INHERITANCE IN THE CARNATION, DIANTHUS CARYOPHYLLUS. IV. THE CHEMISTRY OF FLOWER COLOR VARIATION, I[1]

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Received April 25, 1947

STUDIES on the relationship between the genetical factors for flower color and the structural variations in the pigments responsible for the colors have given promise of being one of the most useful experimental methods for the investigation of many important questions concerning certain aspects of the action and interaction of genes. Since WHELDALE and BASSET (1913, 1914) first called attention to the possibilities of the method and performed some of the first experiments, advances have been rapid, and great impetus was given to such studies by the final elucidation of the structures of most of the common anthrocyanins and the development of rapid and accurate methods for their recognition by ROBINSON and ROBINSON (1931, 1932, 1933, 1934). Excellent summaries of the results obtained up to about 1940 have recently appeared (SCOTT-MONCRIEFF, 1938; LAWRENCE and PRICE, 1940) and no more than a brief review of certain of the findings will be included in the present discussion.

By far the greatest attention has so far been paid to the genetics of structural variations in the anthocyanin[2] pigments and relatively little exact information is available concerning possible similar variations in the anthoxanthins[3] which almost invariably accompany the anthocyanins in the cell sap and which bear a close structural relationship to them. Studies carried out by BEALE, *et al.* (1939) on *Lathyrus odoratus*, HAGIWARA (1931, 1932) on *Pharbitis nil*, LAWRENCE and SCOTT-MONCRIEFF (1935) on *Dahlia variabilis*, SANDO, *et al.* (1935) on *Zea mays*, BEALE, *et al.* (1940) on *Verbena*, WIT (1937) on *Callistephus chinensis*, and SCOTT-MONCRIEFF on *Primula acaulis* (1932) and *Cheiranthus cheiri* and *Tropaeolum majus* (1936), have shown that certain structural variations in the anthocyanin pigments may be regarded as more or less direct expressions of the activity of particular genes. Among these effects are those brought about by genes which control

[1] The present article presents the results of the first part of a study which because of the war had to be suspended. As there may be some time before full scale resumption of the work can be undertaken it has been deemed desirable to publish the results obtained to date.

[2] "Anthocyanin" is the term applied to the naturally-occurring glycosides of the polyhydroxy-2-phenylbenzopyrilium salts which comprise the class of plant pigments responsible for most of the blue to red colors of flowers and fruits.

[3] "Anthoxanthin" is a rather general term applied to sap-pigments other than those of the anthocyanin type. It refers in most cases to flavone derivatives.

(a) the degree of oxidation (hydroxylation) of the 2-phenylbenzopyrilium nucleus,
(b) the methylation of one or more of the hydroxyl groups in the 2-phenyl nucleus,
(c) the number of sugar residues associated with the anthocyanidin nucleus, and their points of attachment,
(d) the concentration of the anthocyanin in the cell-sap,
(e) the pH of the cell-sap,
(f) the presence or absence of co-pigments.

In most of the cases so far examined two or more (complementary) factors have been recognized as necessary for anthocyanin formation.

Few combined genetical and chemical data are available which show a corresponding factorial control of the concentration and structure of the anthoxanthin pigments. Anthoxanthins modify the colors of anthocyanin-pigmented flowers, the nature of the modifying effect being dependent upon the nature of the anthoxanthin and of the anthocyanin and upon their relative concentrations. The bulk of what is known concerning genetical control of anthoxanthin formation is the result of attempts to correlate such "co-pigmentation" effects with factorial differences. Much of this information is unfortunately inexact and some of it has been shown by later work to be erroneous. The chief source of such error or uncertainty appears to be the assumption, which has sometimes been made, that the isolation of a given anthoxanthin (flavone or flavonol) from a certain flower is evidence for the presence of the same anthoxanthin in other flowers, including different color variations, of the same species. There appears to be little justification for such an assumption and no *a priori* reason why the structure of the flavones or flavonols present in a series of color variations of a given species should remain constant from one genotype to another. Indeed, the very nature of the detailed evidence concerning structural variations of the anthocyanins in a series of related genotypes would make it a more reasonable first hypothesis that a constancy of structural type in the anthoxanthins is not to be expected. Whatever the biogenetic relationship between the anthocyanins and flavone derivatives may prove to be, it is reasonable to suppose, in view of their close structural relationship and the consequent probability that their syntheses are closely interrelated, that the same genetic factors which control the amount and structure of the anthocyanins in a series of color variations may have an influence upon the amount and structure of the accompanying anthoxanthins. The evidence so far available has been insufficient to furnish a factual basis for predicting what this influence will prove to be.

Among the questions which arise in this regard are those dealing with the effect of certain genes, which in the cyanic flowers modify the concentration and structure of the anthocyanins, upon the anthoxanthins in flowers lacking the basic complement of genes necessary for the formation of anthocyanins. Let us suppose for example (fig. 1) that a gene *R* is responsible for the formation of a cyanidin (I) derivative while its recessive allele *r* permits the formation of a pelargonidin (II) derivative only. In two white flowers one carrying

R the other carrying *r* will the anthoxanthin (flavonol or flavone) be related to quercetin (III) (or luteolin (IV)) in the one case and to kampferol (V) (or apigenin (VI)) in the other? Will a gene which affects the concentration of anthocyanins in the cyanic flowers exert a corresponding effect upon the concentration of the anthoxanthins in the acyanic flowers?

The answers to questions such as these will undoubtedly furnish a basis upon which to evaluate and to modify existing ideas concerning the synthetic processes by which the flower pigments and structurally related substances

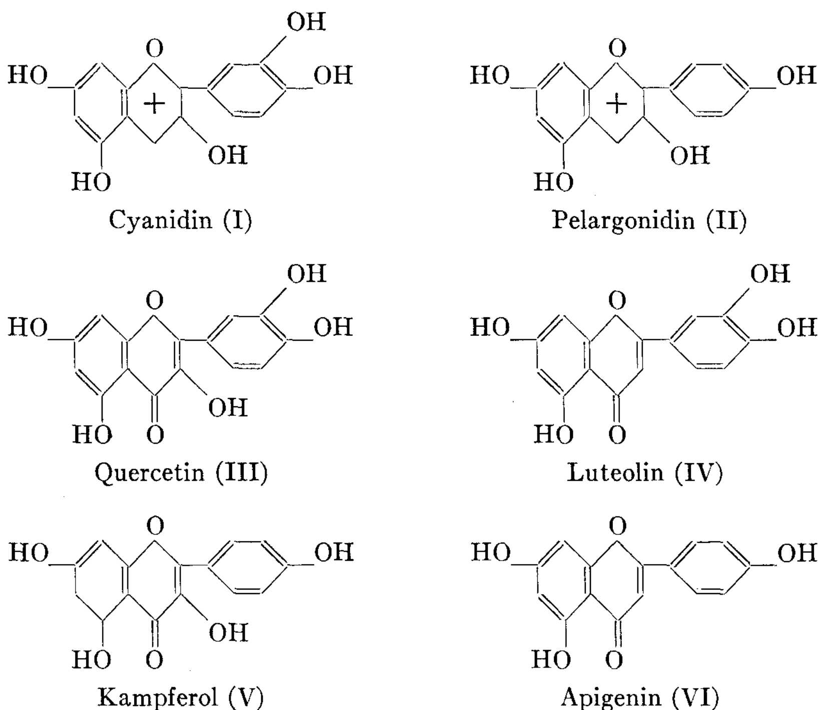

FIGURE 1

are formed. Among the several hypotheses which have been advanced to account for the production of a group of substances so closely related in structure as the anthocyanins and the flavones,[4] two appear to deserve the most serious consideration.

One of these, based largely upon chemical evidence alone (EVEREST, 1918), suggests that the anthocyanins may arise as a result of direct structural modification of a flavone or flavonol by appropriate steps, the last of which is the reduction of a flavonol into the corresponding anthocyanin. The other, resting upon genetical evidence (LAWRENCE and SCOTT-MONCRIEFF, 1935; ROBINSON, 1936), offers the suggestion that the anthocyanins and anthoxanthins arise

[4] The term "flavone" will be used in the generic sense and will frequently refer to flavonols (which are 3-hydroxyflavones) as well.

from a precursor common to both at an early stage but which, in succeeding stages, undergoes modification along two paths, one of which leads to the anthocyanin, the other to the anthoxanthin.

The possibility of direct conversion of flavonol into anthoxyanin is at first sight an attractive one. A comparison of the structures of a representative anthocyanidin (cyanidin (I)) and a typical anthoxanthin (quercetin (III)) shows the simple formal relationship which exists between these classes. The anthocyanidin may be considered as being derived from the flavonol by a reduction of the carbonyl group at C_4 of the latter, a change which gives rise immediately to the anthocyanidin pseudo-base, equivalent, insofar as the state of oxidation is concerned, to the anthocyanidin. It is clear that if any such simple synthetic relationship existed we should expect to find numerous examples of flowers in which the anthocyanin and accompanying anthoxanthin were similarly constituted. Examples of this sort are known but are few in number. SANDO, MILNER and SHERMAN (1935) have found that the purple-husked maize contains a quercetin-3-glucoside and a cyanidin-3-glucoside, and SANDO (1937) has shown that Jonathan apples contain 3-galactosides of both cyanidin and quercetin. EVEREST (1918) has presented inconclusive evidence which indicated that accompanying violanin (a delphinidin derivative) in a so-called black form of the pansy (*Viola tricolor*) is the corresponding flavonol, myricetin. *Rosa gallica*, according to KARRER and SCHWARTZ (1928), contains quercetin-3-rhamnoside and cyanidin-3,5-diglucoside, another example of the correspondence of the aglycones only. In *Antirrhinum majus* both luteolin and apigenin occur in certain color types (WHELDALE and BASSET, 1913, 1914), the related anthocyanidins cyanidin (as a 3-rhamnoglucoside) (SCOTT-MONCRIEFF, 1930) and pelargonidin (as a 2-pentoseglycoside) (SCOTT-MONCRIEFF, 1938) also occurring; but each anthocyanin may be found with either its related or the unrelated flavone. It is to be noted, however, that the identification of the flavones in all of the *Antirrhinum* color-types studied was not carried out. A few other examples showing similar lack of correspondence or partial correspondence make up the bulk of the known cases, and it is important to note, as ROBINSON (1936)has pointed out, that those cases in which complete correspondence of anthocyanin and anthoxanthin have been found have so far been confined to the 3′, 4′-dihydroxy types represented by quercetin and cyanidin, which are by far the commonest structural types of their classes.

The most serious defect in this hypothesis lies in the fact that a lack of correspondence of structural type between the anthocyanin and the flavone derivative in a given flower is the rule, and a more or less complete correspondence, the exception. Numerous specific examples could be cited to show this; a case in point is the observation of BEALE and co-workers (1939) that in *Lathyrus odoratus* the flavonols in highly pigmented types were the same (quercetin and a trace of kampferol) whether the accompanying anthocyanin was derived from pelargonidin, cyanidin or delphinidin (5′-hydroxycyanidin).

Studies on *Dahlia variabilis* by LAWRENCE and SCOTT-MONCRIEFF (1935) have led these authors to suggest that the anthocyanins and anthoxanthins

are derived by parallel rather than by sequential paths from a common, and limited, precursor, and ROBINSON (1936) suggests that this precursor forms one C_6 unit of the final $C_6—C_3—C_6$ structure, the remaining $C_3—C_6$ portion being characteristic of the final substance. Added support for this view is found in the observation that in *Lathyrus* genes have been recognized which result in some cases in a decrease in the amount of anthocyanin and a corresponding increase in the amount of anthoxanthin, and in others in a decrease in the amount of anthoxanthin along with a corresponding increase in anthocyanin (BUXTON, 1932; SCOTT-MONCRIEFF, 1932). However, *Lathyrus* also contains genes which act as specific suppressors of anthoxanthin without affecting the amount of anthocyanin (BEALE, *et al.*, 1939).

An exhaustive criticism of either hypothesis is scarcely justifiable at the present time for two reasons. First, the evidence as to genetically controlled anthoxanthin variations is very scanty, and much of it has been expressed in terms of such generalizations as "anthoxanthins," "copigments" and "ivory" and "yellow flavone," terms which express nothing as to the detailed structure of the substance so designated. Secondly, certain assumptions have occasionally been made concerning the structure of the anthoxanthin present in one color-variation on the basis of a detailed study of another color-variation in the same species (LAWRENCE and SCOTT-MONCRIEFF, 1935).

That such assumptions may lead to error is evident from the observation made in the present work that the flavonol pigment present in some white varieties of the carnation and that present in a bright yellow variety ("Maine Sunshine") are identical. This substance, kampferol (V) (3,5,7,4′-tetrahydroxyflavone), which occurs in the flowers as a glycoside of undetermined nature, is evidently responsible for little or none of the actual pigmentation of the yellow flower. The pigment of the yellow flower is apparently not flavonoid in nature and its character is still unknown. Approximate methods of identification (for example the "ammonia test"),[5] coupled with the bright yellow color of the flowers may have led to the designation of the pigment of this flower as the "yellow flavone" and similar methods applied to the white varieties may have resulted in the conclusion that these contained an "ivory flavone." In both acyanic and anthocyanin-colored variations careful examination is necessary to determine the nature of the flavonoid substances present.

In the cases of the studies on *Dahlia* it is significant to note that what was called in the earlier work the "yellow anthoxanthin" is probably the chalcone, butein, which was later isolated from a yellow form (PRICE, 1939). The provisional and partial structure which had been assumed for the "yellow anthoxanthin" contained the phloroglucinol residue common to most of the anthocyanidins and to the "ivory flavone" which had been isolated earlier; butein, however, contains the resorcinol nucleus, a striking, and certainly important, change from the standpoint of geneticochemical relationships.

[5] Although polyhydroxy flavones and flavonols are not deeply colored their salts are intensely yellow. Consequently, when a flavone-containing white flower petal is exposed to ammonia fumes it becomes yellow; a yellow petal deepens in color.

The point to be emphasized is that very little exact information is available concerning the combined genetic and chemical relationships of pigments other than those of the anthocyanin type. The reasons for this are obvious. The very nature of the anthocyanins lends them to genetical studies since color variations for which they are largely responsible can be easily followed either by inspection of the flowers in the field or with the aid of the simple and accurate tests devised by ROBINSON for the analysis of anthocyanin types on flower-petal extracts. No such ready means for determining either the amount or the nature of the anthoxanthins is yet available. Genetically different white flowers are often difficult or impossible to distinguish in the field and the simple tests available for determining the presence or absence of pigments of the anthoxanthin type can give only an approximate idea of the amount of pigment present and are totally inadequate for determining with any degree of certainty the structure of the compound. If mixtures are present information obtained by simple tests is likely to be more misleading than useful.

Aside from the obvious advantages to be derived from the acquisition of exact information regarding the anthoxanthins present along with the anthocyanins in a colored flower, it seems clear for reasons given earlier that in a species which includes white and yellow (acyanic) color types as well as anthocyanin-colored varieties, the acyanic types deserve equal consideration if an attempt is to be made to correlate chemical structure and pigment concentration with genetical data.

In the course of the investigation of the inheritance of flower color in the carnation (*Dianthus caryophyllus*, Linn.) a genetical analysis of the acyanic (white and yellow) types has been made (MEHLQUIST and GEISSMAN, 1947). Some of these are available for chemical study, and more will be available as their genotypes are established and they can be produced in sufficient quantity.

These genetical studies on color-variation in the carnation have been summarized (MEHLQUIST, 1939) and have recently been described in more detail (MEHLQUIST and GEISSMAN, 1947). The colors fall into three main groups: (1) the acyanic group, composed of the whites and yellows; (2) the transition group in which the color is due to a small amount of pale anthocyanin on yellow background; and (3) the cyanic group in which full anthocyanin colors from salmon to magenta occur. In addition to these there are found variegations which include flaking, striping, shading and the production of various picotee patterns. With one exception (possibly two) the genes which govern the development of these variegations appear to be multiple-allelic to the six main genes governing color variation. These genes have been represented by *Y*, *I*, *A*, *S*, *R* and *M*. So far as the basic genes are concerned the genotypes of the three color groups may be represented thus:

Acyanic group: *Y I a; y I A; y I a; Y i a; y i A; y i a*
Transition group: *Y i A*
Cyanic group: *Y I A*

In the latter two groups the gene pairs *S-s*, *R-r*, and *M-m* bring about modifications in the shade or intensity of the color. Their effects on the members of

the acyanic group are not obvious by inspection of the flowers and it is hoped that chemical studies will reveal any effects which may exist.

The present report is an account of a broad preliminary survey covering determination of (A) the identity of the anthocyanidins and (B) of the anthocyanins of the cyanic flowers (including several variegated forms), (C) some experiments on the application of spectrophotometric and colorimetric methods to the determination of relative and absolute pigment concentrations in crude petal extracts of cyanic forms, (D) an examination of a genetically-controlled variation in pigment concentration in a single color type, and (E) the determination of the identity of the flavones in several selected color forms.

EXPERIMENTAL METHODS AND RESULTS

Most of the flowers used in this study were grown for this purpose in the Ornamental Horticulture experimental gardens of the UNIVERSITY OF CALIFORNIA, Los Angeles. In a few instances flowers of standard commercial varieties obtained in commercial establishments were used. Only such varieties were used whose genotypes were either already known from earlier genetical work or are in the process of being established.

Immediately after collecting the flowers the petals were cut off at the calyx so as to remove the lower, chlorophyll-containing portion of the petal. The discarded portion generally contained little or no anthocyanin. In some cases the fresh petals were used; in others they were dried (a) in air until brittle and then for a short time at 45°C, or (b) in a vacuum desiccator over sulfuric acid. Post-mature and field-dried flowers were not used.

A. Cyanic Varieties—Establishment of the Nature of the Anthocyanidins.

The determination of the anthocyanidin types in the cyanic forms was carried out by the use of the methods developed by ROBINSON and ROBINSON (1931, 1932, 1933, 1934). The fresh petals were ground with sand and extracted with dilute (1 percent) hydrochloric acid. The filtered extract was boiled for about one minute with an equal volume of concentrated hydrochloric acid and the cooled solution extracted first with ether, then with amyl alcohol. The amyl alcohol extract was washed with 1 percent hydrochloric acid, diluted with benzene and the pigment was extracted by washing with 1 percent hydrochloric acid until the benzene layer was nearly colorless. The process was repeated and the final aqueous solution of the anthocyanidin was thoroughly washed with benzene.

The color tests carried out included (a) the color change observed on shaking some of the solution with amyl alcohol, followed by the addition of sodium acetate, (b) the color produced with ferric chloride, (c) extraction with the "cyanidin reagent," and (d) the "oxidation test."

A typical experiment is as follows: To a 1 percent hydrochloric acid extract of *Crimson* petals was added an equal volume of concentrated hydrochloric acid and the solution boiled for about one minute. The cooled solution was washed thoroughly with ether and the anthocyanidin transferred to amyl alcohol. The amyl alcohol layer was separated, diluted with much benzene and

shaken with 1 percent hydrochloric acid. The pigment was again transferred from the aqueous solution to amyl alcohol and again transferred to 1 percent hydrochloric acid. The final aqueous solution was washed thoroughly with benzene. The following tests were performed on samples of the aqueous solution:

1. The solution was shaken with amyl alcohol in the presence of added sodium acetate. The color of the organic layer was red-violet. A drop of ferric chloride solution was added, the mixture shaken and the layers allowed to separate. The amyl alcohol layer was a pure, deep blue.

2. The solution was shaken with "cyanidin reagent" (a mixture of toluene (4 volumes) and cyclohexanol (1 volume)). The organic layer acquired a rose-red color but color still remained in the aqueous layer.

3. A sample of the aqueous solution was shaken with air, half its volume of sodium hydroxide solution (10 percent) was added, followed at once by concentrated hydrochloric acid and amyl alcohol. The pigment was recovered and appeared in the amyl alcohol layer upon shaking the mixture.

These results are characteristic of *cyanidin.*

Only two anthocyanidins have been reported to occur in the carnation color forms previously examined by others (ROBINSON and ROBINSON, 1931; BEALE, *et al.*, 1941) and only two were found in the color forms examined in this work. These are pelargonidin and cyanidin, the first of which is found in the "salmon," "red," "light pink," and "deep pink" forms; and the second in the "lavender," "crimson" and "magenta" forms.

The following color forms were examined (Table 1.):

TABLE 1

COLOR	GENOTYPE	ANTHOCYANIDIN
Salmon	$Y\ I\ A\ s\ r\ m$	Pelargonidin
Red	$Y\ I\ A\ S\ r\ m$	Pelargonidin
Light pink	$Y\ I\ A\ s\ r\ M$	Pelargonidin
Deep pink	$Y\ I\ A\ S\ r\ M$	Pelargonidin
White variegated red (random)	$Y\ I\ a^{var}\ S\ r\ m$	Pelargonidin
White variegated red (picotee)	$Y\ I\ a^{var}\ S\ r\ m\ Pic$	Pelargonidin
Lavender	$Y\ I\ A\ s\ R\ m$	Cyanidin
Crimson	$Y\ I\ A\ S\ R\ m$	Cyanidin
Lavender	$Y\ I\ A\ s\ R\ M$	Cyanidin
Magenta	$Y\ I\ A\ S\ R\ M$	Cyanidin
White variegated crimson (random)	$Y\ I\ a^{var}\ S\ R\ m$	Cyanidin
White variegated crimson (picotee)	$Y\ I\ a^{var}\ S\ R\ m\ Pic$	Cyanidin

It can be said with a considerable degree of certainty that in flowers containing pelargonidin no cyanidin occurs. The highly characteristic blue color produced by the action of ferric chloride on cyanidin solution makes the detection of small amounts of this pigment easy. The pelargonidin-containing extracts studied showed none of this characteristic blue color when treated with ferric chloride. That the cyanidin-containing flowers contain no pelargonidin cannot be inferred by similar observations, since pelargonidin produces no

characteristic color with ferric chloride and small amounts of it in a cyanidin solution would produce no striking modification of the cyanidin reaction. Evidence from another source indicates, however, that in the cyanidin-pigmented magenta (*VIASRM*) carnation the cyanidin (as the glucoside, cyanin) is unaccompanied by pelargonidin derivatives. This conclusion is based upon the observation (see Experimental Part, C.) that spectrophotometric comparison of an unpurified petal extract of magenta carnations with a solution of crystalline cyanin chloride showed that the absorptions of these solutions were practically identical over the range 470–530 mμ.

The ether solutions obtained by the extraction of the hydrolyzed petal extracts, as described above, were evaporated to dryness and the residues taken up in alcohol and treated separately with ferric chloride and with sodium hydroxide solution. Yellow colors with alkali and colors with ferric chloride ranging from brown-green to olive-green were obtained in every case, indicating the presence of substances of the flavone type.

B. Cyanic Varieties—Establishment of the Nature of the Anthocyanins.

ROBINSON and ROBINSON (1931) and BEALE, PRICE and STURGESS (1941) have examined a number of carnation color forms, working with named commercial clones (varieties) of genotypes unknown to or unspecified by them, and have reported the presence in various clones[6] of four anthocyanin types: pelargonidin-3-monoside and -3,5-dimonoside, and cyanidin-3-monoside and -3,5-dimonoside. These observations have been confirmed on known genotypes of the same colors as those tested by ROBINSON and have been extended to some forms not previously examined.

The tests were carried out on freshly collected petals. Samples of these were ground with sand and allowed to stand with 0.5–1 percent hydrochloric acid. The suspensions were all filtered through Super-cel; subsequent treatment of the filtrate varied from case to case. Usually a thorough washing of the aqueous extract with ethyl acetate was the first step, followed in the case of the diglycosides with an amyl alcohol extraction, to remove flavonoid substances. The monoglycosides were purified by removing them from the aqueous extract with a 5 percent solution of picric acid in cyclohexanone and returning them to 1 percent hydrochloric acid after dilution of the cyclohexanone-picric acid solution with petroleum ether. The final dilute hydrochloric acid solutions of the pigments were in every case washed thoroughly with benzene and then with ether, followed by removal of residual dissolved ether under reduced pressure.

The tests employed were those described by ROBINSON and ROBINSON (1931) and by ROSENHEIM (1920), and included (a) observation of the distribution of the pigment between the aqueous solution and amyl and butyl alcohols, with and without the addition of sodium chloride, (b) observation of the color changes brought about by the addition of sodium acetate, sodium carbonate,

[6] The clones (varieties) used by ROBINSON were "Mary Allwood" (salmon); "Brilliant," "Aviator" and "Spectrum" (red); "Improved Ward" (deep pink); "Nigger" and "Topsy" (crimson). Those used by BEALE, *et al.*, were not specified by them.

sodium hydroxide, and ferric chloride solutions to portions of the aqueous extract. In the cases of some of the monoglucosides the test for complex (acylated) sugar residues was performed.

A typical experiment is as follows: A sample of 5.75 g. of the dried, ground petals of the *Scarlet* form was allowed to stand overnight with 30 ml. of 0.5 percent hydrochloric acid. The centrifuged solution was filtered through Super-cel and washed thoroughly with ethyl acetate and with ether. The anthocyanin was extracted with a 5 percent solution of picric acid in cyclohexanone and returned to 1 percent hydrochloric acid after dilution of the cyclohexanone solution with petroleum ether. The resulting aqueous solution was then washed thoroughly with benzene and ether (to remove picric acid) and the last traces of ether removed under diminished pressure.

The final solution was scarlet in color. The pigment was only *slightly* extracted by an equal volume of amyl alcohol but to a considerable extent (not completely) by butyl alcohol. After the addition of salt the pigment was *completely* extracted by butyl alcohol.

The addition of sodium acetate to the solution changed the color to a violet-red, changing to a deeper brownish-red with sodium hydroxide. Ferric chloride did not markedly change the color of the original extract.

The pigment was stable to short treatment at the boiling-point of the solution with sodium hydroxide.

These observations, coupled with identification of the anthocyanidin as pelargonidin in a separate experiment, show that the Scarlet form contains a *pelargonidin-3-monoside.*

The results obtained are as follows (Table 2):

TABLE 2

COLOR	GENOTYPE	ANTHOCYANIDIN	SUGAR-TYPE
Salmon	*Y I A s r m*	Pelargonidin	3-monoside
Red	*Y I A S r m*	Pelargonidin	3-monoside
Light pink	*Y I A s r M*	Pelargonidin	3, 5-dimonoside
Deep pink	*Y I A S r M*	Pelargonidin	3, 5-dimonoside
Lavender	*Y I A s R m*	Cyanidin	3-monoside
Crimson	*Y I A S R m*	Cyanidin	3-monoside
Lavender	*Y I A s R M*	Cyanidin	3, 5-dimonoside
Magenta	*Y I A S R M*	Cyanidin	3, 5-dimonoside
White variegated crimson (random)	$Y\ I\ a^{var}\ S\ R\ m$	Cyanidin	3-monoside
White variegated crimson (picotee)	$Y\ I\ a^{var}\ S\ R\ m\ Pic$	Cyanidin	3-monoside

The nature of the tests carried out does not permit the identification of the sugars present in the anthocyanins. Further, positive proof that such somewhat unusual glycosides as rhamnosides or pentosides were present would have necessitated exact measurements of the distribution coefficients of the pigments between amyl alcohol and water. In view of the findings of ROBINSON

(1931), BEALE (1941) and others it is felt that such refinements were not necessary and that the work of these investigators could be used as the justification for accepting qualitative evidence which agreed with their results.

C. Spectrophotometric and Colorimetric Determination of Pigment Concentrations.

With a view to ultimately extending measurements of pigment concentration to all of the cyanic forms of known genotype, experiments have been performed to determine the relative accuracies of spectrophotometric and colorimetric measurements using crude petal extracts and comparing them with standard solutions of the pure, crystalline anthocyanin the flowers used were known to contain. The flowers chosen were magenta (*YIASRM:* cyanidin dimonoside) and lavender (*YIAsRM:* cyanidin dimonoside). The standard was a sample of crystalline cyanin chloride, isolated from deep-red rose petals and purified by the method of ROBINSON and TODD (1932). The instruments employed were a Beckman Photoelectric Spectrophotometer and a Fisher Photoelectric Colorimeter equipped with a green filter.

A determination of the absorption spectrum of cyanin chloride was carried out by measuring the transmissivities of solutions prepared by diluting with 1 percent hydrochloric acid, a standard stock solution of the crystalline pigment ($C_{27}H_{31}O_{16}Cl \cdot 3H_2O$) in 1 percent hydrochloric acid. Measurements were made over the range 400–540 mμ, using a cell 1.30±0.01 cm in cross-section. The values of ϵ (molar extinction coefficient) were calculated from the expression $\epsilon = \frac{\log I_0/I}{cl}$, where c = moles/liter, l = 1.30 cm. Values of I_0/I below 8.0 were found to deviate significantly from the averages obtained in a series of varying concentrations and were dropped in determining ϵ values. The best average values are tabulated below (Table 3):

TABLE 3

λ(mμ)	$\epsilon \times 10^{-3}$
400	4.40
470	14.30
480	18.75
490	24.20
500	28.45
510	31.15
520	30.60
530	26.00
540	17.50

Magenta. (*YIASRM*) (A) A 1.00 g. sample of the dried petals was ground to a fine powder and extracted with cold 1 percent hydrochloric acid until the petal meal was colorless. The extract was clarified by centrifuging and by filtering it through a layer of Super-cel, and finally made up to 500 ml in a

volumetric flask. The absorptions of four solutions prepared by diluting 0.50, 1.00, 1.50, and 2.00 ml of this stock solution to 10 ml with 1 percent hydrochloric acid were measured in the spectrophotometer. Using the values of ϵ found for cyanin chloride, values for concentration were calculated for each of the four solutions using measurements made at 10 mμ intervals over the range 470–530 mμ. The values found were as follows (Table 4):

TABLE 4

Magenta.

ml stock solution ml 1% HCL	0.5 9.5	1.0 9.0	1.5 8.5	2.0 8.0
λ(mμ)	concentrations found: (moles/liter) $\times 10^6$			
470	3.47	7.38	10.36	13.83
480	3.53	7.31	10.34	13.64
490	3.40	7.07	9.91	13.06
500	3.39	6.87	9.89	13.13
510	3.36	6.80	9.80	13.18
520	3.41	6.89	9.98	13.50
530	3.44	7.03	10.22	13.51
Average:	3.43	7.05	10.07	13.41
Conc. of stock solution (calc.): (moles/l $\times 10^5$)	6.86	7.05	6.72	6.71
Average: 6.84×10^{-5} mole/l				

From these values the pigment concentration in the dry petals is calculated to be 2.40×10^{-2} gm/gm dry petals.

(B) Colorimetric measurements were made on the same magenta stock solution, using as a standard the cyanin chloride solution. Four solutions having concentrations of 1.00, 0.50, 0.25, and 0.125 (stock 1.00) gave the following values in gm pigment/gm dry petals: 2.52, 2.46, 2.44, 2.32 ($\times 10^{-2}$); average 2.43×10^{-2}. These values are in sufficiently close agreement to show that the relatively rapid and convenient colorimetric measurement is capable of an accuracy adequate for the purpose of exact comparisons between different color forms.

Of considerable significance is the close agreement between the individual concentrations calculated for each wave-length in the spectrophotometric measurement. Since these values are calculated from values determined on pure cyanin chloride it is evident that the shape of the absorption curve for the crude petal extract must be almost exactly that of the curve for the pure pigment. The presence of appreciable amounts of pelargonidin glycosides would cause a definite shift in the curve toward shorter wave-lengths. The small deviations which do occur may be due to slight co-pigment effects due to substances other than anthocyanins present in the crude extracts.

Lavender. (*YIAsRM*) (A) Spectrophotometric measurements were made on a crude petal extract (clarified only) prepared from 0.764 gm of dry petals and made up to 100 ml with 1 percent hydrochloric acid. The following figures were obtained (Table 5.):

TABLE 5
Lavender.

ml stock solution	5	5	5	10
ml 1% HCl	15	10	5	5
λ(mμ)	concentration $\times 10^6$ (moles/1)			
490	3.21	4.05	5.96	7.35
500	3.08	4.03	6.00	7.40
510	3.11	4.05	6.02	7.45
515	3.16	4.12	6.11	7.68
520	3.18	4.21	6.29	7.95
530	3.39	4.53	6.84	8.85

From these figures the concentration of the stock solution is calculated to be 12.55×10^{-6} moles/1 and the pigment content of the petals 0.115×10^{-2} gm/gm dry petals.

(B) Colorimetric determination of the concentration of the above stock solution gave a value from which the pigment content of the petals was calculated to be 0.130×10^{-2} gm/gm dry petals. A considerable variation (from 0.152 to 0.124 ($\times 10^{-2}$)) between the values found for various dilutions of the stock solution indicated a considerable deviation of the readings from the standard curve (concentration vs. colorimeter reading) drawn from measurements taken on the pure pigment. A second stock solution prepared from 1.00 gm of dry petals, collected at another time, gave the same average value, 0.130×10^{-2} gm/gm dry petals, and the same variation (0.158 to 0.112 ($\times 10^{-2}$)) over the range of dilutions used.

These variations are interesting in that they show what is probably a co-pigment effect. In the spectrophotometric measurements the absorption markedly increases in the longer wave-lengths (cf. 530 mμ, Table 5), an effect reflected in the "bluing" effect of co-pigments. That a pronounced effect was observed in the colorimetric measurements is probably due to the fact that the green filter used selected a spectral region in which the deviation of the absorption curve of the crude petal extract from that of the pure pigment was becoming marked.

D. Intensity in a Single Color-Type.

There have been segregated in the course of genetic studies three red genotypes differing in shade and identified as *salmon-red*, *standard-red*, and *deep-red*. These are all of the general genotype *YIASrm* and may vary either (a) in having multiple-allelic "*S*" genes, or (b) an inhibition gene (*N*). Qualitative tests have shown that these are all pigmented by pelargonidin and there

is reason to believe that all of them contain this anthocyanidin in 3-monosidic combination.

The pigment concentrations in the *salmon-* and *standard-reds* were determined relative to that in the *deep-red* (=1). Aqueous extracts (1 percent HCl) of weighed amounts of the dried petals were made up to known volumes. Using a Fisher Photoelectric Colorimeter, a standardization curve was drawn using as a standard the *deep-red* solution prepared by making up the extract of 1.00 g of the dried petals to 250 ml with 1 percent HCl. Comparisons of the readings made with solutions of the other two reds made up in the same way gave the following results:

DEEP RED (1.00 G MADE UP TO 250 ML)

SOLUTION	READING	CONCENTRATION
Stock	87.4	1.00
*	74.7	0.50
*	56.2	0.25
*	36.7	0.125
*	21.3	0.0625
*	11.3	0.0312

STANDARD RED (1.00 G MADE UP TO 250 ML)

SOLUTION	READING	CONCENTRATION
Stock	76.6	
*	57.3	
*	37.5	0.526 (ave.)
*	22.4	
*	12.4	
Stock†	76.3	
*	56.2	0.500 (ave.)
*	35.8	
*	20.3	

SALMON-RED (1.00 G MADE UP TO 250 ML)

SOLUTION	READING	CONCENTRATION
Stock	57.5	
*	36.7	0.242 (ave.)
*	20.8	
*	10.5	

* Each solution was prepared by diluting the preceding one to twice its volume with 1 percent HCl.

† Different sample, gathered at another time from different plants.

These results indicate that the pigment concentrations are in the ratio 1:2:4 within the limits of fairly good agreement, in this series of variations. A probable error of up to about 5 percent is indicated in the agreement to that extent between the two samples of standard red, gathered and made up in solution at different times.

E. The Flavone Constituents of Several Selected Color-Forms.

(*A*) *White.* The white flowers used were a mixture of one or more *yIar*, *YIAr* and *YIar* genotypes whose *S-s* and *M-m* constitutions were unknown. The petals were cut off just above the calyx, dried and ground. Extraction of 84 gms (393 gms fresh) with petroleum ether and then with ether resulted in the removal of waxy constituents but these extracts contained no substance soluble in alkali with a yellow color. This shows that the flavone derivatives present in the flowers are all in glycosidic combination.

Extraction of the ether-extracted meal with 95 percent alcohol yielded a brown solution which was evaporated under reduced pressure. The syrupy residue was taken in water and treated with lead acetate. No precipitate was formed, indicating that flavone derivatives having the 3′, 4′-dihydroxy grouping were probably absent. After removal of the lead with hydrogen sulfide the solution was concentrated to 100 ml and refluxed for two hours, with the addition of 1 ml of concentrated sulfuric acid. The brown precipitate was dissolved in alcohol, ether was added and the alcohol was removed by washing with water. Evaporation of the ether layer yielded an oily residue which after solution in alcohol and precipitation with water was obtained as a somewhat amorphous yellow solid weighing 0.55 gm. It gave a yellow solution in alkali, a green-brown color with ferric chloride, and a red color when reduced in alcoholic solution with magnesium and hydrochloric acid. When a solution of this substance in alcohol was treated with aqueous lead acetate some precipitate was obtained. Not precipitated, and recovered from the alcoholic solution, was 0.22 gm, isolated as a yellow, crystalline material, m.p. 245–255°. A part of this substance was sublimed at 200–220°/2mm, yielding two fractions, one melting (with decomposition) at 275–276° (kampferol) and the other at 259–260°. Acetylation of the lower-melting fraction yielded kampferol tetraacetate, m.p. 182–183.5°.

Anal. Calc. for $C_{23}H_{18}O_{10}$: C, 60.78; H, 4.00. Found, C, 61.08, 60.97; H, 4.25, 4.30.

The acetate did not depress the melting point (182–184°) of an authentic sample of kampferol tetraacetate. Acetylation of the remainder of the crude material yielded kampferol acetate, m.p. 182–183°.

The lead precipitate was decomposed with acetic acid and from it were isolated two substances. One of these was crude kampferol which was converted into its acetate, m.p. 182–183.5°. The other was a white, acidic substance, m.p. 354° (dec.). It gave a violet-red color with acetic anhydride-sulfuric acid and a pink color in standing with concentrated sulfuric acid. It appears to be a sapogenin acid.

Anal. Found, C, 72.30, 72.53; H, 9.30, 9.45; $C_{27}H_{42}O_5$ requires C, 72.58; H, 9.50.

These results indicate that kampferol is the only flavone substance present in the white forms tested. No evidence for the presence of any other flavone or flavonol was obtained.

(*B*) *Yellow.* The petals from 500 "Maine Sunshine" (yellow; probable geno-

type $\left(\frac{Y}{y}\ \frac{i^{var}}{i}\ \frac{a^{var}}{a}\ \frac{s}{s}\ \frac{r}{r}\ \frac{M}{M}\right)$ flowers were separated and dried, yielding 250 g. of dry material. A 100 g sample of this was extracted successively with petroleum ether, ether, methanol, ethanol and dilute ethanol (50 percent), and the last three extracts were combined and evaporated to a syrup. The residue was dissolved in 125 ml of water and saturated lead acetate was added until no further precipitate formed. The small amount of dirty grayish-tan precipitate was removed and the filtrate treated with basic lead acetate. The bright yellow precipitate was collected and made up to a suspension in 100 ml of water, 20 ml of 6 N sulfuric acid was added and the mixture was heated on the steam cone for several hours. The lead sulfate was collected and extracted with alcohol and the aqueous solution was extracted with ether. The combined alcohol and ether extracts were washed thoroughly with water and the resulting (alcohol-free) ether solution washed with aqueous sodium bicarbonate, dried and evaporated. A yellow solid was obtained. This was divided into two approximately equal portions. One of these weighed 60 mg and yielded 130 mg of colorless acetate, m.p., after two recrystallizations from alcohol, 183.5–185° (kampferol acetate). The other portion (70 mg) was recrystallized twice from dilute alcohol, forming pale yellow needles, m.p. 272–275° (dec.); it gave the characteristic color-reactions of kampferol with magnesium-hydrochloric acid (scarlet), concentrated sulfuric acid (yellow, strong green fluorescence) and ferric chloride (olive-green).

The precipitate obtained with neutral lead acetate may have contained a trace of quercetin (the magnesium-hydrochloric acid color had a somewhat more bluish tone than that given by kampferol), but none of this could be isolated.

These results are striking proof that the structure of the flavone constituents of acyanic flowers cannot be deduced from the flower color. The term "ivory flavone" and "yellow flavone" are meaningless unless supported by chemical identification of the substances in question. There is now no reason to suppose that one might not find white flowers containing quercetin (or luteolin) and yellow flowers containing kampferol (or apigenin). Possibly the genes *S-s*, *R-r*, and *M-m* are critical in determining the constitution of the flavone constituents in both white and yellow forms. The genotypes necessary for an examination of this possibility have not yet been available but are in process of preparation for future studies.

(*C*) *Crimson.* The commercial variety "Woburn"

$$\left(\text{probable genotype } \frac{Y}{y^{fl}}\ \frac{I}{i}\ \frac{A^{var}}{a}\ \frac{S}{S}\ \frac{R}{r}\ \frac{m}{m}\right)$$

was used. A 100 gm sample of the dried petals was extracted successively with ether, ethyl acetate (which removed only traces of flavones) and methyl alcohol. The alcoholic extract was treated portionwise with lead acetate and then basic lead acetate, discarding the first fractions (violet to blue) which contained the bulk of the anthocyanin. The last two fractions were worked up

separately. The last fraction yielded only dark, brown, tarry materials after hydrolysis and no crystalline substance could be isolated from these.

The middle fraction was hydrolyzed with dilute acid and the liberated flavones taken up in ether. Fractional precipitation of this material with neutral and basic lead acetate yielded two main fractions:

1. Neutral lead acetate. Hydrolysis of this precipitate, isolation of the flavone and acetylation yielded about 100 mg of a crystalline acetate, m.p. 183.5–189°. This was separated by fractional crystallization into five fractions, m.p. 189–193°, 192–193.5°, 192–193.5°, 191–193.5°, and 187–197°. Recrystallization of the first four fractions yielded a product, m.p. 193–194.5°, which was quercetin pentaacetate (mixed m.p. unchanged.).

2. Basic lead acetate. Hydrolysis of this precipitate and acetylation of the ether-soluble material yielded about 10–15 mg of kampferol acetate, m.p. and mixed m.p. 182.5–184° (mixed m.p. with quercetin pentaacetate, from 163°–).

The significance of the presence in this crimson form of quercetin (in much greater amount than kampferol) and the absence of demonstrable amounts of quercetin in the white and yellow forms examined may lie in the presence of the dominant *R* in the crimson form and its absence in the others. It will be recalled that the presence of *R* in the cyanic forms is associated with pigmentation by cyanidin derivatives, while cyanic forms homozygous for *r* contain pelargonidin derivatives. It is possible, therefore, that the factor *R* may be associated with an oxidation of a precursor at an earlier stage than that at wich the final elaboration of the anthocyanin or flavone structure takes place. It will be of considerable importance to determine the structure of the flavones in yellow and white forms carrying the factor *R*.

DISCUSSION

The experiments described in this paper have shown that the flower petals of the carnation may contain the flavonols quercetin and kampferol, as glycosides, and the anthocyanidins cyanidin and pelargonidin (table 1), both as mono- and diglycosides (table 2). No evidence has been obtained that mixtures of cyanidin and pelargonidin derivatives occur in a single flower and in one case (magenta) definite evidence has been obtained that the cyanidin (as cyanin) is not accompanied by any other anthocyanidin derivative. Mixtures of the two flavonols occur in some cyanic and possibly in acyanic forms, but in the few cases studied one of them has predominated, usually to such an extent that only an indication (e.g., by a color-reaction) of the presence of the other is obtained. In every case examined, the cyanic flowers contain flavones. No "albino" flowers—free of both flavone and anthocyanidin derivatives—have occurred among the color forms so far examined. None of the flowers examined appears to contain carotenoid pigments. A yellow form ("Maine Sunshine") contains, along with kampferol, a yellow pigment which is not flavonoid but the nature of which is still undetermined.

The presence of cyanidin is associated with the presence of the gene *R*; when the recessive *r* is present the anthocyanin is derived from pelargonidin (table 1). These genes have the same effect in variegated forms as in full-colored forms.

The genes *M*(*m*) control the degree of glycosidation of the anthocyanidin, the structure of which is determined by *R*(*r*). Flowers having *M* are (3,5-) dimonosides, while flowers with *m* only are monoglycosides (table 2). Again, these conditions obtain in both full-colored and variegated forms. The known "blueing" effect of the *M* factor depends upon this chemical action of the factor, since it has been recognized by many workers that diglycosides produce distinctly bluer tones in flower petals than do monoglycosides of the same anthocyanins.

Evidence from quantitative studies on magenta (*YIASRM*) and lavender (*YIAsRM*) forms, as well as from inspection of flowers in the field, indicates that the *S*(*s*) genes control the amount of anthocyanin, the constitution of which is determined only by the *R*(*r*) and *M*(*m*) genes. The ratio of the pigment concentrations in the magenta (*S*) and lavender (*ss*) forms is about 20:1 (Experimental Part, C). Further data are needed to show whether this same ratio is always associated with this genetical difference, and whether a different ratio is associated with a heterozygous form (*Ss*) compared with an *ss* form.

No information is at hand at the present time concerning the influence of the *S*(*s*) and *M*(*m*) genes on the nature or amount of flavone present in flowers of the acyanic group. Concerning the effect of the *R*(*r*) genes, some information has been obtained (Experimental Part, E). White flowers of several genotypes but all carrying only the recessive *r*, and one bright yellow flower carrying *r* only, contained the same flavonol, kampferol (V). Aside from the bearing this observation has upon the function of *R*(*r*), this result is important in demonstrating that visual differences in acyanic flowers are not necessarily associated with differences in the flavone derivatives they contain. Until acyanic flowers containing the dominant *R* are available for study no more can be said about the influence of this gene in flowers of this type. In view of the additional observation that a crimson form ("Woburn," having *Rr*) contained quercetin (III), along with a much smaller amount of kampferol, it would appear that the *R*(*r*) gene determines the state of oxidation of the flavone derivative in the same way that it determines the structure of the anthocyanidin. It may be significant that "Woburn" is heterozygous for *R* and contained both quercetin and kampferol. On the basis of this very scanty evidence it might be predicted that a flower having the genotype *RR* would contain quercetin only and a scarlet form (*rr*) would contain kampferol only. These predictions must await further experiments for confirmation.

Since the white flowers used contained a mixture of genotypes among which were both *Y* and *y* forms, it can be said that, since only kampferol was found in these, the *Y*(*y*) genes do not directly influence the structure of the 2-phenyl nucleus of the flavone.

It appears that none of the six basic gene pairs so far referred to is solely responsible for the formation of the fundamental C_6–C_3–C_6 skeleton from which the flavones and anthocyanins may be considered to be derived. This conclusion is based largely upon numerous applications of the "ammonia test" to flowers in the field and in the laboratory, in the course of which forms recessive for at least one of all of the basic factors have come under scrutiny. No

acyanic flower has yet been found which does not show qualitative indications of the presence of flavone derivatives.

The results obtained in the present study resemble in general many of those reported by other workers in studies in other flowers. The data at hand, coupled with information obtained from the fundamental genetic studies (MEHLQUIST and GEISSMAN, 1947) may be summarized as follows:

(1) The complementary genes *Y*, *I* and *A* are necessary for full anthocyanin production. Flowers recessive for *Y* and/or *A* are white. Flowers recessive for *I* and carrying the dominant *Y* and *A* genes are lightly anthocyanin-pigmented on a yellow ground and belong to what is called the "transition group." Flowers recessive for *I* and *A* are pure sulfur yellow. Flowers recessive for *I* and *Y* are pale yellow as are the triple recessives *y i a*.

(2) Flowers carrying *Y*, *I* and *A* produce full concentration of anthocyanin color only in the presence of *S*; flowers carrying the recessive allele *s* are pale in color and include the light pink, salmon and lavender shades. The gene *S*, together with one, possibly two, modifying factors, operating as suppressors, appears to determine the exact amount of anthocyanin produced.

(3) The production of cyanidin derivatives through the gene *R* is dominant to the production of pelargonidin derivatives (*r*).

(4) Flowers having the *M* gene produce anthocyanidin diglycosides, while with the recessive allele, *m*, monoglycosides are formed. This gene operates in the same way whether the anthocyanidin is cyanidin or pelargonidin.

(5) Certain flowers carry one or possibly two inhibition or suppression genes which appear to operate in a strictly quantitative manner. Three different red forms, genetically identical except for the dosage of these suppression genes, contained amounts of anthocyanin which were in the ratio 1:2:4.

The points just discussed are recapitulated in table 6.

INTERPRETATION OF RESULTS

The following comments are based upon all of the information so far available concerning inheritance in the carnation; this includes that from the chemical evidence described in the present paper and from genetical data described in two other papers (MEHLQUIST, 1939; MEHLQUIST and GEISSMAN, 1947).

In considering a possible scheme to account in terms of biosynthetical relationships for the chemical effects produced by the various individual color genes, it will be of importance to determine so far as possible what specific role each gene plays in the elaboration of the final structures of the pigments present in a flower.

The function of the genes *S* (together with one of more modifying genes) appears to be solely one of control of the amount of anthocyanin, whereas the nature of the anthocyanin, both in its state of hydroxylation and the number of sugar residues present, is determined only by *R* and *M* and is independent of the allelic state of *S*. Whether *S* controls the concentration of flavone as well is not yet known.

The gene *M* appears from present evidence to be concerned solely with the change from an anthocyanidin monoglycoside to an anthocyanidin diglycoside.

TABLE 6

FLOWER CARRYING*	PLUS GENE	RESULTING COLOR
YA	*I*	Full anthocyanin color on white (ivory) ground
	i	Pale anthocyanin on yellow ground
YI	*A*	Full anthocyanin color
	a	White
IA	*Y*	Full anthocyanin color
	y	White
iA	*Y*	Pale anthocyanin on yellow ground
	y	Pale yellow
Ya	*I*	White
	i	Yellow
yA	*I*	White
	i	Pale yellow
yi	*A*	Pale yellow
	a	Pale yellow
ya	*I*	White
	i	Pale yellow
ia	*Y*	Yellow
	y	Pale yellow
YIA	*S*	Pelargonidin or cyanidin
	s	Pale pelargonidin or pale cyanidin
YIAS	*R*	Cyanidin
	r	Pelargonidin
YIAs	*R*	Pale cyanidin
	r	Pale pelargonidin
YIA (S or s)	*M*	3, 5-Dimonoside
	m	3-Monoside

* Results have been the same whether the plants tested have been homozygous or heterozyous for the dominant genes.

There are two stages in the anthocyanin synthesis at which this gene may operate: (1) it may effect the union of a sugar residue with a preformed anthocyanidin monoglycoside, or (2) it may direct the earlier stages of the synthesis in such a way that the precursor of the final pigment is a polysaccharide which contains the preformed glycosidic linkages, which then remain unchanged throughout the subsequent synthetic processes and appear in the final product.

It may be possible to choose between these alternatives by an examination of the glycosidic linkages existing in the flavones present in two acyanic flowers which differ only in the *M* gene.

Flowers recessive for *Y* produce no anthocyanin except under very favorable conditions (MEHLQUIST and GEISSMAN, 1947). An intermediate allele y^{fl} produces a flush of anthocyanin when it is accompanied by both *I* and *A*, but in the presence of *y* the flowers are pale yellow or white depending upon whether *I* is present in the recessive or dominant condition.

Since flowers recessive for *Y* do produce flavone derivatives, *Y* is not necessary for the formation of the fundamental C_{15} unit from which the final products are formed. It is conceivable that what is at present regarded as the recessive allele of *Y* is really an intermediate in a series of multiple alleles, of which the as yet undiscovered lowest recessive may lack the power of producing the fundamental precursor; at present, however, there is no evidence to support such an assumption. *Y* cannot be responsible for the degree of oxidation (hydroxylation) of the flavonol since flowers dominant for *I* (and carrying *r*) contain kampferol whether *Y* or *y* is present. In the yellow flowers (i.e., recessive for *I*) the intensity of the yellow color is determined by the allelic state of *Y*; that is, *Y*-yellows are deeper in color than *y*-yellows. The meaning of this observation in terms of the chemical constitution of the flower petal is still not clear, since experiments have shown that the bright yellow color of the flowers is due, in part at least, to an ether-soluble, probably non-carotenoid, non-flavonoid pigment of unknown nature. It is probable that the difference in color between pale yellow and a deep yellow form is not due to a higher concentration of flavonol in the latter, since the flavonol present in the deep yellow "Maine Sunshine" is the same as that in certain white forms.

Flowers recessive for *I* still produce anthocyanin in small amounts, but only when both *Y* and *A* are present in the dominant condition. These cases form the faintly-colored transition group. Not enough cases have been examined in sufficient detail to determine whether the allelic state of *I* determines the degree of hydroxylation of the flavones present, but it appears that this is not the case. Evidence based upon visual inspection of various genotypes indicates that *I* does not affect the composition of the anthocyanins; only *S*, *R* and *M* are concerned with that. In short, the chief difference between two flowers differing genetically only in the allelic condition of *I* seems to be that the color of the flower which is recessive for *I* is modified in the direction of yellow, the other pigments being chemically the same in the two cases. The production of the yellow pigment evidently takes place at the expense of anthocyanin: the transition group (*YiA*) are lightly anthocyanin pigmented on a yellow ground, while the cyanic group (*YIA*) show full anthocyanin development on what may be considered a colorless or white (ivory) ground. The gene *I* thus acts as an inhibitor of the process in which some precursor is elaborated into the yellow pigment; when *I* is present in the dominant condition, then, more of the hypothetical precursor is available for the synthesis of anthocyanin, this route being controlled by other genes.

Flowers recessive for *A* like those recessive for *Y*, fail to produce antho-

cyanin. An allele, a^{var}, allows the production of small amounts of anthocyanin in various variegation patterns, but only when *A* is present in the dominant condition can full anthocyanin be produced. The anthocyanin formed when a^{var} is present is chemically the same as that produced in the presence of *A*, if *S*, *R* and *M* are the same in both cases. It is significant to note that the spots or stripes produced in the variegated forms are full-colored areas.

The effect of *A* upon the nature or concentration of the flavones has not been determined. Its function seems to be only that of control of the total amount of anthocyanin. It may act in some synthetic step in which the particular oxidation-level of the C_3 portion of the fundamental carbon skeleton is determined, and is thus a modifying factor operating in some specific oxidation or reduction step upon a supply of precursor already present as the result of the action of other factors.

The gene *S* is necessary for full anthocyanin formation, flowers recessive for *S* containing the same anthocyanin (other factors being the same) as flowers dominant for *S*, but in lower concentration (approximately one-twentieth as much in (*YIAsRM*) as in (*YIASRM*)). Since the flower of the genotype (*YiAs*) is salmon-yellow and that containing (*YiAS*) is orange, it appears that *S* is not concerned in the production of total pigment but, given a sufficient supply of starting material, acts to modify it along a route leading specifically to anthocyanin.

The function of the *R* gene appears to be one of control of the degree of hydroxylation of the 2-phenyl group. Cyanic flowers dominant for *R* always contain cyanidin derivatives; that is true also in the case of flowers carrying variegation factors which produce anthocyanin-pigmented areas upon white backgrounds. Evidence from a limited number of cases indicates that the nature of *R* affects the degree of hydroxylation in the 2-phenyl group of the flavones as well. This may mean that *R* operates at an early stage in the synthetic sequence through which the flavones and anthocyanins are formed, and directs the degree of hydroxylation of some common precursor.

Many questions remain to be answered before all of the suggestions made in the foregoing discussion can be substantiated or elaborated upon. The answers to many of them will undoubtedly be found in a more detailed examination of certain acyanic forms and comparisons of these with suitably constituted cyanic forms.

SUMMARY

1. Chemical studies have shown that the anthocyanidins of cyanic varieties of the carnation are cyanidin (in *R* forms) and pelargonidin (in *rr* forms).

2. The anthocyanins present in cyanic forms are monoglycosides (*mm* forms) and diglycosides (*M* forms).

3. The anthocyanins in the pigmented areas of variegated forms have been found to be controlled by the *R-r* and *M-m* genotype of the flowers in exactly the same way as in the full-colored forms.

4. The flavones present in carnations are kampferol and quercetin, which are probably present in the petals as glycosides. Acyanic forms having *rr* contain kampferol. No acyanic forms having *R* have been examined, but a crimson

form (having *Rr*) was found to contain mostly quercetin along with a small amount of kampferol.

5. All forms studied have contained some flavonoid constituents; no true albinos have been observed.

6. Three scarlet forms, differing in the dosage of an inhibition gene, were found to contain amounts of anthocyanin (pelargonidin monoglycoside) in the ratio 1:2:4.

7. Some suggestions have been made concerning the possible roles of the color genes in the biosynthetic processes by which the pigments are formed.

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