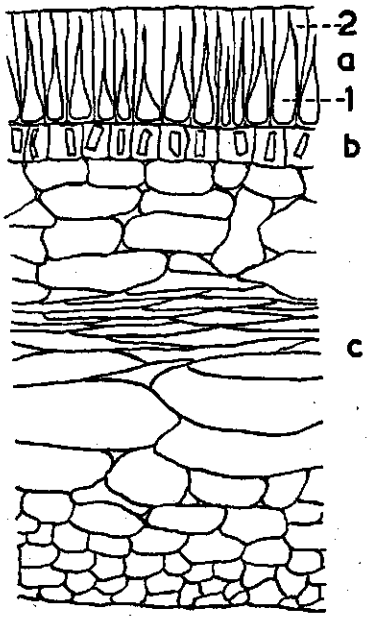


FIG. 2. Cross section through a softened seedcoat.
a: epidermis, 1: lumen, 2: cell wall; *b*: crystal cell layer; *c*: parenchymous layer. In the dry seedcoat the parenchymous layer is collapsed and forms a thin, dense mat underneath the crystal cell layer.

CHAPTER III

THE GENETICAL BASIS OF THE INVESTIGATIONS

1. INTRODUCTION

Extensive research has been carried out over many years on the inheritance of seedcoat colours in *Phaseolus vulgaris*. Between 1900 and 1931 important investigations were carried out by VON TSCHERMAK, EMERSON, SHULL, KAJANUS, NORTON, SHAW, SIRKS, TJEBBES, KOOIMAN, and many other workers. This work yielded a great many results, many of which could not be readily related with each other. A review of this earlier work has been given by KOOIMAN (30).

The various seedcoat colour types of *Phaseolus vulgaris* can be classified as evenly coloured or patterned (striped, mottled, speckled, etc.). In a number of the types only a part of the seedcoat is coloured (evenly or patterned) while the remaining part is completely white. I have investigated the action of genes which, at least when homozygously present, give entirely coloured and unpatterned seedcoats. This class of genes was grouped by KOOIMAN (30) as follows:

I. *Groundgene*; a dominant gene which does not produce any colour by

itself but which must be present for pigment formation to be possible. All plants in which the recessive allele is present in the homozygous condition have a white seedcoat and white flower.

II. *Chromogenous genes*; when present together with the groundgene the dominant alleles of these give rise to a coloured seedcoat.

III. *Colour determiners*; genes which change the colours produced by the combined action of groundgene and one or more chromogenous genes.

Since 1931 the work on bean seedcoat colours has been mainly carried out by LAMPRECHT in Sweden, PRAKKEN in The Netherlands and SMITH in America. The previously published work of PRAKKEN was mainly concerned with genotypes of a series yielding yellow, brown, violet, or black seedcoat colours, while that of LAMPRECHT covered dominant red types as well. SMITH and his coworker investigated a special group of red colours, controlled by recessive genes.

In 1932 LAMPRECHT (31) published the first results of his investigations. In the main independently from results published by previous workers he developed a complicated scheme for the inheritance of the seedcoat colour of *Phaseolus vulgaris*. This scheme was based on data published in a long series of papers the last of which (33) gives a review of a great deal of his work.

By analysing the offspring of a cross, originally made for an investigation into pod characters, PRAKKEN (40) detected colour types and segregations which showed a great resemblance to those mentioned previously by LAMPRECHT. PRAKKEN therefore adopted a number of LAMPRECHT's gene denotations. In 1940 PRAKKEN (42) published the results of further investigations together with a scheme for colour inheritance which was much simpler than that developed by LAMPRECHT. In this paper PRAKKEN also related his findings with those published by KOOIMAN, LAMPRECHT, and SIRKS.

In the following discussion of my own material I shall mainly compare the results obtained with those of LAMPRECHT and PRAKKEN. For homologies with older investigations the reader be referred to Table 1, the larger part of which was adopted from PRAKKEN (42, p. 405).

TABLE 1. Homologous symbols of genes for seedcoat colour in *Phaseolus vulgaris*.

Investigator	Year	Genes									
SHULL	1908	*P*						*B*	*D*		
TSCHERMAK	1912	*A*						*B*	*C*		
SHAW and NORTON	1918	*P*						*G*	*H*	*C*	
KOOIMAN	1920	*A*	*C*	*B*			*D*	*F*	*E*		
SIRKS	1922	*A*	*D*	*B*				*Z*	*L*		
LAMPRECHT	'32–'51	*P*	*J*	*C*	*R*	R^s		*V*	*B*	*G*	
PRAKKEN	'34–'40	*P*	*Sh*	*Cm*			*D*	*V*	*B*	*G*	
SMITH	'39–'48				*R*				*Bl*		*Rk*
FEENSTRA	pres. art.	*P*	*Sh*	*C*	C^r	C^s	*D*	*V*	*B*	*G*	*Rk*

2. GENES DETERMINING THE SEEDCOAT COLOUR

The following genes were taken into account when breeding the material for the chemical analysis.[1])

[1]) The genotypes of most of the varieties which were used as parents had been established by previous investigations (PRAKKEN, unpubl.). Details of the genetical work will be published elsewhere.

2.1. *Ground gene*

All varieties, used as parents in this investigation had coloured seeds. Thus all types possessed the dominant groundgene, called *P* by LAMPRECHT and PRAKKEN.

2.2. *Colour genes*

The name for this group of genes is adopted from LAMPRECHT. It corresponds to KOOIMAN's chromogenous genes; PRAKKEN used the term complementary genes.

2.2.1. Gene pair *Sh–sh*

The dominant allele *Sh* produces a creamish seedcoat with a brown ring around the hilum. The seedcoat has a somewhat glossy appearance, hence the name "shine" gene. During storage of the beans the colour changes slowly into a dark reddish brown. The symbol *Sh* for this gene was adopted from PRAKKEN; LAMPRECHT used the symbol *J*. The latter investigator mentions a second gene, *Ins*, with a similar effect. The material used here showed no evidence for the existence of such a second gene.

2.2.2. Multiple allelic series $C - C^r - C^s - c - c^u$

It must be stressed that the assumption of one multiple allelic series should be considered as provisional. Possibly it later will appear that two or more closely linked loci are involved. In my material these genes always behaved as alleles of the same locus. For the sake of simplicity they will therefore also be denoted as such.

(i) When the gene *C* is homozygously present, the seedcoat is lemon yellow without a hilum ring. The heterozygous combinations $C\,c$ and $C\,c^u$ give a seedcoat, showing mottling of lemon yellow on white. The symbol *C* was adopted from LAMPRECHT, who ascribed the mottling to the heterozygous condition of this gene. PRAKKEN assumed that two genes, *C* and *M* were present; *C* giving the colour and *M* the mottling, the latter by locally suppressing the action of *C*. *C* and *M* were suggested to be almost absolutely linked in the repulsion phase (*Cm* and *cM*), thus giving the mottling only in the heterozygous combination. This hypothesis was made to associate this mottling with the true breeding mottling of other types. PRAKKEN called the strong *C*-colour the dark pattern colour, and the paler colour, caused by the recessive allele *c*, the background colour.

(ii) By C^r is symbolized a gene, originating from the variety 'Canadian Wonder Improved', which, when homozygously present, gives a self-coloured pale pink seedcoat without a hilum ring. Like *C*, the gene C^r gives mottling in the heterozygous combinations $C^r\,c$ and $C^r\,c^u$. TJEBBES (62) and LAMPRECHT (32) ascribed the pink colour to the action of a gene *R*, closely linked with the *C*-locus. The evidence for crossing-over, and thus for the existence of two separate loci, given by these authors, however, was not very convincing.

According to LAMPRECHT the gene *R* belongs to a multiple allelic series, the other alleles of which with one exception give various patterns of red on white. The bottom recessive, *r*, leaves the seedcoat white. In families segregating for *C*- and *R*-alleles TJEBBES mentions 5 recombinants on 6500 plants, while LAMPRECHT, in the F_2 of four crosses between *C r* and *c R* types, found 5 *c r* plants (whites) on a total of 3181 plants. LAMPRECHT calculated a recombination value of $8 \pm 1.76\%$. However, all the so-called cross-over types found by both workers were *sh*-types. No crossing-over was observed among the three times more frequent *Sh*-types. In this connection it should be mentioned that the presence of *Sh* makes the red colour much more pronounced. In both investigations pattern forming alleles of the *R*-locus were involved, and PRAKKEN (pers. comm.) found that in striped and mottled types possessing homozygously the recessive *sh*-gene, the red colour is sometimes even completely lacking, the beans appearing

white. For these reasons it seems probable that TJEBBES' and LAMPRECHT's conclusions regarding recombination were the result of wrong classification.

In our laboratory crossing-over between a gene giving yellow and one giving pink colour has not yet been observed and in this paper the gene, which gives pink, is tentatively considered to be an allele of the *C*-locus; it is called the "dark pattern red" allele and denoted by C^r. The heterozygous type $C\,C^r$ is mainly yellow with a mottling of faint pink spots mainly on the ventral side of the seed.

(iii) The gene C^s gives a pattern of pink stripes on white and behaves as an allele of *C* and C^r. According to LAMPRECHT it belongs to the R-series (see above).

(iv) The gene *c* was described by LAMPRECHT and PRAKKEN as the recessive allele of *C*, and gives a white seedcoat. The genes *Sh* and *c* together give a creamish colour which can be changed by modifying genes (see below).

(v) The gene c^u originated from the variety 'Red Kidney'. Crossing of this variety with *C*- and C^r-types yielded mottled F_1 and the F_2 segregated into 1 dark pattern: 2 mottled: 1 background. The F_1 of 'Red Kidney' with *c*-types was self coloured. From this evidence it was concluded, that 'Red Kidney' possesses a "background" allele. The gene *Sh* together with this allele gives the same creamish colour as it does with *c*. The presence of the modifying genes *G*, *B* and *V* is without influence on this colour however. The allele from 'Red Kidney' therefore is different from *c*; it is called "unchangeable background" allele, and denoted by c^u. Modifying genes can change the hybrid $Sh\,Sh\,c\,c^u$; the latter segregates into 3 changeable: 1 unchangeable. The gene c^u thus appears to be recessive to *c*.

2.2.3. Gene pair $D - d$

PRAKKEN describes the action of a gene *D*, giving a yellow-brown hilum ring, but leaving the remaining part of the seedcoat a faint grayish colour which cannot be changed by modifying genes. PRAKKEN adopted the symbol from KOOIMAN (29) who already mentioned the action of this gene. In the presence of *Sh*, which also produces a yellow-brown hilum ring, the effect of *D* cannot be observed. In the segregating families from which I selected the pure lines for chemical analysis all the *sh*-types were without a yellow-brown hilum ring, and it was concluded, therefore, that the recessive allel of this gene pair, *d*, was present in all the plants.

2.2.4. Interaction of *Sh* with *C* and C^r

The genes *Sh* and *C* together give a canary colour, which is especially pronounced on the ventral side of the seed. The occurrence together of *Sh* and C^r has a very conspicuous effect: the seedcoat shows a dark purplish red colour.

2.3. *Modifying genes*

The name for this group of genes is adopted from LAMPRECHT. PRAKKEN used the same terms; they correspond to KOOIMAN's colour determiners.

Four series of modifying genes were segregating in the material: $V - v^{lae} - v$, $B - b$, $G - g$ and $Rk - rk - rk^d$. The following colour genes or combinations of these genes must at least be present for the modifying genes to have a visible action on seedcoat colour:

C, C^r or $Sh\,c$ for $V - v^{lae} - v$[1]) and $B - b$;

[1]) The gene *V* sometimes can produce a very faint grayish-blue tinge around the hilum together with *sh c*.

Sh C, *Sh* C^r or *Sh c* for $G - g$;
Sh for $Rk - rk - rk^d$.

The colours produced in the presence of the combination *Sh c* are more or less similar to, but paler than those of the corresponding *Sh C*-types; only the latter are described below.

2.3.1. Multiple allelic series $V - v^{lae} - v$

The gene *V* changes the seedcoat colour of types possessing the genes *C* or C^r into a bluish violet. It also produces a violet flower colour and a pale or deep violet tinge on other parts of the plant even when only the groundgene *P* is present. The seedcoat colour is much intensified when the gene *Sh* is simultaneously present.

The alleles v^{lae} and *v* do not differ in visible effect on the seedcoat colour; the difference between them expresses itself in the flower colour: v^{lae} produces a pale lilac, whereas *v* gives a white flower. This multiple allelic series was first described by LAMPRECHT.

2.3.2. Gene pair $B - b$

The gene *B*, together with *C* gives a greenish brown, and together with C^r a pale violet-blue seedcoat colour. When *Sh* is also present the latter colour is intensified to dark blue-violet.

Neither *B* nor *G* (to be mentioned below) produce a yellow-brown hilum ring. This is in agreement with PRAKKEN's conclusions regarding the action of these genes. According to LAMPRECHT, however, both genes do add a yellow-brown hilum ring, even when present together with the groundgene only. PRAKKEN (42) has suggested that LAMPRECHT arrived at this conclusion by failing to recognize the action of the hilum ring gene *D*.

2.3.3. Gene pair $G - g$

The gene *G*, together with *C* or C^r only, has no visible effect. When *Sh* is also present, *G* produces an orange colour with *C*, and a maroon colour with C^r.

2.3.4. Multiple allelic series $Rk - rk - rk^d$

From the variety 'Red Kidney' a recessive gene was derived which gives in the presence of *Sh* a red-brown seedcoat. The previously mentioned effect of the gene *Sh*, namely a creamish seedcoat, is produced in the presence of the dominant allele *Rk*. A third allele of the same locus originated from the variety 'Poepen'. It produces, together with *Sh*, a testaceous seedcoat, which is dominant over red, but recessive to creamish. Genes, having a very similar effect were described by SMITH (58) and SMITH and BECKER MADSEN (59) and denoted *Rk*, *rk*, and rk^d, giving creamish, testaceous, and red-brown, respectively. PRAKKEN (41) in a preliminary note in 1938 had already mentioned the segregation of a recessive red seedcoat together with red veins in the flower petals of the plant; he gave no symbol for this gene however. For the genes in my material I adopted the symbols of SMITH *et al.* It was found that both *rk* and rk^d produce red veins in the flower, irrespective of the presence or absence of *Sh*.

3. GENOTYPIC CONSTITUTION OF THE MATERIAL WHICH WAS CHEMICALLY ANALYSED

The various combinations of the genes mentioned in the previous section produce a large number of phenotypes with regard to the seedcoat colour. Twelve out of these types were chemically analysed. These represented all possible homozygous combinations of the following genes: *Sh–sh*, $C^r–C–c^u$, $V–v^{lae}$.

The twelve types were identical as far as concerned the other loci mentioned in the proceeding sections. This constant part of the genotype was as follows: *P d b g Rk*. The genotypes investigated, their colours, and their origins are given in Table 2.

TABLE 2. Genotype, colour and origin of the pure lines which were chemically analysed.

Number of line	Colour	Varying part of genotype[1])	Origin
F 125	white	*sh* c^u v^{lae}	Citroen *(sh C* v^{lae} *P d b G Rk)* × Red Kidney *(Sh* c^u v^{lae} *P d B g* rk^d*)*
F 111	creamish	*Sh* c^u v^{lae}	Citroen *(sh C* v^{lae} *P d b G Rk)* × Red Kidney *(Sh* c^u v^{lae} *P d B g* rk^d*)*
F 63	lemon yellow	*sh C* v^{lae}	Fiskeby *(Sh C V P d b g Rk)* × Brunetta *(sh C* v^{lae} *P d B g Rk)*
F 191	pale pink	*sh* C^r v^{lae}	Brunetta *(sh C* v^{lae} *P d B g Rk)* × F 175 *(Sh* C^r v^{lae} *P d b g Rk)*
F 125	white	*sh* c^u *V*	F 61 *(sh C V P d b g Rk)* × F 127 *(sh* c^u v^{lae} *P d b g Rk)*
F 47	canary	*Sh C* v^{lae}	Fiskeby *(Sh C V P d b g Rk)* × Brunetta *(sh C* v^{lae} *P d B g Rk)*
F 175	dark purplish red	*Sh* C^r v^{lae}	Canadian Wonder Improved *(Sh* C^r v^{lae} *P d b g Rk)*
F 109	creamish	*Sh* c^u *V*	F 111 *(Sh* c^u v^{lae} *P d b g Rk)* × F 45 *(Sh C V P d b g Rk)*
F 61	violet blue	*sh C V*	Fiskeby *(Sh C V P d b g Rk)* × Brunetta *(sh C* v^{lae} *P d B g Rk)*
F 189	violet blue	*sh* C^r *V*	F 125 *(sh* c^u *V P d b g Rk)* × F 191 *(sh* C^r v^{lae} *P d b g Rk)*
F 45	black violet	*Sh C V*	Citroen *(sh C* v^{lae} *P d b G Rk)* × Poepen *(Sh* C^s *V P d b g rk)*
F 173	dark blue violet	*Sh* C^r *V*	F 175 *(Sh* C^r v^{lae} *P d b g Rk)* × F 109 *(Sh* c^u *V P d b g Rk)*

[1]) The constant part of the genotype was *P d b g Rk*.

In breeding the desired genotypes some special measures had to be taken because of interactions between modifying and colour genes. As was said previously, the genes *B*, *G* and *V* do not show any visible action in c^u-types. Distinction between *V* and v^{lae} always could be made by the flower colour, *V* causing purple, v^{lae} pale lilac flowers. The desired type as regards to *B* and *G* was selected in *C* c^u-types, where the dark pattern of the mottled seedcoats revealed whether the dominant or the recessive alleles were present; selfing then yielded the c^u-types.

Since which allele of the *G*-locus is present in *sh* types cannot be decided even in the presence of *C*, the desired genotypes were established in *Sh sh*-types, and *sh*-types obtained from selfings of the latter.

Differences among the alleles of the *Rk*-locus are not expressed in the seedcoat of *sh*-types, but the flower colour again could be used here, *Rk* giving no coloured veins in the wing petals, whereas the other alleles give more or less deep coloured red veins.

The selected types were propagated by selfing.

CHAPTER IV

METHODS USED IN THE CHEMICAL ANALYSIS

1. Introduction

As was mentioned earlier, paper-chromatography and spectrophotometry are nowadays widely used for the analysis of the pigment mixtures in the chemical-genetical investigations of colour characters. These techniques could be applied succesfully in the present investigation and were extremely valuable since mixtures of compounds, occurring mostly in small amounts, had to be examined.

After the pigments had been extracted from the seedcoats, they were separated by preparative chromatography on thick filter paper. The individual components were identified by paper chromatography where possible. Spectra of the compounds were measured, either in solution or directly on a paper chromatogram, for completion and confirmation of their identification. One compound was obtained in large enough quantity for determination of the elementary composition to be possible.

As a rule only qualitative data about the occurrence of pigments were obtained. However the ratios of the total amounts of the types of pigment (anthocyanins, flavonol glycosides or leuco-anthocyanidins) present in different genotypes, could be estimated by spectrophotometric methods.

2. Isolation of the mixture of flavonoid compounds from the seedcoat

2.1. *Preparation of the seedcoats*

As it was the intention to investigate the pigments in the seedcoat (tissue belonging to the mother plant), this had to be separated from the remainder of the seed (the embryo). In order that the separation could be carried out without laborious and drastic treatment, the beans were harvested when already fully coloured but before they were dry ripe. At this stage the pods were already yellow and half dry but the seedcoats still could easily be pulled from the cotyledons. The seeds were halved to facilitate the separation of the seedcoats.

The parenchymous inner layer and the tough outer layer of the seedcoat of some types were analysed separately. The outer layer consists of the epidermis and the underlying crystal cell layer; the parenchymous tissue could be scraped off with a scalpel. This treatment was carried out under a binocular stereo-microscope.

The seedcoats were stored for some time (up to nine months) in darkness at −15° before they were extracted. Samples of one genotype when analysed qualitatively after both shorter or longer periods of storage yielded identical results. There was some loss of water from the seedcoats, however, and in order to minimise this loss as much as possible the seedcoats were kept in plastic bags.

The question whether the composition of the pigment mixture in the beans which were harvested and treated as mentioned, always corresponds qualitatively with the composition of this mixture in fully dry ripe seeds, cannot be answered. It can be stated, however, that in a number of genotypes corresponding results were obtained from the qualitative analysis of moist seedcoats and of seedcoats which had been prepared by a rather drastic method from seeds which had been harvested fully dry.

The treatment of beans which had been harvested fully dry was as follows. After halving the beans between the cotyledons, the halves were placed in petri dishes with the flat side down and sprayed four times with water or 0.1% aqueous hydrochloric acid within a period of three hours. After standing overnight the seedcoats could then be pulled from the cotyledons. They were dried in an oven at 70° and ground in a mill. No pigments could be extracted from the dry meal with alcoholic solvents, and it was therefore first moistened with water. (2.5 ml per gram).

2.2. *Extraction*

Both methanol or ethanol were used for extraction; for those types which contained anthocyanins the alcohol was acidified with hydrochloric acid (to 0.3%). As a rule three millilitres of liquid were used for one gram of seedcoat material. The seedcoats were macerated under the extraction solvent in a Bühler homogeniser, and the mixture kept at 2–4° for one to three days, with occasional shaking. The liquid was then filtered through a Büchner funnel, and a fresh quantity of extraction solvent was added to the seedcoat debris. The extraction was repeated one to three times until the solvent no longer attained a distinct colour. The successive extracts were then combined.

3. Chromatographic separation

3.1. *General remarks*

Due to the colour of the compounds themselves, spots of anthocyanins can be observed in visible light. Flavonol glycosides have a pale yellow colour, but are readily revealed in U.V. light by their distinct brown or yellow fluorescence. Leuco-anthocyanidins as such have no colour at all. They can be made visible on a chromatogram, however, by spraying with an alcoholic solution of vanillin and hydrochloric acid.

In attempts to chromatograph the mixture of leuco-anthocyanidins with a mixture of butanol, acetic acid and water and with other solvents, the larger part remained in the spot applied on the starting line which became red-brown, while the rest moved as a trail from this spot. This trail, which also had a reddish colour, became larger and more intensely coloured by spraying with vanillin and hydrochloric acid, but no separate spots became visible by this treatment. Presumably the leuco-compounds are converted on the paper to high molecular

Table 3. Solvent mixtures used for chromatographic purposes.

Composition		Mobile phase	Abbreviation
Components	Ratio of vols.		
acetic acid/2*N* aqueous hydrochloric acid	7:3	the homogenous liquid	AH
acetic acid/aqueous hydrochloric acid 37%/water	30:3:10	ibid.	AHW
acetic acid/water	2:98	ibid.	AW 2
ibid.	15:85	ibid.	AW 15
n-butanol/acetic acid/water	6:1:2	ibid.	BAW 612
ibid.	4:1:2	ibid.	BAW 412
n-butanol/2*N* aqueous hydrochloric acid	1:1	top layer	BH 11
ibid.	5:2	ibid.	BH 52
n-butanol/ethanol/1% aqueous ammonia	4:1:5	ibid.	BEN
2% aqueous boric acid			boric acid
m-cresol/acetic acid/water	25:1:24	bottom layer	CAW

substances. The leuco-anthocyanidins were therefore converted to anthocyanidins by heating them with dilute hydrochloric acid. The latter compounds could be separated and identified chromatographically. This method has been widely used for the investigation of leuco-anthocyanins and leuco-anthocyanidins by other workers. When anthocyanins were also present in the extract this method could not be used as such, as these compounds also yielded anthocyanidins on treatment with acid. In the present investigation it was only determined whether leuco-compounds were present or absent in extracts containing anthocyanins, and when these compounds were present, their total amount was estimated (see below, § 5).

The composition of the liquid mixtures, which were used for chromatographic purposes, are given in Table 3. When the components formed no homogeneous liquid, it is also indicated, which layer was used as the mobile phase.

3.2. *Separation of the anthocyanins*

In order to obtain solutions of the individual anthocyanins which were sufficiently pure for identification purposes it was necessary to carry out chromatography in two different solvent systems. BAW 612 was used as the first solvent; the anthocyanin bands sometimes were incompletely separated from each other after the first separation, and moreover sugars were present, originating from the seedcoat and having in BAW about the same Rf-values as the anthocyanins. Boric acid was used in the second separation; monoglycosides have relatively low (0.05–0.20), and dimonoglycosides higher Rf-values (0.25–0.50) in this solvent, while sugars travel very fast ($Rf > 0.80$).

The extract was applied in portions of one ml from a pipette, as a streak, 8 cm from the short side of sheets of thick filter paper Whatman No. 3 or 3 MM (46 × 57 cm). The sheets had previously been washed with 6% aqueous acetic acid to remove impurities which might be present and dried.

The extract had to be concentrated so that the desired quantity of pigment could be applied to the paper without having to streak more than 4 to 8 times. About 80% of the solvent was therefore distilled off *in vacuo* at 25–30°.

After application of the streaks the sheets were placed (maximally eight at a time) in a double-walled chromatography tank and run overnight with BAW 612 by the descending technique. When the solvent front had nearly reached the end of the sheets they were removed, placed in a fume cupboard and dried in a current of air.

The visible anthocyanin bands, differing in Rf-value and often in colour (orange to blue violet), were cut out and eluted with BAW 412 [1]). Each of the solutions obtained was concentrated and at the same time freed from butanol by shaking with an amount of benzene, tenfold the volume of the eluate. The mixture separated into two phases, the smaller aqueous phase containing the pigment. This aqueous phase was collected by centrifuging, the organic layer being subsequently removed in a separating funnel or, in the case of small volumes, by means of a pipette. A few drops of 1% methanolic hydrochloric acid were added to the aqueous solution, since this was found to give better spots when the pigments were rechromatographed. As mentioned previously the pigments were rechromatographed with boric acid as the solvent. Upon elution of the separated pigments with BAW no arabinose was extracted from

[1]) BAW was used for elution after VAN BRAGT (8).

the paper.[1]) After the second separation the anthocyanins were sufficiently pure for their identification.

3.3. *Separation of the flavonol glycosides*

Flavonol glycosides often were present along with the anthocyanins and were separated together with the latter when the extract was chromatographed for the first time. Extracts containing only flavonol glycosides were treated in the same way. The bands were marked under U.V. light [2]), where they gave a brown or faint yellow fluorescence. The sheets were sometimes fumed with ammonia vapour which intensified the fluorescence for better visibility of the bands. The bands were eluted with 70% aqueous ethanol, according to NORDSTRÖM and SWAIN (38), and the eluates evaporated to dryness in air at room temperature.

The flavonol glycosides also had to be chromatographed a second time with a different solvent, in order to obtain them sufficiently pure. They were dissolved in a small amount of 96% ethanol, applied to the paper and run with AW 2 or AW 15. Sometimes the compounds were chromatographed a third time with water as the solvent. The reason for this treatment is given in § 4.1.4.

3.4. *Treatment of the leuco-anthocyanidins*

When an extract was intended for the examination of leuco-anthocyanidins, the compounds were concentrated by the addition of an equal volume of saturated aqueous lead acetate. The lead complexes formed separated out quantitatively as a jelly-like precipitate. The supernatant was poured off, after centrifugation, and the residue was stirred with a small amount of concentrated hydrochloric acid, whereupon a solution of the leuco-anthocyanidins was obtained and separated from the precipitate of lead chloride formed.

By the application of this method the following was achieved: 1° reducing of the volume by evaporation during which the rather unstable leuco-anthocyanidins could undergo changes, was avoided; 2° an aqueous solution was obtained, which was necessary so that extraction with *iso*amyl alcohol could be carried out after conversion to anthocyanidins; 3° sugars which give no precipitate with lead acetate were removed. The solution of leuco-anthocyanidins therefore could be freed from sugars, also originating from the seedcoat, by repeating the precipitation and the redissolving a few times. The removal of these sugars was necessary for the examination of the presence of sugar residues in the leuco-compounds.

The leuco-anthocyanidins were converted into anthocyanidins by adding a tenfold excess of 2*N* aqueous hydrochloric acid to the concentrated solution, and heating it on a boiling water-bath for twenty minutes. In the reaction only a rather small part of the amount of leuco-anthocyanidins is converted into anthocyanidins, the remaining part being converted into red coloured high molecular compounds which are partly insoluble (3).

The precipitate was removed after centrifugation, and the mixture of anthocyanidins extracted from the supernatant with *iso*amyl alcohol. The alcoholic

[1]) For the separation of anthocyanins from sugars, NORDSTRÖM and SWAIN (38) recommended chromatography in *iso*propanol/2*N* aqueous hydrochloric acid, 1 : 1; HARBORNE and SHERRATT (23), stated however, that hydrochloric acid produced arabinose as an artefact from the paper. This observation was confirmed in my experiments.

[2]) The ultra-violet source employed in this work was a Philips HPW 125 W lamp, giving some emission in the visible.

solution was applied to sheets of filter paper and chromatographed as described for anthocyanins, with AHW as the solvent. The bands were eluted with BH 52; the eluates were sufficiently concentrated and pure for further examination.

4. Identification

4.1. *General aspects*

4.1.1. Determination of aglycone and sugars of a glycoside

In order to establish the composition and structure of a glycoside, first an amount of the compound was hydrolysed for the determination of the nature of aglycone and sugars. Secondly the number and position of the sugar residues in the original glycoside molecule were investigated.[1])

The aglycone and sugars obtained on qualitative hydrolysis were determined chromatographically where possible.[2]) Spectra, either of the aglycone or of the glycoside, were measured for confirmation of the structure which had been deduced for the aglycone.

4.1.2. Spectra

The spectrum of an anthocyanidin shows one maximum in the visible region, the wavelength of which is dependent on the number of hydroxyl and methoxyl groups in the molecule. In the normally occurring anthocyanidins the hydrogen atoms at the 3, 5, 7 and 4′ positions are substituted by hydroxyl groups, additional substitution by hydroxyl or methoxyl groups being restricted to the 3′ and 5′ positions of the B-ring. In every natural anthocyanin so far detected the 3-hydroxyl group is substituted by a sugar residue which causes a hypsochromic shift of the wavelength of maximum absorption. Substitution of the 5-hydroxyl group by another sugar residue, which sometimes takes place, does not change this λ_{max} (22). Determination of the wavelength of maximum absorption of an anthocyanin therefore gives information about the structure of the aglycone.

The position of the maximum is only dependent on the number and not on the nature of the substituents in the B-ring, when the spectrum is measured in the absence of metal ions. When isolated by the technique which was used in this investigation the λ_{max} of the compound without substituents in the 3′ and 5′ positions (e.g. pelargonidin glucosides) was $\sim$ 510 mμ. Introduction of a hydroxyl- or methoxyl group in the 3′ position gives a shift to $\sim$ 525 mμ, and substitution of another hydrogen atom, in the 5′ position, gives λ_{max} of $\sim$ 545 mμ. When two hydroxyl groups in orthoposition are present addition of aluminium ions causes a bathochromic shift of 25–40 mμ (18, 49). By this effect the nature of the substituents present in the 3′ and 5′ positions can be established to a certain extent.

Flavonols only absorb in the U.V. region, the spectrum showing two maxima, one between 250 and 280 and the other between 350 and 390 mμ. The wavelengths of maximum absorption are dependent on the number of hydroxyl groups in the molecule, an increasing number causing a shift to longer wavelengths. There is a wider variation in the number and position of the substituents in the group of the flavonols than in that of the anthocyanidins. However, the

[1]) In the literature the occurrence of complex anthocyanins is described, i.e. anthocyanins, esterified by one or more molecules of an organic acid. Such compounds have not been encountered in the material which I have been investigating.

[2]) All the chromatographic identifications were based on comparisons with known specimens on the same chromatograms, since Rf-values tend to vary with changes in the conditions such as temperature, agency of solvent mixtures, etc.

flavonols, which have been detected in the bean seedcoats hitherto (kaempferol, quercetin and myricetin) show a hydroxylation pattern, analogous to that of anthocyanidins, and differ from each other only in the number of hydroxyl groups in the B-ring.

Substitution of a hydroxyl group by a sugar residue results in a hypsochromic shift of the maximum at the longer wavelength. This shift is $\sim$ 15 mμ when substitution occurs at the hydroxyl group in the 3-position, but only $\sim$ 5 mμ, when it occurs at any other hydroxyl group (57). In flavonol glycosides there are more possibilities regarding the position of the sugars than in anthocyanins, and therefore the spectrum of the aglycone must be measured when data concerning its structure are desired. Because of the different effects that substitution of the 3-hydroxyl group and substitution of one of the other hydroxyl groups have on the spectra of flavonols, some information can be derived about the position of the sugar residues in the glycoside from its spectrum in alcohol. More information can be obtained when the spectrum is also measured after the addition of aluminium chloride; a kaempferol-3-glycoside then gives two long wavelength peaks, at 355 and 400 mμ (SWAIN, pers. comm.; 43), while quercetin-3-glycosides and myricetin-3-glycosides show only one maximum, with $\lambda_{max} > 400$ mμ (44).

4.1.3. Determination of the pattern of glycosylation

The determination of the number and position of the sugar residues in the anthocyanins was carried out by chromatographic comparison of the pigments with known specimens.[1]) The flavonol glycosides were converted into anthocyanins, the structure of which were subsequently determined chromatographically. This conversion, which is a reduction, only affects the aglycone moiety, the position of the sugars remaining unchanged. The reaction was carried out electrolytically on a micro scale by a method developed from the procedure which was applied by MASQUELIER and BLANQUET (36) for the reduction of a flavanone.

Spectra of the flavonol glycosides were measured for confirmation of the structure, while in some cases also methylation of the glycoside, followed by hydrolysis, was employed to obtain data about the position of the sugar residues. When a glycoside is methylated all the free hydroxyl groups in the aglycone are converted into methoxyl groups. If the compound is subsequently hydrolysed with dilute hydrochloric acid, the sugars are removed yielding free hydroxyl groups in the positions to which they were attached. The structure of the compound obtained thus gives an indication as to the positions of the sugars in the original glycoside molecule.

4.1.4. Anomalous behaviour of flavonol glycosides

The identification of flavonol glycosides met with difficulties because some-

[1]) Compounds of known structure were either obtained by extraction from flowers of certain plant species, or supplied by other investigators, or obtained from commercial sources.

I am indebted to Dr. J. B. HARBORNE, John Innes Horticultural Institution, Hertford, England, for gifts of malvidin- and petunidin-3-monoglucoside and of delphinidin-3,5-diglucoside, and to Dr. T. SWAIN, Low Temperature Research Station, Cambridge, England, for gifts of pelargonidin- and cyanidin-3-monoglucoside, and of myricetin.

I isolated the 3,5-diglucosides of pelargonidin and cyanidin from the flowers of scarlet *Pelargonium zonale* and red *Rosa sp.* respectively.

Pelargonidin and cyanidin were obtained by hydrolysis of the dimonosides, petunidin and malvidin from the monosides, and delphinidin by hydrolysis of an extract of blue *Viola sp.* flowers.

Quercetin was purchased from LIGHT and Co, Ltd.

times after chromatography in certain solvents two spots were formed which appeared to be due to only one compound. This phenomenon was observed with the 3-monoglucosides of kaempferol, quercetin and myricetin and with another myricetin glucoside, the structure of which could not be fully established.

The phenomenon was first encountered and more closely investigated with kaempferol-3-monoglucoside. This compound was isolated by chromatographic separation with BAW 612 and AW 2 as solvents, and its Rf-values were then determined. When BAW 612, AW 2, AW 15, or 0.1% aqueous hydrochloric acid were used as solvents only one spot occurred. However, on chromatograms run in water, boric acid or CAW, two spots, one strong and the other faint, were found. The faint spot had a higher Rf-value in water and boric acid and a lower Rf-value in CAW then the strong spot. The Rf-values of the strong spot in boric acid and in water showed but little difference from those of the sole spot which was formed in AW 2 and 0.1% aqueous hydrochloric acid.

In order to investigate this anomaly more extensively, a larger amount of kaempferol-3-monoglucoside was chromatographed in water and the two bands eluted in the usual way. The Rf-values of the eluated compounds were then determined in various solvents. On chromatograms developed with water, boric acid and CAW, the component which was present in a larger amount showed Rf-values identical with those of the above mentioned strong spot, while the other component showed Rf-values identical with those of the faint spot. In BAW 612 and AW 2 both components had equal Rf-values. In water, boric acid and CAW the component occurring in the smallest amount thus had a more hydrophilic character. These solvents are distinguished from the others used by the fact that they are less acid or even neutral, and therefore it is possible that a relationship exists between the behaviour of the compound and the acidity of the solvent.

On hydrolysis, both compounds yielded the same aglucone and the same sugar. They were not converted into each other, either spontaneously or during chromatography. However, the "hydrophilic" component was completely converted into the "non-hydrophilic" one by incubation with a 0.25 *N* methanolic sodium hydroxide solution for a few seconds at room temperature, followed by neutralization of the solution with methanolic hydrochloric acid. The "non-hydrophilic" substance was recovered unchanged after this treatment.

The other flavonol glucosides mentioned above also showed segregation into "hydrophilic" and "non-hydrophilic" components, the latter always being present in the larger amount. The "hydrophilic" component of each of these pigments was similarly converted into the "non-hydrophilic" one by treatment with base.

Analogous formation of double spots has been observed by other investigators with a number of different compounds including amino alcohols, diamines and diamino acids (64), sugars (5) and sodium salts of orthophosphoric acid (10). The term "multi spots" has been used. A conclusive explanation of the cause of the phenomenon in these cases has not been given by any of the investigators, although it is probable that formation of mono- and di-acid salts caused the two spots of the amines, while in the case of the phosphoric acid salts the occurrence of different ionic forms which did not equilibrate on the paper may have played a rôle.

In the present investigation the phenomenon was not further investigated. In Chapter V, where the results of the chemical analysis are given, a substance which showed this abnormal behaviour will be indicated by "showing

multi-spots", while the Rf-values of the "non-hydrophilic" component only will be given. The determinations to establish the position of the sugars in the compounds were always carried out on the latter component. Rf-values of both components of the flavonol glucosides in the solvents in which these values were normally determined for the identification are given in Table 4.

TABLE 4. Rf-values of "multi-spots", observed in the chromatography of a number of flavonol-glucosides in various solvents.

Compound	Component	Rf-value in		
		BAW 612	CAW	boric acid
kaempferol-3-monoglucoside	non-hydrophilic	0.61	0.64	0.14
	hydrophilic	0.61	0.38	0.50
quercetin-3-monoglucoside	non-hydrophilic	0.47	0.38	0.19
	hydrophilic	0.47	0.18	0.57
myricetin-3-monoglucoside	non-hydrophilic	0.30	0.16	0.13
	hydrophilic	0.30	0.05	0.46
myricetin-glucoside	non-hydrophilic	0.15	0.17	0.19
	hydrophilic	0.15	0.07	0.54

4.1.5. Examination of the leuco-anthocyanidins

The nature of the anthocyanidins obtained by heating the leuco-anthocyanidins with hydrochloric acid were determined chromatographically and their spectra were measured for confirmation of the presumed structure.

As mentioned earlier the leuco-compounds could not be separated as such and therefore the individual compounds could not be analysed for the possible presence of glycosidic sugars. However, any sugars actually present in the leuco-compounds would be freed when the mixture of leuco-compounds was treated with hydrochloric acid to form the sugarfree anthocyanidins. A solution of the leuco-compounds, from which free sugars originating from the seedcoat had been removed, was therefore heated with hydrochloric acid and then analysed for sugars. When sugars were found, it was not of course certain that they originated from the leuco-compounds, since other substances including glycosides form insoluble lead complexes when treated with lead acetate solution.

As was mentioned previously, the leuco-anthocyanidins could not be easily analysed qualitatively when anthocyanins were also present in an extract. In this case the presence of leuco-anthocyanidins was determined by comparing the optical densities at the wavelength of maximum absorption of an extract before and after hydrolysis. At the pH chosen, the absorption of anthocyanins and of anthocyanidins which originate from these compounds are almost equal. When leuco-anthocyanidins are present, however, the optical density is higher after the hydrolysis because in this case anthocyanidins, originating from the leuco-compounds, contribute to the absorption.

4.1.6. Restricted identification

When in a seedcoat colour type a compound (anthocyanin, flavonol glycoside or leuco-anthocyanidin) was encountered which had not previously been found in another colour type, and a sufficient amount of it was available, the appropriate determinations for the identification were all carried out. When a com-

pound was isolated showing Rf-values identical with those of a substance which had previously been isolated from another phenotype, and the results of the chromatographic determination of the aglycone and the sugars were in agreement, identification was based on these data only. The identification of an anthocyanidin obtained from a leuco-anthocyanidin in such a case was solely based on its Rf-values.

4.2. *Methods*

4.2.1. Identification of anthocyanins

An amount of the compound was hydrolysed by adding an equal volume of 2*N* aqueous sulphuric acid to a part of the solution and heating the mixture on a boiling water-bath for one hour.

The aglycone was extracted from the mixture with *iso*amyl alcohol and chromatographed with AHW and BH 11 as solvents.

Before the identity of the sugars present in the aqueous layer could be determined the acid had to be removed, because it affected the Rf-values of the sugars, and moreover it yielded arabinose by interaction with the paper, even when present in very small amounts. Methyl-di-*n*-octylamine was used for this purpose according to the method of HARBORNE and SHERRATT (23). About 2 ml of the hydrolysate, from which the anthocyanidins had been removed, was extracted with three successive amounts of 4 ml of a solution of the amine in chloroform (10% w/v). The aqueous solution was then evaporated to dryness *in vacuo* over potassium hydroxide. The residue was dissolved in two drops of water in a small test tube and again shaken with 0.5 ml of amine solution to be sure that no trace of acid was left. The sugars in the top layer were chromatographed in BAW 612, CAW, and BEN. Since in the latter solvent the Rf-values were influenced by the treatment, the sugars used for comparison were also treated with the amine solution.

Aniline phosphate in water-saturated *n*-butanol was used as a spray to reveal the position of the sugars on the chromatograms.

The determination of the wavelength of maximum absorption of the anthocyanin was carried out in a spot on a paper chromatogram using a method which was first applied by BRADFIELD and FLOOD (7). A small strip containing the spot was cut from a chromatogram which had been developed in BAW 612. It was placed in a double folded metal strip, which could be slipped in the cuvette holder of the spectrophotometer[1]). An opening in the metal strip allowed the light to go through the paper. A blank strip which had been cut from the same chromatogram from a position alongside the spot to be measured was used as a reference.

It was desirable to work under standardised conditions, because the position of the λ_{max} is influenced by pH. The paper strips were therefore kept overnight in an desiccator over concentrated acetic acid, and then for a few hours *in vacuo* at room temperature, before the spectrum was measured. After the spectrum had been determined, the strips were sprayed with 0.1 *M* aqueous aluminium sulphate solution, dried and the spectrum then remeasured.

The chromatographic comparison of an anthocyanin with known specimens was carried out with BAW 612, CAW, and occasionally AH, as solvents.

4.2.2. Identification of flavonol glycosides

A portion of the compound was hydrolysed by dissolving it in 2% aqueous sulphuric acid, and heating the solution for half an hour on a boiling water-bath.

The aglycone was extracted from the hydrolysate with *iso*amyl alcohol and the Rf-values determined in BAW 612, CAW and AHW. The sugars were examined as described previously for the analysis of anthocyanins.

In order to determine the spectrum of the aglycone, an amount of the glycoside was hydrolysed as before and the aglycone extracted from the hydrolysate with ethyl acetate; the extract was washed twice with water and then evaporated to dryness. The residue was dissolved in absolute ethanol and the spectrum was measured in this solution.

For the reduction of a flavonol glycoside to an anthocyanin the following method was developed. A glass tube, inside diameter 5 mm, length 50 mm, was closed at one end with a rubber stopper and filled with mercury up to 7 mm from the top. A thin steel wire, connected with the negative pole of a rectifier (6 V, 0.5 A), went through the stopper into the mercury. From a micro-burette 0.01 ml of 2*N* aqueous sulphuric acid was added on top of the mercury followed by 0.05 ml of an alcoholic solution of the flavonol glycoside, and the two liquids mixed by shaking. A platinum wire was arranged so that it just dipped into the solution and

[1]) A Unicam S.P. 500 spectrophotometer was used.

was connected with the positive pole of the rectifier. The current was passed for 5 minutes, and according to the flavonol glycoside that was treated the solution developed an orange to violet colour. This colour changed into dirty gray when the treatment was continued for too long a period indicating that the anthocyanins, which were first formed, decomposed. By means of a small pipette the solution was transferred to a small test tube provided with a glass stopper. After adding 0.007 ml of 2*N* aqueous barium chloride solution the tube was shaken and then centrifuged. The compounds in the supernatant were then examined chromatographically.

Sometimes, together with the main anthocyanin spot and a spot of unchanged flavonol glycoside, a faint spot developed in the form of a trail with a lower Rf-value than the anthocyanin. As this trail could never be related to any known anthocyanin, whereas the main spot always could, this phenomenon was not further investigated and only the main spot taken into account for identification purposes.

For the determination of the spectrum an amount of the glycoside was dissolved in absolute ethanol.[1]) After the spectrum had been measured, 0.6% ethanolic aluminium chloride solution was added, 0.2 ml for every ml of pigment solution, the final aluminium chloride concentration being 0.1%. The blank was treated in the same way and the spectrum measured again.

The methylation of a glycoside and the subsequent hydrolysis of the methylated compound was carried out as follows: 40 mg of the glycoside was dissolved in 20 ml of dry acetone, and 4 g of dry potassium carbonate and 2 ml of freshly destilled dimethyl sulphate added. The mixture was refluxed for four hours and the potassium carbonate filtered off and washed with dry acetone. The two solutions were combined and the acetone removed *in vacuo*. The oily residue remaining was refluxed for one and a half hours with 5 ml of 2*N* aqueous hydrochloric acid. The resulting light yellow precipitate was separated from the acid by filtration, washed with water, and dried *in vacuo* over phosphorus pentoxide. Its melting point was determined after recrystallization from aqueous ethanol.

4.2.3. Identification of leuco-anthocyanidins

Solvents AH and BH 11 were used for the chromatographic identification of the anthocyanidins obtained from the leuco-anthocyanidins as described above.

The spectrum of the anthocyanidin was measured in a solution obtained by eluting a band from a paper chromatogram with 0.36 *N* methanolic hydrochloric acid.

In order to examine whether sugars were present in the leuco-compounds, the mixture of these substances was purified three times by precipitation with lead acetate and then heated with dilute mineral acid; after extraction of the anthocyanidins from the mixture with *iso*amyl alcohol, the resulting aqueous solution was examined for the presence of sugars, as previously described.

In order to establish the presence or absence of leuco-anthocyanidins in an extract containing anthocyanins, a portion of this extract was diluted tenfold with 2*N* aqueous hydrochloric acid. A part of the solution was heated in a test tube on a boiling water-bath for 20 minutes. The reaction was carried out in darkness to prevent decolourization of the anthocyanidins formed. After cooling, the heated solution was readjusted to its original volume by addition of water. The optical densities of the heated and the non-heated solutions were then measured.

5. Estimations of pigment contents

The present investigation was primarily started in order to obtain qualitative data about the effects of the gene actions.

When the pigment mixtures obtained from the various seedcoat colour types were analysed chromatographically, however, some ideas were obtained as to the relative amounts of compounds which were present. Thus, while analysing types containing anthocyanins it appeared that differences in seedcoat colour between *sh*-types and the corresponding *Sh*-types were caused by differences in total anthocyanin content rather than by differences in the composition of the anthocyanin mixtures. In order to obtain data about the magnitude of these differences the optical densities at the wavelength of maximum absorption of

[1]) The eluate of a strip with the same Rf-value as the pigment, cut from a blank sheet which had been irrigated with the same solvent used for separation, was evaporated to dryness. The residue was dissolved in absolute ethanol and yielded the reference in the above determination.

extracts of the types in question were measured. In order to investigate whether the gene *Sh* also influenced flavonol glycoside production, the optical densities of extracts of two yellow types were measured.

It was essential to be informed as to whether the leuco-anthocyanidin content was influenced by the simultaneous formation of anthocyanins and flavonol glycosides. The amounts of leuco-compounds, present in a number of *Sh*-types therefore were compared. As the compositions of the mixture of leuco-anthocyanidins in some of these types were not known, it was necessary to employ a method in which the structure of the B-ring did not influence the results. Use was made of the fact that leuco-anthocyanidins, owing to the structure of the A-ring and of the heterocyclic part of the molecules, give a red compound with vanillin and hydrochloric acid, which has a spectrum showing a well defined peak at 500 mμ. Extracts treated with an excess of ethanolic vanillin solution and concentrated aqueous hydrochloric acid were compared by measuring the optical density at 500 mμ of the derivatives formed.

Proof that optical density was directly proportional to leuco-anthocyanidin concentration in our experiments was obtained as follows. An extract was made from seedcoats containing only leuco-anthocyanidins, by extracting 0.5 mg of seedcoat with 15 ml of methanol. 0.04 milliliter of this extract was diluted with methanol to a volume of 1 ml. To this solution were added 1 ml of a solution of 3 g of vanillin in 8 ml of ethanol, and 1 ml of 25% aqueous hydrochloric acid and the optical density was measured. The same determination was carried out with solutions, prepared by diluting 0.07 ml and 0.10 ml of the extract to 1 ml respectively. The values of optical density plotted against the amount of extract give a straight line which goes through the origin (fig. 3).

Because it was necessary to measure the amounts of leuco-anthocyanidins by this method in the presence of flavonol glycosides and anthocyanins, their influence on the optical density in the above determination was examined. Fla-

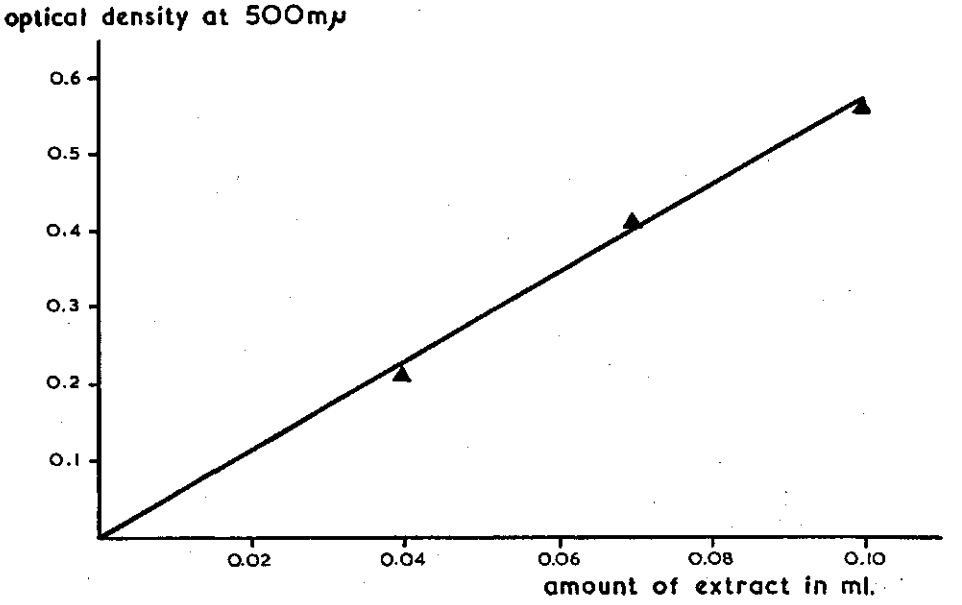


FIG. 3. Relation between leuco-anthocyanidin concentration and optical density at 500 mμ, after treatment of the solution with vanillin solution and concentrated hydrochloric acid.

vonol glycosides, as expected, were found to have no effect as they neither absorb at 500 mμ nor change the absorption due to leuco-anthocyanidins. Anthocyanins have an appreciable absorption at 500 mμ, but the magnitude of this was found not to be changed by the addition of vanillin. The absorption due to leuco-anthocyanidins was also not changed by the presence of anthocyanins. The leuco-anthocyanidin content therefore could be evaluated in the presence of anthocyanins by calculating the difference between the optical density of an amount of extract to which ethanolic vanillin solution had been added and the optical density of an amount of extract to which only ethanol had been added.

Evidence for the validity of the above method was obtained as follows. The optical densities of two acid solutions of equal anthocyanin concentration, one of which also contained vanillin, were measured and appeared to be identical. Furthermore the optical density of a solution, obtained by mixing an amount of extract containing anthocyanins with an amount of extract containing only leuco-anthocyanidins, after addition of vanillin, proved to be practically identical with the sum of the optical densities, measured separately for the same amount of anthocyanins and leuco-anthocyanidins (Table 5).

TABLE 5. Effect of the presence of anthocyanins when measuring the optical density of an extract after treatment with vanillin and hydrochloric acid.

Extract containing	Optical density at 500 mμ	
1) anthocyanins only	0.106	sum 0.575
2) leuco-anthocyanidins only	0.469	
3) mixture of 1) and 2)		0.550

CHAPTER V

RESULTS OF THE CHEMICAL ANALYSIS

1. PRELIMINARY REMARKS

As was mentioned previously, fifteen compounds isolated from the bean seedcoats have been identified. Their structures are given in Fig. 4, I–XV. Three other substances (XVI–XVIII) which were met with and whose structure could only partly be established are not included in this figure.

The pigments isolated from each genotype are indicated in the tables of results by the number of the type, followed by either 'a' (anthocyanins), 'f' (flavonol glycosides), or 'l-ad' (leuco-anthocyanidins), and by a figure. This figure indicates the order in which the compounds separated on the first chromatograms. The lowest number refers to the compound with the highest Rf-value.

In the tables giving the results a compound which occurred as the main component is indicated by +++ or ××× ; ++ and ×× stand for secondary components still present in reasonable amounts; and + and × for compounds which were present in small amounts. The comparison was based on an estimation of the intensity of the bands in the first chromatographic separation, and therefore only holds within a group of compounds of the same kind. Different marks are therefore used for anthocyanins, flavonol glycosides and leuco-anthocyanidins, when these compounds occur together.

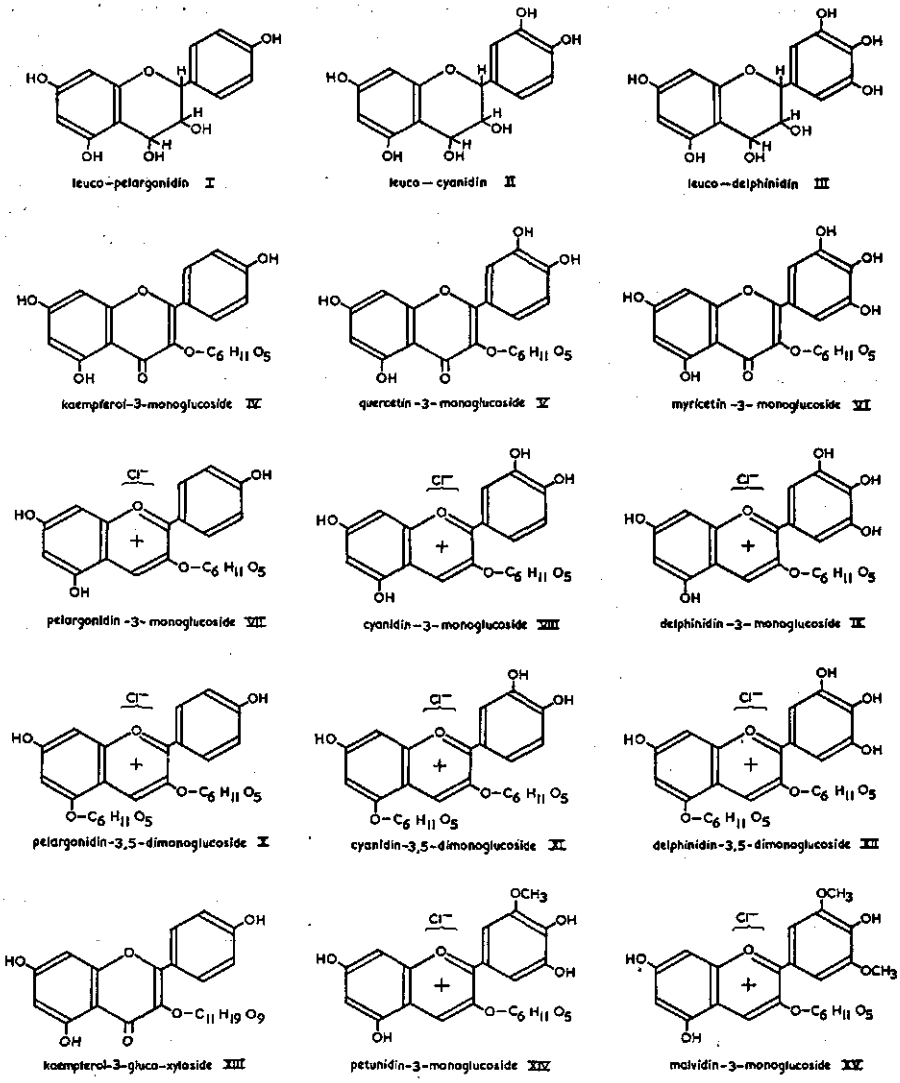


FIG. 4. Structures of flavonoid compounds, isolated from various seedcoat colour types of *Phaseolus vulgaris*. The flavan-3,4-diol structure for the leuco-anthocyanidins has been given after KING *et al.* (28), BAUER *et al.* (4), and SWAIN (60). Work by these investigators has shown this structure to be more probable for leuco-anthocyanidins than the flavan-2,3,4-triol structure, which was originally also presumed for these compounds.

2. RESULTS OF THE QUALITATIVE ANALYSIS

2.1. *Type* sh c^u v^{lae}, *white (F 127)*

No anthocyanins, flavonol glycosides or leuco-anthocyanidins could be isolated from this type.

2.2. *Type* Sh c^u v^{lae}, *creamish (F 111)*

Two leuco-anthocyanidins were found to be present in this type, and were

identified as shown in Table 6. Both anthocyanins and flavonol glycosides were found to be absent from this type.

TABLE 6. Identity of the leuco-anthocyanidins in type *Sh* c^u v^{lae}.

Compound	Identified as	Structure	Relative amount present
F 111 l-ad_1	leuco-pelargonidin	I	+
F 111 l-ad_2	leuco-cyanidin	II	+ + +

The above findings were based on the identification of two anthocyanidins which were isolated from the red solution obtained by boiling the initially yellow extract with hydrochloric acid, and the fact that no sugars were found in this solution. The epidermis contained the larger part of the leuco-compounds, while a lesser amount was present in the underlying parenchymous layer. The data on which the identification of the anthocyanidins were based are given in Table 7.

TABLE 7. Identification of the anthocyanidins, obtained from leuco-anthocyanidins in type *Sh* c^u v^{lae}.

Compound	Rf-value in		λ_{max}*)
	AHW	BH 11	
F 111 ad_1	0.66	0.77	524 mμ
pelargonidin	0.66	0.77	524 mμ
F 111 ad_2	0.48	0.67	539 mμ
cyanidin	0.48	0.67	539 mμ

*) Solvent was 0.36*N* methanolic hydrochloric acid.

2.3. *Type* sh C v^{lae}, *lemon yellow* (*F 63*)

From this type two flavonol glycosides were isolated, which were identified as shown in Table 8. No anthocyanins or leuco-anthocyanidins were found.

TABLE 8. Identity of the flavonol glycosides in type *sh C* v^{lae}.

Compound	Identified as	Structure	Relative amount present
F 63 f_1	kaempferol-3-monoglucoside	IV	+ + +
F 63 f_2	kaempferol-3-gluco-xyloside	XIII	+ +

The pigments from this type were isolated in the usual way, and also by a procedure described by GAGE *et al.* (13), and ICE and WENDER (24). In this method the pigment mixture was first purified and concentrated on a column of the ion-exchange resin Amberlite IRC 50-H; the components were then separated on columns of Magnesol, a synthetic magnesium silicate. As a result of this larger amounts of material were available for study, especially of the main compound.

The identification of the compound F 63 f_1 [1]) was based on the following data. M.p. 175–178.5°, after recrystallization from 5% aqueous acetic acid. On further heating the melt solidified and melted finally at 215–218°. [2]) In the literature the m.p. of kaempferol-3-monoglucoside is given as 173–175° and 178° (14, 37).

[1]) Showing multi-spots.

[2]) In a preliminary note (12) only this last m.p. was mentioned.

Analysis[1]): Found C: 54.0%; H: 5.1%. Calculated for kaempferol monoglucoside monohydrate ($C_{21}H_{20}O_{11} . H_2O$) C: 54.08%; H: 4.75%. After drying at 100°C and 0.05 mm Hg: Found C: 56.0%; H: 4.6%. Calculated for kaempferol monoglucoside C: 56.25%; H: 4.50%.

Hydrolysis of 20 mg of the glucoside yielded 12.3 mg of an aglucone m.p. 273–275°, after recrystallization from 50% aqueous ethanol. (m.p. kaempferol 276–277° (39)). Theoretical yield of kaempferol monohydrate after hydrolysis 13.0 mg. The determination of the absorption spectrum of the aglucone in 96% ethanol yielded the data, given in Table 9.

TABLE 9. Absorption maxima of the aglucone of F 63 f_1, compared with those of kaempferol.

Compound	Maxima			
	1		2	
	λ_{max}	E	λ_{max}	E
aglucone from F 63 f_1	268 mμ	4.2	368 mμ	4.3
kaempferol, according to (57)	267,5 mμ	4.1	370 mμ	4.3

The sugar proved to be glucose by paper chromatography. It was concluded from the following determinations that the glucose residue was linked in the 3-position. Reduction of the glucoside yielded an anthocyanin which had identical Rf-values to that of pelargonidin-3-monoglucoside on chromatograms developed in BAW and CAW. The kaempferol glucoside showed maximal absorption at the wavelengths given in Table 10.

TABLE 10. Absorption maxima of kaempferol-3-monoglucoside.

Compound	λ_{max} in	
	ethanol	0.1% ethanolic aluminium chloride
F 63 f_1	268, 354 mμ	276, 348, 400 mμ
kaempferol-3-monoglucoside according to (43)	266, 353 mμ	274, 354, 400 mμ

Methylation of 40 mg of the glucoside, followed by hydrolysation yielded 17 mg of a compound m.p. 147–148°, after recrystallization from aqueous ethanol. Kaempferol-4',5,7-trimethylether, the compound expected, has m.p. 149–150° (21). The spectrum of the methyl ether obtained was in agreement with that recorded by SKARZYNSKI for the above kaempferol-trimethylether (Table 11).

TABLE 11. Absorption maxima of the reaction product from F 63 f_1, kaempferol-4',5,7-trimethylether, when measured in ethanol.

Compound	λ_{max}
reaction product of F 63 f_1	259, 355 mμ
kaempferol-4',5,7-trimethylether, (57)	256, 355 mμ

[1]) The micro-analyses were carried out by Mr. W. P. COMBÉ in the Laboratory of Organic Chemistry of the Agricultural University.

The second glycoside, F 63 f_2, was isolated in a smaller amount. On hydrolysis it gave kaempferol, glucose and xylose. The identification of the aglycone was based on identity of its Rf-values and spectrum with that of the aglucone of F 63 f_1. Partial hydrolysis of the glycoside, in 1% aqueous hydrochloric acid for $2\frac{1}{2}$ min. at 100°, yielded a compound having the same Rf-values as the kaempferol-3-monoglucoside. The spectrum of the compound F 63 f_2 in ethanol and ethanolic aluminium chloride solution was indistinguishable from that of the kaempferol monoglucoside; methylation and subsequent hydrolysis yielded a compound, which was shown by mixed melting point to be identical with the kaempferol-4',5,7-trimethylether obtained previously.

From the above data the substance F 63 f_2 was identified as kaempferol-3-gluco-xyloside, the sequence within the molecule being kaempferol–glucose–xylose.

2.4. *Type* sh C^r v^{lae}, *pale pink (F 191)*

From this type four anthocyanins and two flavonol glycosides were isolated. They were identified as given in Table 12, the identification being based on the data given in Tables 13 and 14. Leuco-anthocyanidins were absent from this colour type.

TABLE 12. Identity of anthocyanins and flavonol glycosides from type *sh* C^r v^{lae}.

Compound	Identified as	Structure	Relative amount present
F 191 a_1	pelargonidin-3-monoglucoside	VII	+ + +
F 191 a_2	cyanidin-3-monoglucoside	VIII	+
F 191 a_3	pelargonidin-3,5-diglucoside	X	+
F 191 a_4	cyanidin-3,5-diglucoside?	XI	trace
F 191 f_1	kaempferol-3-monoglucoside	IV	×
F 191 f_2	kaempferol-glycoside	XVI	trace

TABLE 13. Data for the identification of anthocyanins from type *sh* C^r v^{lae}.

Compound	Rf-value of				λ_{max}		Sugar detected
	glycoside in		aglycone in		on plain paper	after spraying with $Al_2(SO_4)_3$	
	BAW	CAW	AHW	BH11			
F 191 a_1 pelargonidin-3-monoglucoside	0.43 0.46	0.68 0.68	0.73 0.73	0.82 0.82	510 mμ	510 mμ	glucose
F 191 a_2 cyanidin-3-monoglucoside	0.20 0.19	0.38 0.38	0.50 0.50	0.69 0.69			glucose
F 191 a_3 pelargonidin-3,5-diglucoside	0.18*) 0.18*)	0.41*) 0.41*)	0.70 0.70	0.80 0.80			glucose
F 191 a_4 cyanidin-3,5-diglucoside	0.10 0.09	0.20 0.20	0.45 0.45	0.60 0.60			?

*) Showing characteristic orange-yellow fluorescence in U.V. light.

TABLE 14. Data for the identification of flavonol glycosides from type *sh* C^r v^{lae}.

Compound	Rf-value of						Sugar detected
	glycoside in			aglycone in			
	BAW 612	CAW	boric acid	BAW 612	CAW	AHW	
F 191 f_1*)	0.71	0.77	0.17	0.81	0.63	0.55	glucose
F 63 f_1 = kaempferol-3-monoglucoside	0.71	0.77	0.17	0.81	0.63	0.56	
F 191 f_2	0.43	0.56	0.02°)	0.82	0.56	0.55	?
kaempferol				0.82	0.56	0.56	

*) Showing multi-spots.
°) Showing bright yellow fluorescence in U.V. light.

Neither the nature of the sugars of F 191 f_2, nor their position could be determined. However, the low Rf-value and bright yellow fluorescence on chromatograms, developed in boric acid indicate that the hydroxyl group in the 3-position must be free (44).

2.5. *Type* sh c^u V, *white* (*F 125*)

No anthocyanins, flavonol glycosides or leuco-anthocyanidins were isolated.

2.6. *Type* Sh C v^{lae}, *canary* (*F 47*)

This type contains flavonol glycosides and leuco-anthocyanidins. Their identity is given in Table 15. The identification of the flavonol glycosides was based on the data given in Table 16; that of the anthocyanidins, obtained from the leuco-compounds, on the data given in Table 17.

TABLE 15. Identity of flavonol glycosides and leuco-anthocyanidins from type *Sh C* v^{lae}.

Compound	Identified as	Structure	Relative amount present
F 47 f_1	kaempferol-3-monoglucoside	IV	+ + +
F 47 f_2	quercetin-3-monoglucoside	V	+
F 47 1-ad_1	leuco-pelargonidin	I	×
F 47 1-ad_2	leuco-cyanidin	II	× × ×

TABLE 16. Identification of the flavonol glycosides from type *Sh C* v^{lae}.

Compound	Rf-value of						Sugar detected
	glycoside in			aglycone in			
	BAW 612	CAW	boric acid	BAW 612	CAW	AHW	
F 47 f_1*)	0.67	0.61	0.14	0.80	0.62	0.48	glucose
F 63 f_1 = kaempferol-3-monoglucoside	0.67	0.61	0.14	0.80	0.62	0.48	
F 47 f_2	0.48	0.39	0.27	0.64	0.20	0.35	glucose
F 45 f_2 = quercetin-3-monoglucoside	0.48	0.39	0.27	0.62	0.18	0.35	

*) showing multi-spots.

TABLE 17. Identification of the anthocyanidins, obtained from the leuco-anthocyanidins from type *Sh C* ^{plae}.

Compound	Rf-value in	
	AHW	BH 11
F 47 ad_1	0.65	0.85
pelargonidin	0.65	0.85
F 47 ad_2	0.49	0.74
cyanidin	0.49	0.74

The lead salts precipitated from the extract yielded a small amount of glucose after hydrolyses with hydrochloric acid. This was not unexpected, however, as the quercetin monoglucoside, owing to its ortho-dihydroxy structure, would be precipitated by lead acetate along with the leuco-compounds. By comparison

TABLE 18. Identity of anthocyanins and flavonol glycosides from type *Sh C*^{r} ^{plae}.

Compound	Identified as	Structure	Relative amount present
F 175 a_1	pelargonidin-3-monoglucoside	VII	+ + +
F 175 a_2	cyanidin-3-monoglucoside	VIII	+ +
F 175 a_3	pelargonidin-3,5-diglucoside	X	+
F 175 a_4	delphinidin-3-monoglucoside?	IX	trace
F 175 a_5	cyanidin-3,5-diglucoside	XI	+
F 175 f_1	kaempferol-3-monoglucoside	IV	× × ×
F 175 f_2	quercetin-3-monoglucoside	V	×
F 175 f_3	kaempferol-3-gluco-xyloside?	XIII	trace
F 175 f_4	kaempferol-glycoside = F 191 f_2	XVI	trace

TABLE 19. Identification of anthocyanins from type *Sh C*^{r} ^{plae}.

Compound	Rf-value of				λ_{max}		Sugar detected
	glycoside in		aglycone in		on plain paper	after spraying with $Al_2(SO_4)_3$	
	BAW 612	CAW	AHW	BH 11			
F 175 a_1 pelargonidin-3-monoglucoside	0.39	0.60	0.72	0.86			glucose
	0.38	0.60	0.72	0.86			
F 175 a_2 cyanidin-3-monoglucoside	0.20	0.40	0.48	0.67	525 mμ	555 mμ	glucose
	0.20	0.40	0.48	0.67			
F 175 a_3 pelargonidin-3,5-diglucoside	0.17*)	0.36*)	0.72	0.86	510 mμ	510 mμ	glucose
	0.18*)	0.37*)	0.72	0.86			
F 175 a_4 delphinidin-3-monoglucoside	0.10	0.15	0.27	0.29			?
	0.10	0.15	0.27	0.29			
F 175 a_5 cyanidin-3,5-diglucoside	0.10	0.19	0.45	0.67	525 mμ	555 mμ	glucose
	0.10	0.19	0.45	0.67			

*) showing characteristic orange-yellow fluorescende in U.V. light.

with the results of the analysis of type *Sh* c^u v^{lae} (§ 2.2) it was assumed therefore, that the leuco-compounds in type *Sh C* v^{lae} were sugar free.

2.7. *Type* Sh C^r v^{lae}, *dark purplish red (F 175)*

Five anthocyanins and four flavonol glycosides were isolated from this genotype; their identity is given in Table 18, the identification being based on the data given in Tables 19 and 20. Leuco-anthocyanidins were present, but they were not qualitatively analysed.

TABLE 20. Identification of flavonol glycosides from type *Sh* C^r v^{lae}.

Compound	Rf-value of						Sugar detected
	glycoside in			aglycone in			
	BAW 612	CAW	boric acid	BAW 612	CAW	AHW	
F 175 f_1*)	0.69	0.67	0.16	0.80	0.62	0.48	glucose
F 63 f_1 = kaempferol-3-monoglucoside	0.69	0.67	0.16	0.79	0.62	0.48	
F 175 f_2	0.49	0.38	0.26	0.60	0.23	0.35	glucose
F 45 f_2 = quercetin-3-monoglucoside	0.51	0.38	0.27	0.60	0.24	0.35	
F 175 f_3	0.49	0.38	0.26	0.79	0.60	0.54	?
F 63 f_2 = kaempferol-3-gluco-xyloside	0.49	0.38	0.29	0.79	0.60	0.55	
F 175 f_4	0.40	0.52	0.02°)	0.81			?
F 191 f_2	0.40	0.52	0.02°)	0.81			

*) Showing multi-spots.
°) Showing bright yellow fluorescence in U.V. light.

2.8. *Type* Sh c^u V, *creamish (F 109)*

This type contains three leuco-anthocyanidins, the identity of which is given in Table 21. The identification of the anthocyanidins, obtained from these leuco-anthocyanidins is given in Table 22; the leuco-compounds proved to be sugar free. Neither anthocyanins nor flavonol glycosides were present in this type.

TABLE 21. Identity of leuco-anthocyanidins, obtained from type *Sh* c^u *V.*

Compound	Identified as	Structure	Relative amount present
F 109 1-ad_1	leuco-pelargonidin	I	+
F 109 1-ad_2	leuco-cyanidin	II	+ + +
F 109 1-ad_3	leuco-delphinidin	III	+ + +

TABLE 22. Identification of the anthocyanidins, obtained from leuco-anthocyanidins from type *Sh* c^u *V.*

Compound	Rf-value in		λ_{max}
	AHW	BH 11	
F 109 ad_1	0.68	0.77	
pelargonidin	0.68	0.77	
F 109 ad_2	0.49	0.67	
cyanidin	0.49	0.67	
F 109 ad_3	0.31	0.39	551 mμ
delphinidin	0.30	0.38	551 mμ

The three leuco-anthocyanidins were present in the epidermis as well as in the parenchymous inner layer.

2.9. *Type* sh C V, *violet blue (F 61)*

Three anthocyanins and five flavonol glycosides were isolated from this type. Their identities are given in Table 23. This genotype contained no leuco-anthocyanidins.

TABLE 23. Identity of anthocyanins and flavonol glycosides from type *sh C V.*

Compound	Identified as	Structure	Relative amount present
F 61 a_1	malvidin-3-monoglucoside	XV	+
F 61 a_2	petunidin-3-monoglucoside	XIV	+ +
F 61 a_3	delphinidin-3-monoglucoside	IX	+ + +
F 61 f_1	kaempferol-3-monoglucoside	IV	+
F 61 f_2	quercetin-3-monoglucoside	V	+ +
F 61 f_3	kaempferol-3-gluco-xyloside	XIII	trace
F 61 f_4	myricetin-3-monoglucoside	VI	+ + +
F 61 f_5	myricetin-glycoside	XVII	trace

The identification of the anthocyanins was based on the data given in Table 24.

TABLE 24. Identification of anthocyanins from type *sh C V.*

Compound	Rf-value of				λ_{max}		Sugar detected
	glycoside in			aglycone in	on plain paper	after spraying with $Al_2(SO_4)_3$	
	BAW 612	CAW	AH	AHW			
F 61 a_1 malvidin-3-monoglucoside	0.27 0.28	0.85 0.86	0.76 0.76	0.62 0.61	550 mμ	553 mμ	glucose
F 61 a_2 petunidin-3-monoglucoside	0.20 0.18	0.50 0.52	0.63 0.63	0.45 0.45	550 mμ	575 mμ	glucose
F 61 a_3 delphinidin-3-monoglucoside	0.11 0.11	0.14 0.14	0.52 0.52	0.30 0.29	540 mμ	580 mμ	glucose

The identification of the flavonol glycosides was based on the data, given in Tables 25 and 26 and that of F 61 f_4 also on the fact that this compound yielded delphinidin-3-monoglucoside on reduction.

The position of the sugar residues in the compound F 61 f_5 could not be established. The substance showed brown fluorescence on chromatograms in U.V. light. This suggests that one of the glucose residues was present in the 3-position (15). No indications could be obtained as to the position of the second sugar residue, which must be present to account for the difference to F 61 f_4, owing to the very small amount of the compound which was available.

In a preliminary note (12) it was stated that three flavonol glycosides had been isolated from an extract of this type, the main compound being myricetin-3-

TABLE 25. Rf-values of, and sugars from flavonol glycosides from type *sh C V*.

Compound	Rf-value of						Sugar detected
	glycoside in			aglycone in			
	BAW 612	CAW	boric acid	BAW 612	CAW	AHW	
F 61 f_1	0.63	0.58	0.14	0.79	0.57	0.55	glucose
F 63 f_1 = kaempferol-3- monoglucoside	0.63	0.58	0.14	0.79	0.57	0.56	
F 61 f_2	0.47	0.36	0.18	0.61	0.23	0.35	glucose
F 45 f_2 = quercetin-3-monoglucoside	0.47	0.36	0.18	0.63	0.24	0.35	
F 61 f_3	0.43	0.39	0.24	0.79	0.49*)	0.54	?
F 63 f_3 = kaempferol-3-gluco-xyloside	0.43	0.38	0.28	0.79	0.57	0.56	
F 61 f_4 °)	0.33	0.14	0.15	0.35	0.05	0.24	glucose
myricetin				0.35	0.05	0.24	
F 61 f_5	0.20	0.13	0.21	0.35	0.05	0.22	glucose
myricetin				0.35	0.05	0.24	

*) Faint spot. In the solvent CAW, it was often noticed that the Rf-value was dependent on the amount of substance that was applied to the chromatogram, the Rf-value increasing with the amount.
°) Gives delphinidin-3-monoglucoside on reduction.

TABLE 26. Absorption maxima of compound F 61 f_4.

Compound	λ_{max} in	
	ethanol	0.1% ethanolic aluminium chloride
aglycone of F 61 f_4	376 mμ	
myricetin	378 mμ	
F 61 f_4	365 mμ	420 mμ
myricetin-monoglucoside, probably the 3-monoglucoside according to (44).	366 mμ	420 mμ

monoglucoside, while the other two were present in too low amounts to be identified. It was mentioned, however, that the Rf-values of these other glycosides suggested that they were possibly compounds related in hydroxylation and methylation pattern of the B-ring to petunidin and malvidin. When more material became available this suggestion appeared to be untrue, since no methoxyl groups were present in the compounds, and they have now been identified as glucosides of quercetin and kaempferol. However, it did appear that still two other flavonol glycosides were present in this genotype.

2.10. *Type* sh C^r V, *blueish violet* (*F 189*)

Four anthocyanins were isolated from this type; their identities are given in Table 27. No flavonol glycosides could be isolated, and leuco-anthocyanidins were likewise absent.

TABLE 27. Identity of anthocyanins from type *sh* C^r *V*.

Compound	Identified as	Structure	Relative amount present
F 189 a_1	pelargonidin-3-monoglucoside	VII	trace
F 189 a_2	cyanidin-3-monoglucoside	VIII	trace
F 189 a_3	delphinidin-3-monoglucoside	IX	+ + +
F 189 a_4	delphinidin-3,5-diglucoside	XII	+

The identification of the anthocyanins was based on the data given in Table 28. Further data regarding compounds F 189 a_1 and a_2 could not be obtained owing to the low amounts present. As these compounds also occurred in other types possessing the gene C^r, the information available was considered sufficient to identify the compounds as given in Table 27.

TABLE 28. Identification of anthocyanins from type *sh* C^r *V*.

Compound	Rf-value of				Sugar detected
	glycoside in		aglycone in		
	BAW 612	CAW	AHW	BH 11	
F 189 a_1 pelargonidin-3-monoglucoside	0.36 0.37	0.69 0.69			
F 189 a_2 cyanidin-3-monoglucoside	0.21 0.20	0.38 0.39	0.50 0.50	0.69 0.69	
F 189 a_3 delphinidin-3-monoglucoside	0.11 0.11	0.14 0.14	0.32 0.31	0.35 0.35	glucose
F 189 a_4 delphinidin-3,5-diglucoside	0.06 0.04	0.08 0.08	0.30 0.31	0.35 0.35	glucose

2.11. *Type* Sh C V, *black violet* (*F 45*)

Five anthocyanins and four flavonol glycosides could be isolated from this type. Their identities are given in Table 29. Leuco-anthocyanidins were also present, but were not qualitatively analysed.

TABLE 29. Identity of anthocyanins and flavonol glycosides from type *Sh C V*.

Compound	Identified as	Structure	Relative amount present
F 45 a_1	malvidin-3-monoglucoside	XV	+
F 45 a_2	petunidin-3-monoglucoside	XIV	+ +
F 45 a_3	delphinidin-3-monoglucoside	IX	+ + +
F 45 a_4	petunidin-glycoside?	XVIII	trace
F 45 a_5	delphinidin-3,5-diglucoside	XII	+ +
F 45 f_1	kaempferol-3-monoglucoside	IV	×
F 45 f_2	quercetin-3-monoglucoside	V	× ×
F 45 f_3	myricetin-3-monoglucoside	VI	× × ×
F 45 f_4	myricetin-glucoside	XVII ?	×

The identification of the anthocyanins was based on the data, given in Table 30.

F 45 a_4 possibly was petunidin-3,5-diglucoside. No specimen of known structure was available, however, to test this assumption.

TABLE 30. Identification of the anthocyanins from type *Sh C V.*

Compound	Rf-value of				Sugar detected
	glycoside in			aglycone in	
	BAW 612	CAW	AH	AHW	
F 45 a_1	0.24	0.84	0.78	0.62	glucose
malvidin-3-monoglucoside	0.24	0.83	0.78	0.62	
F 45 a_2	0.18	0.52	0.67	0.46	glucose
petunidin-3-monoglucoside	0.15	0.53	0.67	0.47	
F 45 a_3	0.12	0.18	0.50	0.32	glucose
delphinidin-3-monoglucoside	0.12	0.18	0.51	0.32	
F 45 a_4	0.05	0.33	0.68	0.43	?
petunidin				0.45	
F 45 a_5	0.04	0.08	0.56	0.30	glucose
delphinidin-3,5-diglucoside	0.04	0.08	0.53	0.32	

The identification of the flavonol glycosides was based on the data given in Tables 31 and 32, while the structure of F 45 f_2 was also deduced from reduction of this compound to cyanidin-3-monoglucoside. The compound F 45 f_4 was probably identical with F 61 f_5 (see Table 25), but there was not a sufficient amount of the latter compound available to prove this. The quantity of F 45 f_4 was too low for further investigation of its structure.

TABLE 31. Rf-values of, and sugars from flavonol glycosides from type *Sh C V.*

Compound	Rf-value of						Sugar detected
	glycoside in			aglycone in			
	BAW 612	CAW	boric acid	BAW 612	CAW	AHW	
F 45 f_1*)	0.62	0.64	0.14	0.80	0.56	0.53	glucose
F 63 f_1 = kaempferol-3-monoglucoside	0.61	0.64	0.14	0.80	0.56	0.53	
F 45 f_2*) °)	0.47	0.38	0.19	0.67	0.25	0.38	glucose
quercetin				0.67	0.24	0.36	
F 45 f_3*)	0.30	0.16	0.13	0.34	0.07	0.25	glucose
F 61 f_3 = myricetin-3-monoglucoside	0.31	0.15	0.13	0.32	0.07	0.25	
F 45 f_4*)	0.15	0.17	0.19	0.30	0.07	0.25	glucose
myricetin				0.32	0.07	0.25	

*) Showing multi-spots.
°) Gives cyanidin-3-monoglucoside on reduction.

TABLE 32. Absorption maxima of compound F 45 f_2.

Compound	λ_{max} in	
	ethanol	0.1% ethanolic aluminium chloride
aglycone of F 45 f_2	372 mμ	
quercetin	372 mμ	
F 45 f_2	361 mμ	405 mμ
quercetin-3-monoglucoside*)	362 mμ	410 mμ

*) Data, obtained by SWAIN, and given by ROBERTS *et al.* (44).

2.12. *Type* Sh C^r V, *dark blue violet* (*F 173*)

Six anthocyanins were isolated from this type, but no flavonol glycosides. Leuco-anthocyanidins were present, but were not analysed qualitatively. The identities of the anthocyanins are given in Table 33, the identification being based on the data, given in Table 34.

TABLE 33. Identity of anthocyanins from type *Sh C^r V*.

Compound	Identified as	Structure	Relative amount present
F 173 a_1	pelargonidin-3-monoglucoside	VII	+ +
F 173 a_2	cyanidin-3-monoglucoside	VIII	+ +
F 173 a_3	pelargonidin-3,5-diglucoside	X	+
F 173 a_4	delphinidin-3-monoglucoside	IX	+ + +
F 173 a_5	cyanidin-3,5-diglucoside	XI	+
F 173 a_6	delphinidin-3,5-diglucoside	XII	+ +

TABLE 34. Identification of anthocyanins from type *Sh C^r V*.

Compound	Rf-value of				Sugar detected
	glycoside in		aglycone in		
	BAW 612	CAW	AHW	BH 11	
F 173 a_1	0.34	0.70	0.68	0.77	glucose
pelargonidin-3-monoglucoside	0.34	0.70	0.68	0.77	
F 173 a_2	0.17	0.40	0.50	0.71	glucose
cyanidin-3-monoglucoside	0.17	0.40	0.50	0.71	
F 173 a_3	0.13	0.40	0.68	0.77	glucose
pelargonidin-3,5-diglucoside	0.13	0.40	0.68	0.77	
F 173 a_4	0.06	0.17	0.32	0.38	glucose
delphinidin-3-monoglucoside	0.07	0.17	0.32	0.36	
F 173 a_5	0.06	0.21	0.50	0.71	glucose
cyanidin-3,5-diglucoside	0.06	0.21	0.50	0.71	
F 173 a_6	0.03	0.05	0.32	0.38	glucose
delphinidin-3,5-diglucoside	0.03	0.05	0.32	0.36	

5. Results of the quantitative estimations of pigment contents

In order to measure anthocyanin contents, extracts were made of 0.5 g of seedcoat material in 50 ml of 1% methanolic hydrochloric acid. The final dilutions were chosen so as to give reasonable readings on the spectrophotometer (between 0.1 and 0.5). The ratio of the anthocyanin contents was calculated from the ratio of the optical densities adjusted for dilution. The determinations were carried out in duplicate; the results are given in Table 35.

Table 35. Estimations of relative amounts of anthocyanins present in pairs of seedcoat colour types only differing for the gene pair *Sh - sh*.

Type	Dilution of extract	Wavelength of maximum absorption	Optical density		Relative amount of anthocyanins
			measured	average	
sh C^r v^{lae} (F 191)	1 ×	515 mμ	0.206; 0.267	0.24	1.0
Sh C^r v^{lae} (F 175)	5 ×	515 mμ	0.264; 0.290	0.28	5.8
sh C V (F 61)	2 ×	540 mμ	0.194; 0.230	0.21	1.0
Sh C V (F 45)	6 ×	540 mμ	0.267; 0.326	0.30	4.3

The estimation of flavonol content was carried out in a similar way to that used for the anthocyanin content; the results are given in Table 36.

Table 36. Estimation of relative amount of flavonol glycosides, present in two seedcoat colour types, differing for the gene pair *Sh - sh*.

Type	Dilution of extract	Wavelength of maximum absorption	Optical density		Relative amount of flavonol glycosides
			measured	average	
sh C v^{lae} (F 63)	5 ×	350 mμ	0.480; 0.425	0.45	1.0
Sh C v^{lae} (F 47)	5 ×	350 mμ	0.355; 0.339	0.35	0.8

In order to estimate leuco-anthocyanidin contents, extracts were made of 0.5 g of seedcoat material in 15 ml of methanol. 0.07 Milliliter of each extract was diluted with methanol to 1 ml and 1 ml of ethanolic vanillin solution (3 g of vanillin in 8 ml of ethanol) together with 1 ml of 25% aqueous hydrochloric acid were added. Optical densities were measured at 500 mμ, the correction for

Table 37. Estimation of relative amounts of leuco-anthocyanidins present in the *Sh*-types.

Type	Optical density				leuco-anthocyanidins	Relative amount of leuco-anthocyanidins
	measured	average	correction for anthocyanins			
			measured	average		
Sh c^u v^{lae} (F 111)	0.472; 0.548	0.51	–	–	0.51	1.0
Sh C v^{lae} (F 47)	0.624; 0.641	0.63	–	–	0.63	1.2
Sh C^r v^{lae} (F 175)	0.659; 0.673	0.67	0.068; 0.082	0.08	0.59	1.2
Sh c^u *V* (F 109)	0.299; 0.316	0.31	–	–	0.31	0.6
Sh C V (F 45)	0.178; 0.181	0.18	0.007; 0.012	0.01	0.17	0.3
Sh C^r *V* (F 173)	0.303; 0.262	0.28	0.054; 0.059	0.06	0.22	0.4

anthocyanins being determined by measuring the optical density of an equal amount of extract to which 1 ml of ethanol had been added instead of the vanillin solution. The determinations were carried out in duplicate; the results are given in Table 37.

CHAPTER VI

DISCUSSION

1. Introduction

The results obtained from the chemical analysis point to the existence of a close interrelationship between the processes controlled by the genes under investigation. It is therefore impossible to discuss the effects of substitution at one locus separately from the situation at the other loci studied.

The genic background, as far as concerned the other most important colour influencing genes was identical in all the types: *P d b g Rk*. Owing to the presence of the dominant groundgene *P* most of the gene substitutions investigated had visible effects on the seedcoat colour. However, due to the presence of the recessive colour gene *d*, the triple recessive type *sh* c^{u} v^{lae} was colourless (= white). The recessive alleles of *B* and *G* and the dominant gene *Rk*, all belonging to the group of the modifying genes, were chosen because the alternative alleles of these genes produce darker colours which are possibly the result of a change in the products formed by the action of the genes under investigation.

It should be kept in mind that pure lines were investigated which had been selected by eye. Thus it is possible that, other than with respect to the genes investigated, these lines differed with regard to genes having actions more or less undetectable by visual screening, but which still influenced the pigment mixture in the seedcoat, either qualitatively or quantitatively. Moreover the further possibility exists that although it has been concluded that the same gene is present in a number of varieties (based on the visual analysis of the progeny of crosses) in fact different alleles of the same locus were present having a different effect which could only be detected by chemical analysis.

These disadvantages of working with pure lines were accepted, because for the development of methods suitable for this material, it was necessary to have at my disposal larger amounts of each colour type. Work on segregating progenies will be carried out in the future to test whether the differences which are ascribed in this paper to substitution of one gene indeed are monogenic.

In order to facilitate the discussion of the effects of the various gene substitutions the results of the qualitative analysis have been brought together in Table 38, pages 38 and 39.

2. The gene pair $V - v^{lae}$

The effects of substitution at the *C*-locus, and the interactions between the *C*- and the *Sh*-locus are to a large extent determined by the gene pair $V - v^{lae}$; this gene pair, therefore, is dealt with first.

Comparison of the v^{lae}-types with the *V*-types (upper half of each column with the whole column in Table 38) shows that substitution of v^{lae} by *V* influences the structure of the B-ring of leuco-anthocyanidins, flavonol glycosides

and anthocyanins formed under control of the genes at the other loci. In the presence of v^{lae}, with one exception only compounds with one or two hydroxyl groups in the B-ring are formed.[1]) In the presence of the allele *V*, substances with three substituents in the B-ring are also synthesized, which as a rule then form the main components of the mixtures. The fact, to be described below, that the effects of the actions of other genes are depending on the *number* of hydroxyl groups, suggests that this number is determined *before* these genes perform their action. This means that the genes at the *V*-locus act at an earlier stage in the biosynthesis of the pigments than the genes at the *C*- and *Sh*-loci. The simplest assumption is that in the synthesis of these compounds a common precursor exists in which one or two hydrogen atoms of the benzene ring which ultimately becomes the B-ring in the flavonoids are replaced by hydroxyl groups as a result of the action of *V*.

Introduction of an hydroxyl group in the B-ring of different pigments present in the same tissue by the action of one dominant gene has been described previously by other workers. It was found with anthocyanins and flavonol glycosides in the flowers of *Antirrhinum majus* (11, 19, 55), *Dianthus caryophyllus* (17), *Solanum phureja* and *Primula sinensis* (55), with anthocyanins and leuco-anthocyanidins in the seeds of *Zea mays* (9) and in the flowers of *Impatiens balsamina* (1).

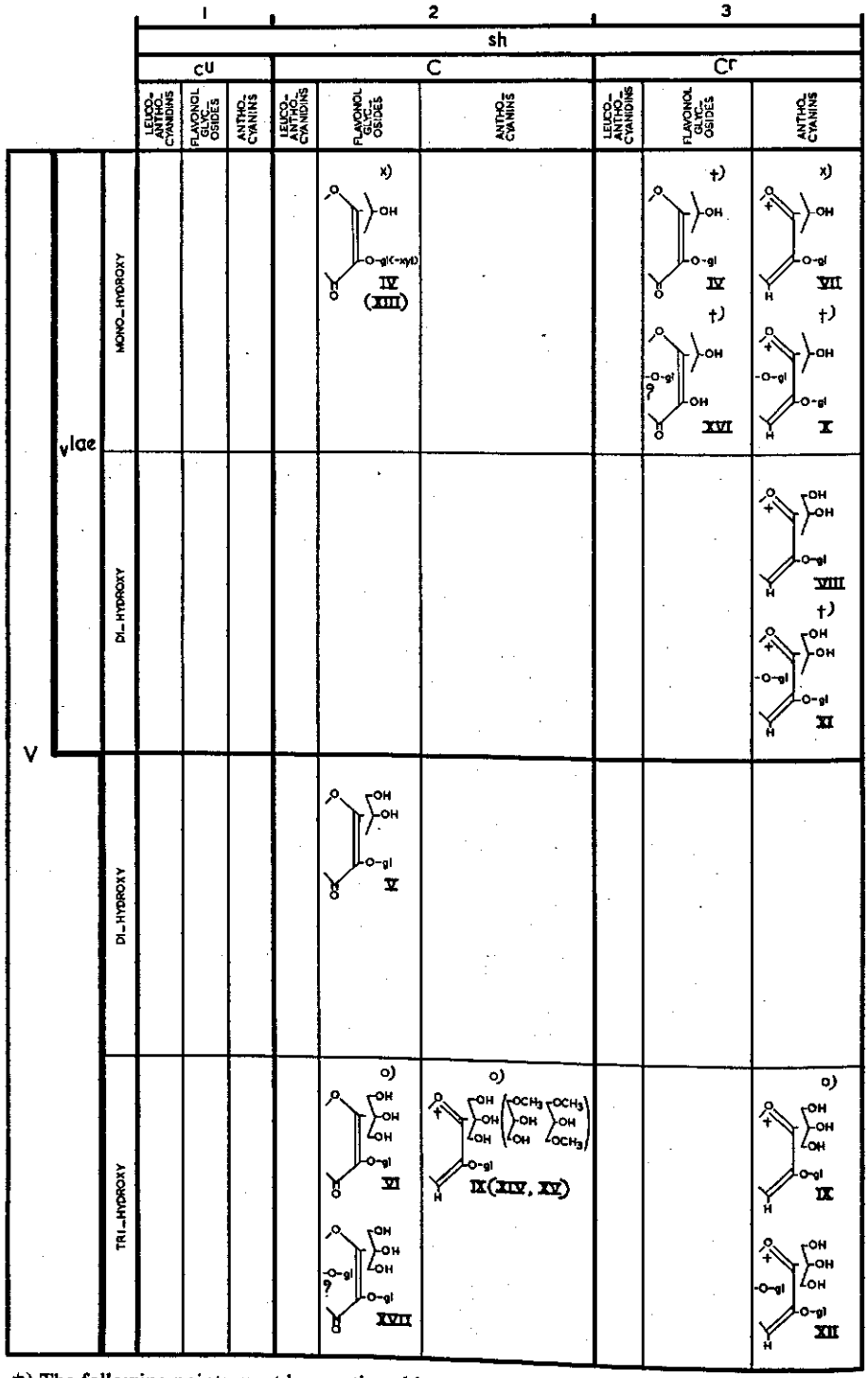
The exception mentioned above of the rule that v^{lae}-types only contain compounds with one or two hydroxyl groups in the B-ring is formed by the type *Sh* C^r v^{lae}, in which a trace of delphinidin-3-monoglucoside was detected. The explanation possibly comes from the fact that v^{lae} is not the most recessive allele of the *V*-locus. The bottom recessive allele is *v*. As was mentioned previously no difference was found between v^{lae} and *v* as concerns the effect on the seedcoat colour. In the flower, however, v^{lae} and *v* differ in their effect, *v* giving a white flower, whereas in the presence of v^{lae} a pale lilac colour is produced. The pigment giving this colour proved to be delphinidin-3-monoglucoside, the same compound as was present in larger amounts in the flower of *V*-types. The large amount of anthocyanin produced in the seedcoat of type *Sh* C^r v^{lae} (as a result of the influence of *Sh* and C^r) may allow the gene v^{lae} here also to express its ability to produce a small amount of a trihydroxy compound. This trihydroxy compound then should be absent in type *Sh* C^r *v*. Future investigation will show whether this assumption is correct; if so, it means that at least in certain genetical backgrounds v^{lae} and *v* have different effects on seedcoat pigmentation.

3. THE MULTIPLE ALLELIC SERIES $C^r - C - c^u$

The alleles *C* and C^r of the *C*-locus promote the formation of anthocyanins and flavonol glycosides (Table 38, cols. 2, 3, 5 and 6). In the presence of c^u none of these compounds is formed. (Table 38, cols. 1 and 4). The anthocyanins and flavonol glycosides formed have identical substitution patterns of the A-ring and, apart from the occurrence of methylated anthocyanins, show the same variation with respect to the hydroxylation pattern of the B-ring. It appears, however, that in the formation of the above compounds there not only exists a relation with the situation at the *C*-locus, but also with the situations at the *V*- and the *Sh*-loci.

The results of the analysis of the *Sh*-types will be dealt with first (Table 38,

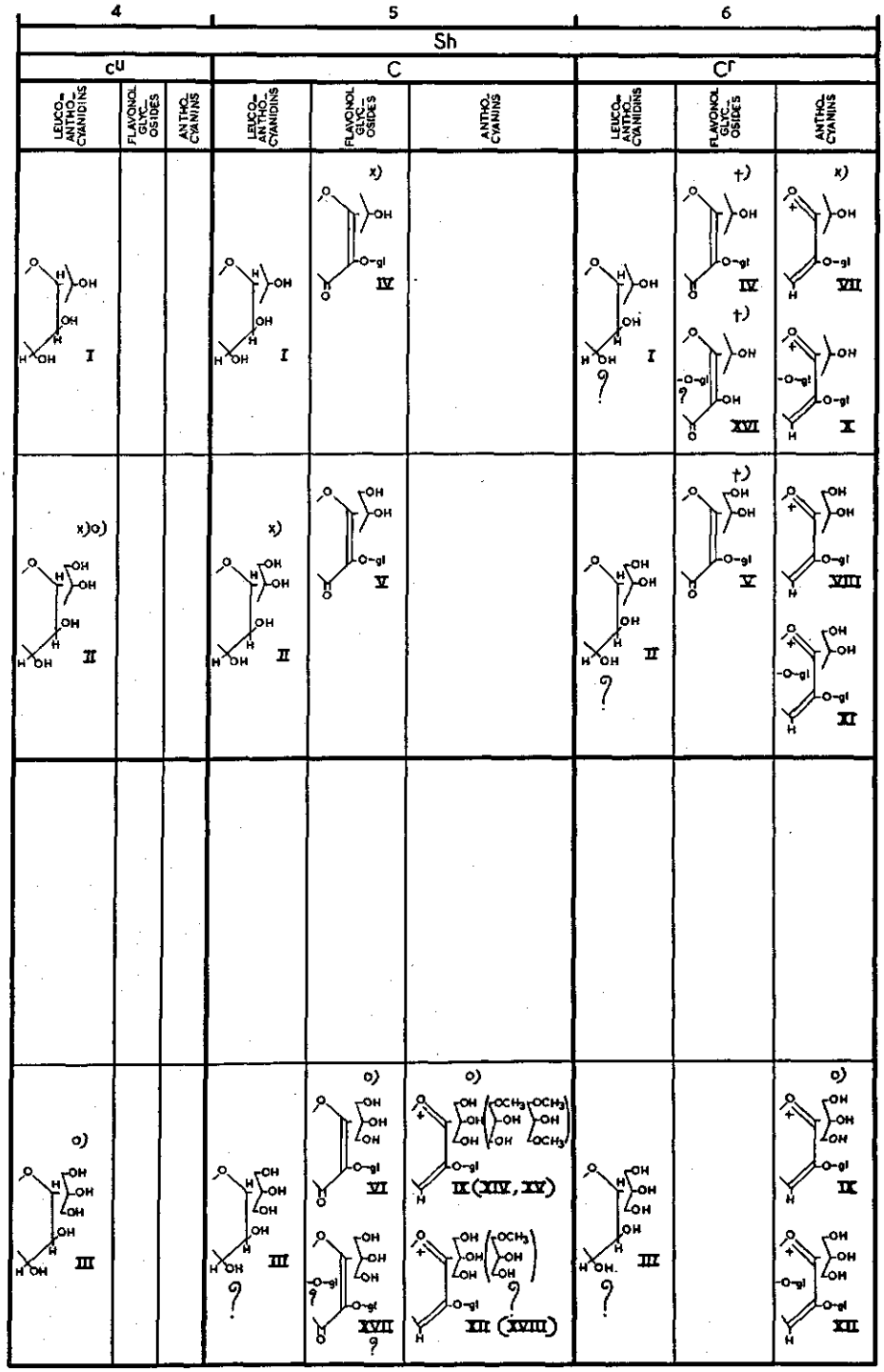
[1]) For the exception in type *Sh* C^r v^{lae} see below.



*) The following points must be mentioned in connection with this table.

1. Only the varying part of the aglycones has been drawn. > stands for the B-ring, representing the 3′, 4′ and 5′ positions.
2. The upper half of each column represents the v^{lae}-type; the trace of delphinidin-3-monoglucoside found in type *Sh* C^r v^{lae} has been ommitted. The whole column represents the *V*-type; compounds only present in the v^{lae}-type but not in the corresponding *V*-type are marked with †). The main compound in each v^{lae}-type is indicated by ×), that in the *V*-type by °).
3. A sugar residue, when not attached to the 3-position, is represented by -O-gl. When it con-

analysis of the twelve types investigated.[1])



cerns an anthocyanin this indicates that the sugar residue is in the 5-position (in compound XII, col. 5, -0-gl has been erroneously ommitted). Some flavonol glycosides could not be fully identified as to the number and position of their sugar residues. With the compounds concerned this has been indicated by -0-gl?.

4. The identity of most of the leuco-anthocyanidins mentioned in cols. 5 and 6 is based on the assumption that the mixtures of these compounds present in the types represented in the columns consist of the same components as were found to be present in the types of column 4. This has been indicated by? with the compounds concerned. Also compounds XVII and XVIII, col. 5, have been marked with ? because their structures have only been tentatively established.

cols. 5 and 6; see point 2 for †). In the presence of *C* v^{lae} only flavonol glycosides are formed, mainly with one, but to a lesser extent with two hydroxyl groups in the B-ring. In the presence of *C V* flavonol glycosides with one, two, but mainly three hydroxyl groups, and anthocyanins only with three hydroxyl groups in the B-ring are formed. In the presence of C^r v^{lae} both flavonol glycosides and anthocyanins with mainly one, and in lower amounts two, hydroxyl groups are formed. Finally, in the presence of C^r *V* only anthocyanins are formed, with one, two, but mainly three hydroxyl groups in the B-ring.

In the presence of the gene *sh* the four gene combinations have analogous effects; however, in this case no flavonol glycosides with two hydroxyl groups in the B-ring are formed when v^{lae} is present (Table 38, cols. 2 and 3).

From the foregoing it appears that a shift from flavonol glycoside towards anthocyanin synthesis always accompanies the appearance of the trihydroxy structure. In the case when *C* is present, this shift is from flavonol glycosides only to flavonol glycosides plus anthocyanins; when C^r is present, there is a shift from flavonol glycosides and anthocyanins to the latter compounds only.

In order to seek an explanation for these phenomena it is necessary to consider the possible routes of formation of the compounds in question. There are *a priori* four possibilities regarding the formation in the same tissue of an anthocyanin and a flavonol glycoside with a corresponding hydroxylation pattern, *viz.*:

1. They are synthesized from different precursors, by different reactions catalyzed by different enzymes.
2. They are synthesized sequentially by two reactions catalyzed by two enzymes; one compound acting as precursor for the other, e.g. the flavonol (glycoside) being the precursor for the anthocyanidin (glycoside).
3. They are synthesized from closely related precursors by the same kind of reaction, catalyzed by one enzyme.
4. They are formed by parallel synthesis from one precursor, by two reactions, controlled by two different enzymes.

The existance of the interrelationships between the formation of anthocyanins and flavonol glycosides mentioned above makes the first improbable. The results of the analysis of *sh* v^{lae} types (Table 38, cols. 2 and 3, upper halves) indicate that the assumption given in point 2 is probably also not right. In type *sh C* v^{lae} only flavonol glycosides were found, whereas from type *sh* C^r v^{lae} flavonol glycosides and anthocyanins could be isolated. If it were assumed, therefore, that the gene *C* does not produce an enzyme which converts flavonol glycosides with one or two hydroxyl groups in the B-ring into anthocyanins, while the gene C^r produces such an enzyme, one would expect to find quercetin glucoside in type *sh C* v^{lae} since cyanidin glucoside was isolated from type *sh* C^r v^{lae}. This is not the case, however, and therefore the assumption of sequential synthesis seems less probable.

The results of the chemical analysis allow no definite choice between the third fourth possibilities. However, the existence of a relatively large number of alleles with different qualitative effects which all seem to belong to the *C*-locus, as was found in the genetical analysis, suggests that this locus is a complex one. In this case the hypothesis that *C* and C^r each produce two enzymes (point 4) is an attractive one. Competition of these enzymes for the common substrate[1]),

[1]) An analogous hypothesis regarding the formation of anthocyanins and flavonol glycosides based on results of chemical-genetical work with *Dahlia variabilis* has been made earlier by LAWRENCE and SCOTT-MONCRIEFF (34).

together with two additional assumptions may then account for the phenomena observed. The additional assumptions are:

1. The "common substrate" really consists of three kinds of molecule, differing only in the number of hydroxyl groups in the benzene ring which subsequently forms the B-ring in the flavonoid pigments. This number of hydroxyl groups, one, two or three, which is controlled by the genes at the *V*-locus according to the hypothesis given in § VI.2, determines the suitability of the molecule to act as a precursor for the formation of anthocyanins. A trihydroxy structure is assumed to be more suitable for the synthesis of anthocyanins than the mono- or dihydroxy structures.
2. The anthocyanin-forming enzyme produced by C^r is assumed to have a greater activity than that formed by *C*, and also to be capable of catalysing the production of anthocyanins with one and two hydroxyl groups in the B-ring; the gene *C* on the other hand can only produce anthocyanins, when the precursor with the trihydroxy structure is available.

According to these assumptions *C*, in the presence of v^{lae}, should only produce flavonol glycosides, because of lack of a precursor with the trihydroxy structure; C^r, in this case, should produce anthocyanins as well, with a consequent decrease in the production of flavonol glycosides. No quantitative measurements of the flavonol glycoside content in the C^r v^{lae}-types were carried out, but the amounts of the flavonol glycosides, which were isolated in the qualitative analysis, strongly suggest that the flavonol glycoside contents of these types were lower than those of the corresponding *C* v^{lae}-types.

When, by the action of *V*, mainly precursor with the trihydroxy structure is available, *C* should produce anthocyanins with the trihydroxy structure along with flavonol glycosides also having this structure, but the precursors with one and with two hydroxyl groups still can be converted only into flavonol glycosides. When C^r is present together with *V*, owing to the greater activity of the enzyme catalyzing anthocyanin synthesis and the greater suitability of the precursor with three hydroxyl groups for conversion into such compounds, the whole amount of this precursor should be converted into anthocyanins, while the rather small amounts of precursors with one or two hydroxyl groups would be expected to be converted into such small amounts of flavonol glycosides that these compounds might be no more detectable. Since these effects were found in practice (Table 38) it is probable that the assumptions made are true.

These relations between the synthesis of different structures (of the heterocyclic part of the molecule), specific for anthocyanidins or flavonols, and the number of hydroxyl groups in the B-ring have not previously been described in other plants, although genes for anthocyanin production or for the simultaneous production of anthocyanins and flavonol glycosides have been found in a number of species e.g. *Antirrhinum majus* (11, 19), *Cyclamen persicum* (52), *Dianthus caryophyllus* (20) and *Impatiens balsamina* (1).

We must now turn our attention to the effect of *C* on the methylation of part of the anthocyanins, the occurrence, in some of the types, of small amounts of anthocyanidin-3,5-diglucosides, and the abnormal chromatographic behaviour of flavonol glucosides.

The majority of the pigments contain in the B-ring only hydroxyl groups. Some of the anthocyanins produced in the *C*-types, however, contain one or two methoxyl groups in this ring, although the flavonol glycosides also produced in these types only possess hydroxyl groups. The occurrence of methylated

anthocyanins along with unmethylated flavonol glycosides has been reported previously in plants, e.g. in *Solanum phureja* and *Primula sinensis* (55), and it has been suggested that it is an indication that methylation occurs as a late stage in the synthesis. In a number of cases, however, a special gene was shown to be responsible for methylation. Nevertheless a separate gene pair controlling methylation in *Phaseolus vulgaris* has not been found, but its existence is not impossible. Chemical analysis of the families segregating for $C - C^r$ will be necessary to establish whether the methylation is either determined at the *C*-locus, or by a separate gene pair. It is interesting to note that in the seedcoat of *Phaseolus vulgaris* the unmethylated anthocyanins (delphinidin glucosides) form the major components of the anthocyanin mixture, while in the flowers of other plants where such methylation occurs, the major part of the anthocyanins is usually present in the methylated form. No explanation can be offered at present for this difference.

Like methylation, the introduction of an extra sugar residue in the 5-position only affects a small portion of the total amount of anthocyanins formed in the seedcoats. In the major part of the anthocyanins formed, only the hydroxyl group at the 3-position is replaced by a sugar residue. Gene-controlled introduction of an extra sugar residue in anthocyanins has been noted in various plant species, e.g. *Callistephus* (66), *Cyclamen* (52), *Dianthus* (20) and *Streptocarpus* (35). If in the bean seedcoat a separate gene is likewise responsible for the occurrence of the 3,5-diglucosides, this gene must be present in all the C^r-types investigated and also in type *Sh C V*. It could not be established whether the flavonol glycosides were affected in the same way. In those genotypes where anthocyanidin-3,5-diglucosides occurred flavonol glycosides were always found, differing from the 3-glucosides which were formed predominantly. The exact structure of these compounds, however, could not be determined.

The differences found between the genotypes with regard to the chromatographic behaviour of some of the flavonol glucosides ("multi-spots") are not included in Table 38. The separation into "hydrophilic" and "non-hydrophilic" components was only found in some of the genotypes. As the nature of the phenomenon is still unknown it is possible that the anomalous behaviour results from the treatment the beans receive after harvesting, and therefore the fact that it was only found in some of the genotypes may have been caused by an unintentional difference in the treatment. The further possibility that the "hydrophilic" and the "non-hydrophilic" components were already present in the plants, and that differences among the genotypes have a genetical basis must be kept in mind. Since there was no correlation found with the situations at one of the loci studied, a gene or genes other than those investigated here must be responsible if the phenomenon is in fact genotypically determined.

4. THE GENE PAIR *Sh – sh*

Substitution of the gene *sh* by its allele *Sh* has a twofold effect: the formation of leuco-anthocyanidins; and a strong stimulation of anthocyanin-production and probably in most cases of the production of flavonol glycosides also. In type *Sh* c^u v^{lae} only leuco-anthocyanidins are produced. (Table 38, col. 4). The main anthocyanidin obtained by boiling the mixture of leuco-anthocyanidins with mineral acid was cyanidin, but a very small amount of pelargonidin was formed as well. Only a portion of the total leuco-compounds was converted

into anthocyanidins, the remainder forming high molecular substances which were not further analysed. However, assuming that equal parts of each leuco-anthocyanidin were converted into an anthocyanidin, it was concluded that leuco-cyanidin was the main compound in type *Sh* c^u v^{lae}.

The analysis showed that the leuco-compounds were sugar free; thus they should be called leuco-anthocyanidins. Other investigators have used the term leuco-anthocyanin, without making it clear, whether or not the compounds investigated did contain sugars. Thus it is recorded that leuco-anthocyanins are present in the seedcoats of *Phaseolus multiflorus, Lathyrus odoratus* (47), and *Arachis hypogaea* (61). In a preliminary paper FEENSTRA (12) mentioned that, as far as he knew, the gene *Sh* found in *Phaseolus vulgaris* was the first one reported which is responsible for the production of leuco-anthocyanidins. A short time afterwards SEYFFERT (53) showed that a gene is present in *Silene armeria*, which produces leuco-cyanidin in the flower.

The effect of *Sh* on anthocyanin production is readily observed in the types possessing the gene C^r, or the genes *C* and *V*. Qualitative analysis demonstrated that anthocyanin mixtures of approximately equal composition were present in types *Sh* C^r v^{lae} and *sh* C^r v^{lae}, but quantitative comparison showed that the total anthocyanin content present in the former type was about 5.8 times larger than that present in the latter (Table 35). The flavonol contents of both types were not measured, but the qualitative analysis gave the impression that the amount of flavonol glycosides present in type *Sh* C^r v^{lae} was also higher than that present in type *sh* C^r v^{lae}.

Leuco-anthocyanidins were also present in type *Sh* C^r v^{lae}. They were not qualitatively analysed but an estimation of the total amount present showed that this was at least not less than the amount present in type *Sh* c^u v^{lae} (Table 37).

The main anthocyanin in the types *Sh* C^r v^{lae} and *sh* C^r v^{lae} is pelargonidin-3-monoglucoside. Thus *Sh*, in the presence of v^{lae}, causes a larger production of anthocyanins with one hydroxyl group in the B-ring and, as was mentioned above, the formation of leuco-anthocyanidins with mainly two hydroxyl groups in the B-ring. The possibility exists of course, that a gene very closely linked to *Sh*, rather than *Sh* itself, is responsible for the stimulation of the anthocyanin production. The close structural relationship of the leuco-compounds and the pigments suggests, however, that there is in fact a direct relation between the gene *Sh* and the production of anthocyanin. Moreover, in the presence of *V*, the larger anthocyanin production is accompanied by a decrease in the amount of leuco-anthocyanidins formed.

Assuming that the gene *Sh* itself influences the production of anthocyanins and flavonol glycosides, the simplest explanation is that it forms compounds which are intermediates in the biosynthesis of these pigments. Because the presence of *Sh* also leads to the formation of leuco-anthocyanidins, the question arises as to whether the latter compounds might be the intermediates in question. Another possibility is that *Sh* forms compounds which are precursors of leuco-anthocyanidins as well as anthocyanins and flavonol glycosides. The difference, in the presence of v^{lae}, between the hydroxylation pattern of anthocyanins and flavonol glycosides on the one hand and that of the leuco-anthocyanidin on the other, together with the results of the estimations of leuco-anthocyanidin contents which, if they can be taken to represent the actual situation fairly well, indicate that the increase in the production of anthocyanins and flavonol glycosides is not accompanied by a decrease in the production of

leuco-anthocyanidins, points to the second possibility as being more correct. If *Sh* does control an intermediate step in the production of all three kinds of flavonoid compound, this implies two things:

a. In the presence of v^{lae}, *Sh* must form compounds both with one and with two hydroxyl groups in the 'pro-B'-ring (= benzene ring which ultimately becomes the B-ring in the flavonoid compounds). The compound with one hydroxyl group is used mainly for the production of anthocyanins and flavonol glycosides, and the dihydroxy compound mainly for the production of leuco-anthocyanidins.

b. The formation of leuco-anthocyanidins must need at least one more reaction after the one controlled by *Sh*; the gene promoting this reaction must be homozygously present in all the *Sh*-types.[1]) This gene then acts (via an enzyme produced) preferably on the substrate with dihydroxy structure.

The hypothesis may be summarized as follows. In the presence of v^{lae}, *Sh* forms compounds with one and with two hydroxyl groups in the pro-B-ring. The compound with monohydroxy structure is used mainly for the production of anthocyanins and flavonol glycosides, and the compound with dihydroxy structure mainly for the synthesis of leuco-anthocyanidins, thus competition for a common substrate is absent. The values obtained in the estimation of leuco-anthocyanidin contents point to the presence of even higher amounts of these compounds in types *Sh C* v^{lae} and *Sh* C^r v^{lae}, compared with that in type *Sh* c^u v^{lae}. If this difference is significant, it might be explained by the assumption that when the precursor with one hydroxyl group in the pro-B-ring is not used for the production of anthocyanins or flavonol glycosides it acts as a competitive inhibitor in the formation of the leuco-anthocyanidins.

The situation becomes different in the presence of the gene *V*. Large amounts of anthocyanins, flavonol glycosides and leuco-anthocyanidins with the same (trihydroxy) structure of the B-ring are then formed. In this case a large part of the total precursors produced by *Sh* must have the trihydroxy structure and among the reactions leading to the formation of the various flavonoid compounds there may be competition for the common substrate. The amount of leuco-anthocyanidins which could be isolated from type *Sh* c^u *V* was much lower than that isolated from type *Sh* c^u v^{lae} (Table 37). With the assumptions mentioned above, this may be due either to a smaller production of the intermediate with three hydroxyl groups by *Sh*, or to a smaller production of leuco-delphinidin by the gene(s) forming leuco-anthocyanidins. The anthocyanin content in type *Sh C V* shows a considerable increase over that in type *sh C V* (Table 35), but the amount of leuco-anthocyanidins isolated from type *Sh C V* was still lower than that of type *Sh* c^u *V* (Table 37). According to the hypothesis, this decrease in the amount of leuco-anthocyanidins formed may be due to a decrease in the amount of trihydroxy substrate available for the production of the leuco-compound due to the simultaneous formation of anthocyanins and flavonol glycosides from this substrate. A smaller amount of leuco-anthocyanidins was also isolated from type *Sh* C^r *V* than from type *Sh* c^u *V*. The data obtained for type *Sh* C^r *V* are not exactly comparable to those of the

[1]) It may be asked, whether the gene *Ins* mentioned by LAMPRECHT may be identical with this or, when more genes are involved, with one of these genes. This question must be answered negatively. According to LAMPRECHT the two gene pairs *Sh–sh (J–j)* and *Ins–ins* when segregating together give a 15 : 1 ratio of creamish to white, thus showing polymery, whereas in our case both genes have a complementary action, which would express itself in a 9 : 7 ratio.

other types, however, as the plants had been grown under different circumstances.

Fig. 5 shows the hypothesis in the form of a diagram. For the sake of simplicity specific differences between *C* and C^r with respect to the production of anthocyanins and flavonol glycosides have been ignored. *X* represents the unknown gene(s) for leuco-anthocyanidin production which are present in all *Sh*-types; *P* represents the precursor which can be modified by *V*; Q is the intermediate produced by *Sh*. The index figures refer to the number of hydroxyl groups in the pro-B- and B-rings. Not too much importance should be attached to the thickness of the arrows, it merely indicates whether a large or a small amount of compound is formed by the reaction concerned.

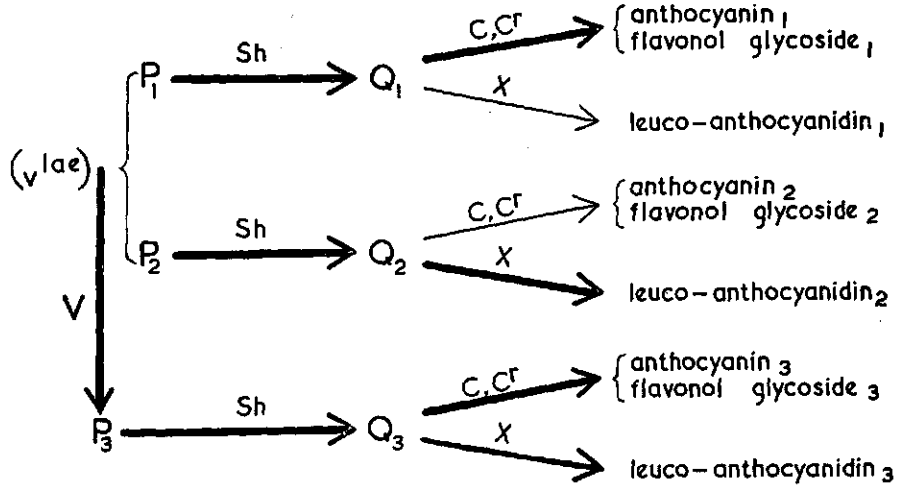


FIG. 5. Scheme of the hypothetical actions of the genes *V*, *Sh*, *C* and C^r in seedcoat pigmentation. For symbols, see text.

Two points must still be discussed in connection with the gene pair *Sh* – *sh*, namely the production of anthocyanins and flavonol glycosides in *sh*-types, and the phenotypic effects of the presence of leuco-anthocyanidins.

Although the phenomena mentioned above support the assumption that the dominant gene *Sh* produces intermediates in the formation of anthocyanins and flavonol glycosides, these compounds are also formed in the presence of the recessive allele *sh* (Table 38, cols. 2 and 3). As a rule the pigments are formed in much lower amounts then in the corresponding *Sh*-types, but in type *sh C* v^{lae} the flavonol content is even higher than that in type *Sh C* v^{lae} (Table 36). In the former type a rather large amount of kaempferol-3-glucoxyloside is produced, a compound which is either not formed in other types, or is present in only very small amounts. Analysis of the flowers of various types showed that this bioside is the main flavonol glycoside in this plant organ. Moreover, as mentioned previously, delphinidin-3-monoglucoside is present in the flower of v^{lae}- and *V*-types. Substitution of *sh* by *Sh* has no effect on the flower colour, and no leuco-anthocyanidins could be isolated from the flowers of either *Sh*-types or *sh*-types. The plant thus seems to possess a mechanism for the formation of anthocyanins and flavonol glycosides which can act without

the intervention of a step like that which has been suggested is catalyzed by the gene *Sh*. It would appear possible that in the absence of the dominant gene *Sh*, this mechanism is also responsible for the formation of anthocyanins and flavonol glycosides in the seedcoat.

The phenotypic effects of the presence of leuco-anthocyanidins are most obvious in the types which possess only these compounds. The characteristics of the dry ripe seeds of type *Sh* c^{u} v^{lae} which have been mentioned in Chapter III § 2.2.1., are the creamish colour, the shiny surface and the darkening of the colour after the beans have been harvested. How are these characters connected with the occurrence of leuco-anthocyanidins, which are themselves colourless compounds? The incompletely dry seeds when harvested for chemical analysis are colourless, and the leuco-compounds were detected both in the epidermis proper and in the underlying parenchymous layers. Whether the compounds in the parenchymous layer are synthesized *in situ*, or originate from the epidermis and diffuse into the underlying tissue must be left open. When the beans dry, the parenchymous layer shrivels to a thin dense mat. This mat is white in *sh*-types, but in *Sh*-types which are harvested in the dry state, it is yellow-brown. It seems probable that during the process of drying, which presumably is accompanied by the death of the parenchymous tissue, the unstable leuco-anthocyanidins are converted into compounds responsible for the yellow-brown colour. The cystal-cell layer and the epidermis (fig. 2) have thick cell walls, and when observed through a binocular stereo-microscope these layers have a glass-like appearance. The underlying layer shines through the upper layer to give the creamish colour.

The glassiness of the upper layer is more conspicious when the underlayer is coloured than when it is white. This phenomenon was also observed when a section of the upper-layer which had been freed from underlying tissue was examined on either dark or white paper surfaces. It made no difference whether the upper-layer was taken from an *sh*-type or from a *Sh*-type. It was concluded, therefore, that the glossy appearance of *Sh*-types results from the presence of a coloured layer under the glasslike upper layer, the latter being present in all the seedcoat colour types of *Phaseolus vulgaris*.

The instability of leuco-anthocyanidins appears to be the reason for the darkening of the *Sh*-types after they have been harvested. Reddish-brown substances are present in the lumina of the epidermic cells of darkened beans. In Chapter IV it was mentioned that when leuco-anthocyanidins are exposed to the air they are quickly converted into red-brown substances of high molecular weight. This reaction presumably also takes place, albeit slowly, in the palissade cells of the dry ripe seedcoat after harvesting. Exclusion of light and oxygen prevents the darkening of the beans. In the incompletely dry beans the presence of an antioxidant might be the reason why the leuco-anthocyanidins do not discolour. When this antioxidant is lost during the drying oxidation can take place.

In types which, instead of the dominant allele *Rk*, possess one of the alleles *rk* or rk^{d}, a red colour develops even in the immature beans when the gene *Sh* is present. The colour of a dry rk^{d}-type shows a great resemblance to that of the corresponding strongly darkened *Rk*-type. The *Rk*-locus may have something to do with the production of the antioxidant, *Rk* producing the normal amount, *rk* a smaller, and rk^{d} giving an even smaller amount or possibly none at all.

One may aks, how the results obtained in the present investigations fit in with what is known about the biosynthesis of the flavonoid compounds.

The present knowledge concerning this biosynthesis is briefly summarized as follows.

The B-ring and the adjacent C_3-moiety of the flavonoid molecule are formed from a derivative of phenylpropane (C_6–C_3-compound). Phenyl alanine, cinnamic acid and *para*hydroxy cinnamic acid have all been used as precursors in the formation of this part of the molecule. The C_6–C_3-compound is presumably synthesized from carbohydrates, via the formation of shikimic acid (3,4,5-trihydroxy-1-*cyclo*hexene-1-carboxylic acid).

The A-ring is probably not built into the molecule as one unit (e.g. phloroglucinol) as was presumed formerly, but arises from three acetate units probably by acyl condensation, the first of these units reacting with the terminal carbon atom of the C_3-chain in the C_6–C_3-compound. By dehydration and subsequent oxidative ring closure the structure, characteristic of the various flavonoid compounds, is finally synthesized. This account of the synthesis is based on both physiological and chemical studies with use of C_{14}-labelled compounds.

It is not known with certainty whether modifications of the hydroxylation pattern of the B-ring are already made in the C_9-compounds or are elaborated only when the synthesis of C_{15}-compounds is complete. Most data are consistent with the hypothesis that the hydroxylation pattern of the B-ring is already established prior to the formation of the C_{15}-structure. For example, the detection of different flavonoid compounds in the same tissue all possessing the same hydroxylation pattern in the B-ring, and genetical investigations which have shown that a modification of the structure of this ring which affects more than one compound may be determined by a single gene.

It is not known whether flavonoid compounds that have different structures in the heterocyclic part of the molecule, are synthesized sequentially (one acting as precursor for the other), or whether each compound is synthesized from its own C_9-compound. The former possibility has hitherto attracted most attention. For instance BOGORAD (6) has given a hypothetical scheme for the biogenetic relationship among flavonoid compounds, which for the larger part is based on the assumption of sequential formation. In this scheme it is presumed that leuco-anthocyanidins are precursors of anthocyanidins. This assumption is based on the observation, which has been made in a number of cases, that an increase in the amount of anthocyanins in a tissue is accompanied by a decrease in the amount of leuco-compounds, and *vice versa*.

The old controversy of parallel versus sequential formation of flavonols and anthocyanidins has not yet been settled definitely. However, the theory of parallel synthesis from a common precursor, as originally formulated by LAWRENCE and SCOTT-MONCRIEFF (34) appears to have most supporters nowadays.

The results of the present investigation support the hypothesis that the hydroxylation pattern of the B-ring is determined at an early stage in the synthesis, at least prior to the establishment of the final structure of the heterocyclic part of the flavonoid compounds. However, no conclusion can be drawn from the data as to whether the structure of the heterocyclic part of the molecule is

mogelijke homozygote combinaties, in de vorm van zuivere lijnen, werden onderzocht. De zuivere lijnen waren op het oog geselecteerd uit F_2-families. In elk type was het basis gen *P*, dat zelf geen kleur geeft, doch welks aanwezigheid noodzakelijk is om andere genen een zichtbaar effect op de zaadhuidkleur te doen hebben, homozygoot aanwezig.

2. Het onderzoek was er in de eerste plaats op gericht kwalitatieve gegevens te verkrijgen over het voorkomen van kleurstoffen in de genoemde typen, terwijl daarnaast een aantal schattingen van kleurstof-hoeveelheden uitgevoerd werden. Anthocyaninen, flavonol-glycosiden en leuco-anthocyanidinen, alle stoffen, behorende tot de groep der flavonoide verbindingen, werden in het materiaal aangetroffen. In totaal werden met behulp van papierchromatografie 18 verschillende verbindingen geïsoleerd, waarvan er 15 papierchromatografisch en met behulp van absorptie-spectrofotometrie geïdentificeerd konden worden. De structuren van deze verbindingen zijn weergegeven in fig. 1 en 4, blz. 3 en 23 en de resultaten van de kwalitatieve analyse der twaalf typen zijn samengevat in tabel 38, blz. 38 en 39.

II. Conclusies betreffende de werking der genen

1. Het genenpaar $V - v^{lae}$ controleert het aantal hydroxylgroepen dat aanwezig is in de B-ring der gevormde verbindingen. Bij aanwezigheid van v^{lae} worden verbindingen met één en met twee hydroxylgroepen gevormd, in aanwezigheid van *V* worden voornamelijk verbindingen met drie hydroxylgroepen gesynthetiseerd. Het is aannemelijk te stellen, dat de werking van *V* plaats heeft op een precursor, waaruit alle genoemde verbindingen worden gesynthetiseerd. Genenparen met een analoge werking zijn reeds eerder in andere plantensoorten aangetroffen. In het huidige onderzoek is gebleken, dat het aantal hydroxylgroepen in de B-ring van invloed is op de werking van andere genen, die de structuur van het heterocyclische gedeelte in de gevormde verbindingen bepalen.

2. De multiple allele serie $C^r - C - c^u$ bepaalt de vorming van anthocyaninen en/of flavonol-glycosiden. Bij aanwezigheid van c^u wordt geen van deze verbindingen gevormd. Het gen *C* doet flavonol-glycosiden en anthocyaninen ontstaan, de laatste echter slechts wanneer verbindingen met drie hydroxylgroepen in de B-ring gevormd worden. Het gen C^r doet ook flavonol-glycosiden en anthocyaninen ontstaan, eerstgenoemde verbindingen echter alleen dan in aantoonbare hoeveelheden als geen verbindingen met de trihydroxy-structuur gevormd worden. Een hypothese wordt opgesteld ter verklaring van deze verschijnselen, welke nog niet eerder in enige plantensoort werden waargenomen.

3. Het genenpaar *Sh – sh* bepaalt het voorkomen van leuco-anthocyanidinen, en heeft invloed op de hoeveelheden van anthocyaninen en flavonol-glycosiden die gevormd worden. Bij aanwezigheid van het gen *sh* worden geen leuco-anthocyanidinen gevormd, en is in de regel de productie van anthocyaninen en flavonol-glycosiden laag. Aanwezigheid van het gen *Sh* leidt tot de produktie van leuco-anthocyanidinen, en stimuleert de produktie van anthocyaninen en flavonol-glycosiden, als deze plaats heeft ten gevolge van de aanwezigheid van *C* of C^r. Of de gelijktijdige vorming van deze kleurstoffen leidt tot een toename dan wel tot een afname van de gevormde hoeveelheid leuco-anthocyanidinen lijkt af te hangen van het aantal hydroxylgroepen in de B-ring (bepaald door $V - v^{lae}$). Ter verklaring wordt aangenomen, dat de kleurstoffen en de leuco-

verbindingen naast elkaar gevormd worden, uit dezelfde precursor, of uit precursors welke alleen verschillen in het aantal hydroxylgroepen in de benzeen ring, die later de B-ring der flavonoide verbindingen vormt (de 'pro-B'-ring).

Dit onderzoek was het eerste, waarin een gen gevonden werd dat voor de vorming van leuco-anthocyanidinen verantwoordelijk is.

De samenhang van de in dit onderzoek gevonden resultaten met wat bekend is over de biosynthese der flavonoide verbindingen wordt in het kort besproken.

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