CHEMICAL ASPECTS OF THE INHERITANCE OF FLOWER COLOR IN *IMPATIENS BALSAMINA* L.

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RECENT applications of chromatographic techniques (BATE-SMITH 1948, 1949) have facilitated identification of anthocyanin pigments and related compounds in micro quantities, thus permitting extension of the combined genetic and biochemical work initiated by SCOTT-MONCRIEFF (1931) and extended by many others. The genetic studies by the late D. W. DAVIS and his associates (1958) on the inheritance of flower color in *Impatiens balsamina* provide a material which seems particularly well suited for such studies. The balsams have a short life cycle, large flowers, and rather precise differentiation of color classes. Various flower colors exhibited in this species have been ascribed previously to the presence of glycosides of three anthocyanidins: pelargonidin, peonidin and malvidin, (BEALE *et al.* 1941; HAYASHI *et al.* 1953; FORSYTH and SIMMONDS 1954) but no association of the pigment content with the genetic structure of the plant was made in these studies. The species is one of a family of herbaceous plants which is known to produce leucoanthocyanins (BATE-SMITH 1954) and flavonols (HAYASHI *et al.* 1953); hence an intensive genetical and biochemical study may reveal the metabolic interrelations of several classes of similar compounds.

This paper deals with the correlation between genotype and complement of both anthocyanins and leucoanthocyanins. The data are still limited but allow some conclusions as to stages in the biosynthesis of the anthocyanins.

MATERIALS AND METHODS

Genetic material: One of the authors (R.A.) enjoyed the privilege of working with PROFESSOR DAVIS for several years preceding his death and was able to preserve stocks from which all color types were regained. All plants analyzed were of the noncream series, *i.e.*, *WW*, and only one of each of the several alleles designated *P^r* or *P^s* by DAVIS (1958) was employed in the biochemical studies.

Twelve potential phenotypes are recognized as consequence of interaction of the genes *L*, *l*, *H*, *h*, *P^r*, *P^s*, and *p*, although some, especially *L-H-P^s* and *L-hh-P^s*, can scarcely be distinguished without knowledge of the genetic history. In certain of the paler color classes, there is an obvious quantitative difference between the heterozygote and the dominant homozygote, although no qualitative differences

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in pigment complement have been detected which can be ascribed to gene dosage. In subsequent portions of this paper the dominant homozygote and the heterozygote will be considered equivalent and genotype will be indicated by a single symbol for each locus.

A breeding program is in progress which should ultimately yield isogenic stocks, but analyses presented here were performed on less highly refined material. With few exceptions the pigment complements have been confirmed on segregants of crosses between multiple heterozygotes and an inbred white stock. The exceptions include those genotypes which involve P^s in association with other dominant genes.

Methods of pigment analysis: Three separate methods of pigment extraction have been employed at various stages in the qualitative analysis of the pigments present in the various phenotypes. Since the principal complication of the present study lies in the distinction between derivatives of leucoanthocyanins and those of anthocyanins, each of the methods is described below.

In initial determinations the desired tissue, either fresh or frozen, was boiled for five to ten minutes in one percent HCl, then filtered. An equal volume of concentrated HCl was added to the filtrate and the mixture boiled again for five to 15 minutes. After another filtration, the acid solutions were exhaustively extracted with ether to remove flavonol and other substances which complicated the chromatograms. (The ether extraction was omitted in later assays.) The anthocyanidins were partitioned into a small volume of isoamyl alcohol and applied to chromatograms. These amyl alcohol solutions contained mixtures of anthocyanidins derived from conversion of leucoanthocyanins as well as from hydrolysis of anthocyanins.

Preliminary tests revealed that repeated extraction by boiling with small quantities of 0.04 percent HCl in 95 percent ethanol without maceration of the flower parts would remove only traces of leucoanthocyanins from white flowers yet would completely remove visible pigments from intensely colored flowers. The residual tissue contained considerable leucoanthocyanin which could be converted with unknown efficiency to anthocyanidin and extracted by boiling with 50 percent HCl. These anthocyanidins were partitioned into isoamyl alcohol and applied to chromatograms in determining the class of leucoanthocyanins present in the tissue. The ethanolic extracts, which contained the anthocyanins, were evaporated to near dryness, then hydrolyzed with 50 percent HCl and partitioned into isoamyl alcohol. The aglycones revealed in these latter extracts by chromatography were not entirely reliable as indicators of the anthocyanins present since traces of anthocyanidin were obtained from completely white flowers.

To assay the anthocyanidins present as visible derivatives, the tissues were extracted with one percent HCl in 95 percent ethanol. The extracts were concentrated over a steam table, then applied directly as long streaks and developed in the t-butanol or ethyl acetate solvents (see below). The center of each anthocyanin band was cut from the chromatogram and the pigments eluted from the

paper by allowing one percent HCl in 95 percent ethanol to flow the length of the strip. The eluates were concentrated over steam, hydrolyzed, partitioned into isoamyl alcohol and applied as a solution of the aglycones. Since preliminary tests had shown that at least the bulk of the leucoanthocyanin was immobile in the t-butanol solvent, aglycones determined in this fashion were considered to be derived from anthocyanins. This method has been successful only with the stronger bands of the more intensely colored forms. Anthocyanins of the more weakly pigmented phenotypes have been identified by comparison on the same chromatogram with anthocyanins from the more intensely pigmented flowers.

Pigments were applied as short streaks to Whatman #1 filter paper from isoamyl alcohol or from one percent HCl in 95 percent ethanol depending on the extraction method. The chromatographic technique was essentially the ascending method described by WILLIAMS and KIRBY (1948). Three solvent mixtures were employed for anthocyanidin identification. A mixture of t-butanol, acetic acid, and water (3:1:1) forms a single phase and provides moderate resolution of both anthocyanins and anthocyanidins. Where several anthocyanidins are present in the same extract, however, overlapping may occur, hence other solvents or diagnostic color tests must be employed simultaneously. The Forestal solvent described by BATE-SMITH (1954) gives excellent resolution, while a mixture of m-cresol, acetic acid, and water (55:7:6) accelerates the methylated pigments and permits identification where complex mixtures of pigments occur. Representative Rf values for the principal anthocyanidins in each of these solvents are listed in Table 1.

TABLE 1
Average Rf values of anthocyanidins in three solvent mixtures

	t-butanol: acetic acid: water (3:1:1)	m-cresol: acetic acid: water (55:7:6)	acetic acid: hydrochloric acid: water (30:3:10)
pelargonidin	.64	.63	.72
peonidin	.47	.75	.73
cyanidin	.41	.33	.53
malvidin	.36	.85	.67
petunidin	.30	.48	.50
delphinidin	.18	.12	.32

Extracts of intensely colored flowers yield several anthocyanins. In order to determine the number of anthocyanins present in such extracts, a complex solvent was developed consisting of a mixture of ethyl acetate, t-butanol, acetic acid and water (5:4:1:3). This mixture sweeps flavonols, naphthoquinones, and other interfering substances to the top of the paper while providing excellent definition and separation of the derivatives of individual anthocyanidins. Derivatives of unlike anthocyanidins are moderately well separated. Thus magenta flowers (*LHP*), which yield two anthocyanidins from anthocyanins, provide five distinct bands in chromatograms of unhydrolyzed extracts. Peonidin and

cyanidin derivatives overlap in this solvent but are distinguishable by their fluorescence and their color after exposure to AlCl_3 .

Since Rf values fluctuate with ambient conditions, all identifications were based ultimately on comparisons with anthocyanidins obtained from species which had been investigated by other authors. After considerable selection, the following were chosen as useful sources of standard anthocyanidins: *Pelargonium zonale*—pelargonidin with other pigments; *Salvia splendens*—pelargonidin; *Centaurea cyanus* (deep blue)—cyanidin; *Viola papilionaceae* or *Delphinium ajacis* (blue)—delphinidin; *Peonia lactiflora* (deep red)—peonidin; *Parthenocissus quinquefolia* (fruits)—malvidin; and *Petunia hybrida* var. Alderman—petunidin with malvidin. Quantities of these were frozen and extracted as needed.

While certain of the ROBINSONS' tests (1931) were employed in early stages, the only reagent used regularly was two percent AlCl_3 (ethanolic). When sprayed on chromatograms this accentuates color differences. Pigments containing orthohydroxyl groups (cyanidin, delphinidin, and petunidin) turn blue whereas others are relatively unaffected.

Certain genotypes control the production of flowers which are distinctly bi-colored. While in some cases this can be attributed to differences in the concentration of the same pigment, in others the difference in hue suggests that the pigment complements are different. Preliminary tests of pink flowers (*lhP*^r) revealed real differences in the anthocyanidins present, hence in all subsequent analyses petals and sepals were examined separately. The single upper petal which is exposed in the bud and is somewhat intermediate in color was omitted from both samples.

RESULTS

Leucoanthocyanins: Anthocyanidins in extracts of buds (Table 2, column a) were derived entirely from leucoanthocyanins since care was taken to exclude any buds which had begun to show visible pigmentation. Leucoanthocyanins are present in both sepals and petals of the earliest bud stages which could be dissected (about 3 mm length) and occur also in other tissues. The buds analyzed by chromatography ranged from six to nine millimeters in length and were not separated into size classes.

Cyanidin and delphinidin were obtained from buds of all phenotypes with cyanidin generally present in excess. Variations in the relative quantity of delphinidin could not be associated with the presence or absence of any single gene. The dominant allele at the *H* locus, however, is perfectly correlated with the presence of a leucoanthocyanin which yields pelargonidin. No qualitative differences between sepals and petals of colorless buds have been detected.

Leucoanthocyanins of mature pigmented flowers were examined after prior removal of the visible pigments through alcoholic extraction. The leucopigments of mature flower parts (Table 2, column d) seem identical with those of immature buds with the one exception that delphinidin has not been obtained from the sepals of pink flowers (*lhP*^r). Cyanidin was abundant in these extracts hence it is possible that delphinidin was overlooked. Only traces of delphinidin were

TABLE 2

Anthocyanidins from genetic races of Impatiens balsamina by various extraction methods

PETALS							
Genotype	Phenotype	Colorless buds	Mature flowers				Anthocyanidins from anthocyanins
		(a)	(b)	(c)	(d)	(e)	(f)
<i>lhp</i>	White	C,D	C,D	C	C,D		none
<i>Lhp</i>	Pale lavender	C,D	C,D,M		C,D		M
<i>lHp</i>	Pale pink	P,C,D	P	P	P,C,D		P
<i>LHp</i>	Pale pink-lav.	P,C,D	P,D,M	P,M	P,C,D		P,M
<i>lhP^g</i>	Pale pink	C,D	P,C	P,C	C,D		P
<i>LhP^g</i>	Lavender	C,D	M	M,C(tr.),D	C,D		M
<i>lHP^g</i>	Rose	P,C,D	P	P	P,C,D		P
<i>LHP^g</i>	Lavender	P,C,D	P,M	P,C(tr.), D(tr.),M	P,C,D		P,M
<i>lhP^r</i>	Pink	C,D	P,Peon.	P,C	C,D(tr.)	P	P,Peon.(?)
<i>LhP^r</i>	Purple	C,D	M	M	C,D	M	M
<i>lHP^r</i>	Red	P,C,D	P	P	P,C,D	P	P
<i>LHP^r</i>	Magenta	P,C,D	P,M	P,M	P,C,D	P,M	P,M

SEPALs							
Genotype	Phenotype	Colorless buds	Mature flowers				Anthocyanidins from anthocyanins
		(a)	(b)	(c)	(d)	(e)	(f)
<i>lhp</i>	White	C,D	C,D	C	C,D		none
<i>Lhp</i>	White(?)	C,D			C,D		none(?)
<i>lHp</i>	White(?)	P,C,D	P,C	no yield	P,C,D		none(?)
<i>LHp</i>	White(?)	P,C,D	C,D,M	no yield	P,C,D		M(?)
<i>lhP^g</i>	Pale pink	C,D	C	C,Peon.	C,D(tr.)		Peon.
<i>LhP^g</i>	Pale lavender	C,D	M	M,C(tr.)	C,D		M
<i>lHP^g</i>	Pale pink	P,C,D	P,Peon.	P,C	P,C,D(tr.)		P,Peon.
<i>LHP^g</i>	Pale lavender	P,C,D	M	C,D(tr.), M(tr.)	P,C,D		M
<i>lhP^r</i>	Deep pink	C,D	C,Peon.	C,Peon.	C	C,Peon.	C,Peon.
<i>LhP^r</i>	Lavender	C,D	M	C,M	C,D(tr.)	M	M
<i>lHP^r</i>	Pink	P,C,D	P,Peon	P,C	P,C,D	P,C,Peon.	P,C,Peon.
<i>LHP^r</i>	Lavender	P,C,D	M	C(tr.),M	P,C,D		M

The pigments in columns (a) and (b) were extracted with aqueous one percent HCl. Pigments of column (c) were obtained through extraction with ethanolic 0.04 percent HCl, and the colorless residual tissue yielded the pigments of column (d) after boiling with 50 percent HCl. Column (e) lists the aglycones obtained by hydrolysis of anthocyanins eluted from chromatograms. Column (f) lists the anthocyanidins believed to have been recovered from visible pigments in the flowers.

Specific anthocyanidins are represented by the following symbols in the table: pelargonidin = P; cyanidin = C; delphinidin = D; malvidin = M; peonidin = Peon.

present in similar extracts from petals of the same flowers. There seems no explanation for its absence which may be directly attributed to the gene *P^r* since corresponding combinations involving the alleles *P^g* and *p* contain leucodelphinidin as do all other combinations involving *P^r*.

Data from stem material are incomplete. Simultaneous extraction and hydrol-

ysis in boiling 50 percent HCl yields cyanidin from all genotypes tested and a nearly equal quantity of pelargonidin from several types containing *H*. A trace of pelargonidin may be present in *lhP^r* stems, and definite traces of delphinidin appear on chromatograms developed in t-butanol. Thus the pattern of extractives from stems analyzed to date follows generally that of the colorless buds. Since *lhP^r* stems are red, the pelargonidin in these may be in the form of an anthocyanin rather than a leucoanthocyanin. Pelargonidin is probably derived in part from anthocyanin in *LHP^r* and *LHP^r* as well as *lhP^r*.

Anthocyanidins from anthocyanins: Aqueous solvents removed considerable leucoanthocyanins from tissues. Alcoholic solvents removed less, but nevertheless traces of anthocyanidin from this source could still be obtained in extracts of white flowers. The presence of an anthocyanidin in total extracts which was not represented among the leucoanthocyanins, however, has been considered an indication of its presence as an anthocyanin, especially where this is also indicated by the color of the flower (in Table 2, compare columns b and c with d). Reliable evidence that an anthocyanidin was derived from an anthocyanin was also gained through chromatography of the glycosides followed by elution, hydrolysis, and rechromatography of the aglycones (Table 2, column e). Direct comparison of anthocyanins extends the information to paler flowers where bands were not sufficiently strong for elution and hydrolysis (see Table 3). The anthocyanidins considered present in each genotype as anthocyanins are summarized in the final column of Table 2.

Malvidin occurs in extracts only when *L* is present in the genotype. Pelargonidin always occurs when *H* is present, but is also found in petals when only *P^r* or *P^s* are present. The pelargonidin producing action of *P^r* (or *P^s*) is masked by the presence of *L*, although *L* is not epistatic to *H*.

In the sepals, both *P^r* and *P^s* govern the production of peonidin derivatives. Cyanidin derivatives occur with *P^r* but have not been detected with *P^s*. These products are apparently eliminated with the introduction of *L* into the genotype but are not eliminated by *H*.

No single gene is essential to anthocyanin formation, for only the multiple recessive is truly white. Of the alleles at the '*p*' locus, *P^r* invokes more intense pigmentation than occurs in corresponding combinations involving *P^s*, and combinations involving *P^s* produce more pigment than those involving *p*. This effect is reflected in the stem where *P^r* brings about distinct red coloration through at least the lower-most internode, *P^s* provides a faint rose flush in seedling stages and *p* has no apparent effect.

Anthocyanins: Each anthocyanidin is represented in balsams by two or more derivatives. These have been classified through chromatography in the ethyl acetate solvent without prior hydrolysis and are listed for each genotype in Table 3. The major bands of all combinations involving *P^r* have been eluted, hydrolyzed, and rechromatographed as aglycones. The bands labeled pelargonidin 3 and cyanidin 2 have not been successfully eluted in sufficient quantity for analysis, but have been assigned on the basis of fluorescence and color after spraying

TABLE 3

Anthocyanins from genetic strains of Impatiens balsamina

Genotype	Pelargonidin			Peonidin		Cyanidin			Malvidin	
	1	2	3	1	2	1	2	3	1	2
Petals										
<i>lhp</i>	..	..	..	..	..	..	..	..	..	..
<i>Lhp</i>	..	..	..	..	..	..	..	..	+	..
<i>lHp</i>	+	+	..	..	..	..	..	..	..	..
<i>LHp</i>	..	..	..	..	..	..	..	..	+	..
<i>lhPg</i>	+	+	..	..	..	..	..	..	..	..
<i>LhPg</i>	..	..	..	..	..	..	..	..	+	+
<i>lHPg</i>	+	+	+	..	..	..	..	..	..	..
<i>LHPg</i>	+	+	..	..	..	..	..	..	+	+
<i>lhPr</i>	+	+	..	..	..	..	..	..	..	..
<i>LhPr</i>	..	..	..	..	..	..	..	..	+	+
<i>lHPr</i>	+	+	+	..	..	..	..	..	..	..
<i>LHPr</i>	+	+	+	..	..	..	..	..	+	+
Sepals										
<i>lhp</i>	..	..	..	..	..	..	..	..	..	..
<i>Lhp</i>	..	..	..	..	..	..	..	..	..	..
<i>lHp</i>	..	..	..	..	..	..	..	..	..	..
<i>LHp</i>	..	..	..	..	..	..	..	..	..	..
<i>lhPg</i>	..	..	..	+	+	..	..	..	..	..
<i>LhPg</i>	..	..	..	..	..	..	..	..	+	..
<i>lHPg</i>	+	+	..	..	..	..	..	..	..	..
<i>LHPg</i>	..	..	..	..	..	..	..	..	+	..
<i>lhPr</i>	..	..	..	+	+	+	+	+	..	..
<i>LhPr</i>	..	..	..	..	..	..	..	..	+	+
<i>lHPr</i>	+	+	..	+	..	+	..	..	..	..
<i>LHPr</i>	..	..	..	..	..	..	..	..	+	+

with AlCl_3 . Neither of these is likely to be confused with derivatives of other anthocyanidins known to occur in the same flower parts. In addition, the band labeled pelargonidin one appears to be doubled in some chromatograms of extracts of petals of *lhPr*, *lhPg*, and *lHPr*. This suggests the presence of another pelargonidin derivative.

The anthocyanins present in plants carrying *P^s* or *p* have been matched with the more prominent components of the corresponding classes involving *P^r*. Where bands are absent in chromatograms of extracts of the more weakly pigmented flowers, the missing bands are always the minor components of extracts of intensely pigmented forms. The absence of such bands suggests a failure to achieve sufficiently concentrated extracts rather than absence of the specific derivatives in the flowers. It is noteworthy, however, that pelargonidin is present in lower concentrations relative to malvidin in the combinations *LHp* and *LHP^s* than in *LHP^r*. In *LHp*, pelargonidin derivatives were not detected among the anthocyanins although the color of the petals suggests their presence.

DISCUSSION

Genetic regulation of flower color in *Impatiens balsamina* influences the quantity and nature of the anthocyanidin nucleus rather than the nature of the glycosidic or acyl groups attached or such factors as copigmentation and pH of the cell sap. The genes must, therefore, affect specific stages in the synthesis of the anthocyanidins and perhaps of related flavonoids.

The classical hypothesis concerning the origin of these compounds was developed by ROBINSON (1935). A polyphenol resembling phloroglucinol is assumed to combine with a polyhydroxyphenylpropane derivative to yield a precursor resembling I. An alternative proposal by BIRCH and DONOVAN (1953) involves head to tail condensation of three acetate units with an appropriate C_6-C_3 unit. In this way the fused phenyl (A) ring formation would not involve free phloroglucinol.

Strong support for the "acetate theory" has come from recent work. WATKINS *et al.* (1957) have evidence that neither mesoinositol or phloroglucinol is incorporated into rutin in buckwheat, and WEYGAND *et al.* (1957) found that C^{14} -labeled mesoinositol was not incorporated into rubrobrassicin in red cabbage seedlings. Watkins also reported results from the feeding of sodium acetate-1- C^{14} and sodium acetate-2- C^{14} which indicate that labeled acetate is selectively incorporated into the A ring. Finally, GRISEBACH (1957) who likewise used sodium acetate-1- C^{14} and sodium acetate-2- C^{14} found that, in red cabbage seedlings the pattern of C^{14} present in the A ring of rubrobrassicin represents the pattern which would be expected on the basis of the "acetate theory." This pathway could also yield a compound such as I in Figure 1. A cyclic form of this compound would bear a close resemblance to melacacidin (II), a leucoanthocyanin isolated from wood of *Acacia melanoxylon* by KING and BOTTOMLEY (1953). Melacacidin yields an anthocyanidin which is not known in nature, but other leucoanthocyanins are thought to be similar in structure (BATE-SMITH 1953; SWAIN 1954).

The leucoanthocyanins of the balsam have never been characterized. By analogy they are perhaps flavandiols, like melacacidin, with the hydroxylation patterns of pelargonidin, cyanidin, and delphinidin. Through appropriate dehydration and oxidation or reduction the various flavonoid compounds, including flavonols like quercetin (III) and anthocyanidin (IV) could arise. The leucoanthocyanins, however, are not necessarily *in vivo* precursors of the anthocyanins and may represent a class of compounds independently derived from a common precursor resembling I. The five anthocyanidins derived from balsam tissues differ only in the groups appended to the free phenyl ring (IV,B).

In the balsams the series p , P^s , and P^r leads to increased anthocyanin production in the order listed when alone or in corresponding combinations with L and H . Since several anthocyanidins occur in plants carrying P^r or P^s as the only dominant gene and since it is unlikely that the locus would affect both the total anthocyanin production and the specific hydroxylation or methoxylation pattern, the gene action must allow anthocyanin production from fragments of appropriate structure which are present at the time of synthesis. The distinction be-

FIGURE 1.—Compounds of interest in the consideration of the biosynthesis of flavonoids: I Hypothetical precursor of the flavonoid nucleus; II melacacidin; III quercetin; IV anthocyanidin.

tween various alleles grouped as P^s or as P^r by DAVIS (1958) lies largely in the extent and pattern of anthocyanin production, so it is probable that even qualitative differences among alleles of p reflect differences in the time or extent of gene action rather than specific regulation of ring substitution.

Several possibilities as to the nature of the specific chemical effect of the dominant alleles of p are suggested, but a choice between these awaits more data on quantitative relations between the anthocyanins and other flavonoid compounds. Another complicating factor, frequently overlooked, is that theoretical competition between groups of compounds for a common moiety may not actually occur due to differences in time of synthesis of the different types. For example, in the balsam leucoanthocyanins appear in buds much earlier than anthocyanins and somewhat earlier than flavonols.

The genes H and P^r must have both an additive and synergistic action. The total pelargonidin production in petals of plants carrying H and P^r is considerably greater than the sum of the production in lHp and lhP^r , yet those pigments of sepals which are characteristic of lhP^r persist in lHP^r . Addition of L to the complement eliminates the products of P^r but does not eliminate the enhanced pelargonidin production associated with the synergism of H and P^r . H

must, therefore, make available for anthocyanin synthesis a precursor which is susceptible to the reactions promoted by *Pr* but which is not formed at the expense of the precursors available to *Pr* in the absence of *H*.

At present the stage in the biosynthesis of flavonoids at which the hydroxylation or methoxylation pattern is established is unknown. Moreover, it is uncertain whether the leucoanthocyanins are formed independently from a common precursor or are able to serve directly as precursors for the anthocyanins. In balsams *H*, but not *Pr*, favors production of the pelargonidin configuration in both leucoanthocyanins and anthocyanins, but this could represent modification of a common precursor before completion of the flavonoid nucleus. Similar correlation between anthocyanin and leucoanthocyanin structure has been reported previously by COE (1955) in maize and BEALE *et al.* (1939) in sweat pea. If leucoanthocyanins served regularly as precursors for anthocyanins this relation would be expected to hold generally, yet leucoanthocyanins are commonly absent in herbaceous plants (BATE-SMITH 1954) and in the balsams the presence of a leucodelphinidin in buds is not necessarily associated with the production of the corresponding anthocyanidins (delphinidin, malvidin, or petunidin). Moreover, the production of anthocyanins might be accompanied by a decrease in the corresponding leucoanthocyanins as has been reported in bananas (SIMMONDS 1954) and Eucalyptus (HILLIS 1956). The apparent absence of leucoanthocyanin derivatives in aqueous one percent HCl extracts of intensely colored flowers (Table 2) caused the authors to support the concept that leucoanthocyanins were in this case the immediate precursors of anthocyanins (ALSTON and HAGEN 1955). Aqueous solvents, however, remove only a portion of the leucoanthocyanins from balsam tissues and these are obscured on chromatograms by the much greater quantities of anthocyanidins derived from anthocyanins. The application of methods which convert the leucoanthocyanins to anthocyanidins simultaneously with extraction from the tissues, especially after prior removal of the anthocyanins, has revealed the presence of leucoanthocyanins in mature, heavily pigmented flower parts. Attempts to assay the quantities of leucoanthocyanins present at various stages of developing buds have yielded erratic results.

L also affects the substitution of the B ring. Its action differs from that of *H*, however, in two ways: (1) *L* does not affect the nature, or, conspicuously, the relative quantities of leucoanthocyanins present, and (2) *L* through competition or modification eliminates from flowers the pigments produced by *Pr* (or *P^s*) in the absence of *L*. The interpretation is complicated by absence of direct information as to whether the methyl ethers are formed by reactions equivalent to methoxylation or to methylation. If *L* provides a methoxylated precursor, this must be unavailable for leucoanthocyanin synthesis either through late appearance in the buds or through specific exclusion from the reactions involved. Methoxyl leucoanthocyanins are uncommon in nature although BATE-SMITH (1954) has reported a leucopigment which yields an anthocyanidin resembling peonidin.

The genetic system in *Impatiens balsamina* resembles closely that in *Primula*

sinensis as described by SCOTT-MONCRIEFF (1936). In that species, *v* induces pale flowers and green stems, pure white flowers being obtained only when other genes regulating anthocyanin structure are recessive. In the absence of other dominant genes, *V* allows red stems and pelargonidin derivatives in the flowers. *Dz* produces slight pelargonidin pigmentation with *v* and much more with *V*. *K* replaces the pelargonidin produced by *V* with malvidin, but when both *K* and *Dz* are present pelargonidin and malvidin occur together. Thus *V* of *Primula sinensis* is the equivalent of *P'* of the balsams, *K* matches *L*, and *Dz* has the action of *H*. Other genes of *Primula sinensis* may find their counterparts in *Impatiens balsamina* with *w* which controls a cream background color (but not a copigmentation) and *F* which introduces white spotting of colored petals.

Primula sinensis has recently been re-examined by DAYTON, HARBOURNE, and SHERRAT (1956) who find petunidin and delphinidin as well as malvidin in plants carrying *K*. This does not alter the resemblance to *Impatiens balsamina* but suggests that *K* of *Primula* and, perhaps, *L* of *Impatiens* act to introduce the hydroxyl substituent in the free phenyl ring, the methyl ether formation being independent of the action of these genes.

The effect of the anthocyanin genes on other flavonoids is not yet clear. Identification of the flavonol complement is complicated by a considerable excess of one component, probably kaempferol, which obscures others present in the extracts.

In addition to flavonols, other flavonoid compounds occur and these exhibit changes in relative quantity during bud development.

SUMMARY

Flowers of *Impatiens balsamina* exhibit a wide range of colors controlled by genes at three loci. Each color class yields two or more anthocyanins which may be converted to anthocyanidins by boiling with strong acids. The aglycones can be identified readily through paper chromatography. Both aqueous and alcoholic extracts, however, contain leuco-anthocyanins which yield anthocyanidins under the same conditions which hydrolyze the anthocyanins.

The leucoanthocyanins of balsams have been freed of anthocyanins by extraction of fresh flower parts with alcoholic solvents. The colorless residual tissue releases anthocyanidins derived from leucoanthocyanins when boiled in strongly acid solution. All genotypes yield cyanidin and delphinidin under these conditions from petals and sepals and from immature, colorless buds as well as from mature pigmented flowers. In addition a leucoanthocyanin which yields pelargonidin is present whenever the genotype includes the gene *H*.

Since extracts containing anthocyanins generally include leucoanthocyanins, anthocyanidins obtained by boiling the extracts with acid are of mixed origin. Where the anthocyanidins differ from the derivatives of leucoanthocyanins known to be present, they must be derived from anthocyanins, but where leucoanthocyanins and visible pigments yield the same aglycone, the anthocyanins must be separated by chromatography, eluted, hydrolyzed, and the derived

aglycones identified by chromatography. For technical reasons the latter method has been successful only with the major components of intensely pigmented flowers, although the identifications may be extended to weakly pigmented flowers by directly matching on chromatograms the anthocyanins of the several color classes.

In this manner, ten anthocyanins derived from four anthocyanidins have been identified in the twelve phenotypes available. Only the multiple recessive lacks visible pigment. The genes *H* and *L* lead to the production in petals of small quantities of pelargonidin and malvidin derivatives respectively. *P^r* as the only dominant pigmentation gene introduces pelargonidin into the petals and cyanidin and peonidin into the sepals. *P^s*, an allele of *P^r*, is similar in its action although colors produced are less intense and cyanidin does not appear as a visible pigment.

When *H* and *P^r* occur together, production of pelargonidin derivatives is much increased, although cyanidin and peonidin in addition to pelargonidin occur in the sepals. *L* is completely epistatic to *P^r*, malvidin derivatives being produced to the exclusion of pelargonidin, cyanidin, and peonidin. *L* is not epistatic to *H* and when both dominant genes are present flowers yield pelargonidin and malvidin from the petals. Sepals contain only malvidin, however. The presence of *P^r*, *P^s*, or *p* affects only the color intensity in all combinations involving *L*. The pattern of pigment inheritance in *Impatiens balsamina* is quite similar to that in *Primula sinensis*.

Genes at the *p* locus apparently regulate a general reaction in anthocyanin synthesis whereas *H* and *L* control specific substitutions in the ring structure. The control of both leucoanthocyanin and anthocyanin structure by the single gene, *H*, implies a close association of the syntheses of the two classes of compounds. While the nature of the relationship is limited to relatively few possibilities, these cannot be resolved by qualitative studies. On the other hand quantitative analyses of flavonoid components during bud development should permit ultimate definition of the biochemical effects of the genes at the three loci.

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