A BIOCHEMICAL BASIS FOR THE PSEUDO-ALLELIC ANTHOCYANIN SERIES IN GOSSYPIUM

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INTRODUCTION

PSEUDO-ALLELISM describes a situation where two members of a supposedly allelic series give a complementary hybrid when crossed together. Typically the parental types are intermediate members of the "series" and the complementary hybrid resembles the top dominant or wild type. The clearest example of this situation is probably the one described by McCLINTOCK (1944) in the case of certain chlorophyll abnormalities in maize. Phenotypically a "series" of four members, normal green, yellow green, pale yellow, and white, were distinguished whose dominance relationships could be expressed as follows:—

(1) Normal green→yellow green→white

(2) Normal green→pale yellow→white

However, the heterozygote, yellow green/pale yellow, gave a complementary hybrid—it was indistinguishable phenotypically from the top dominant, normal green. By a very neat cytological analysis, McCLINTOCK was able to show that the pale yellow and white "alleles" were in reality homozygous deficiencies, the former adjacent to, and the latter including the normal green/yellow green locus. Consequently in the hybrid, pale yellow/yellow green, the deficient section in the "pale yellow" chromosome was covered by the "yellow green" chromosome thus enabling the dominant normal green gene, carried by the "pale yellow" chromosome, to gain full expression.

The successful analysis of the above case in maize gives rise to two points of considerable genetic interest. In the first place it suggests a probable explanation of all complex series of multiple alleles whose members cannot be arranged in a simple quantitative series and in which complementary heterozygotes are found. For example in Gossypium, it provides a reasonable explanation of complementary hybrids found in the "anthocyanin," and "crinkle" series (YU and CHANG, in press; HUTCHINSON 1946). Secondly it may have considerable bearing on such problems as have been recently described by DUNN and CASPARI (1945) in the mouse, where genes having similar though not identical effects are located in nearly adjacent positions on the same chromosome. The origin and significance of such gene complexes is still an open question. Do they arise as duplications (repeats) of an originally single locus? DUNN and CASPARI, in the case of the Brachy-Fused-Kinky complex in the mouse, did not favor this interpretation since they pointed out that the respective loci, though close, were not adjacent, and embryological

studies revealed far greater differences in the development of the three abnormal types than might have been expected from the appearance of the adult animals.

In several respects the anthocyanin complex in Gossypium offers more favorable material for an attack on this problem. The effects on the phenotype are relatively restricted *in character*, and the respective loci cross over with a very low frequency. Furthermore the considerable work previously carried out on the chemistry of the anthocyanin pigments in several plant genera, ROBINSON and ROBINSON (1934 and earlier papers), LAWRENCE and SCOTT-MONCRIEFF (1935), SCOTT-MONCRIEFF (1936), and others, make possible a more detailed comparative study of the action of the genes concerned. The aim of the present investigation was to compare the actions of two genes at adjacent loci on the production of anthocyanin pigment in the flower petal, and to attempt to decide between the two possibilities (1) that the two loci control successive and similar but not identical mechanisms, (2) that they control chemically unrelated steps in a common chain reaction. It is clear that the former possibility would suggest a high degree of homology and therefore favor the hypothesis that the two loci have evolved from an "ancestral" type by duplication.

GENETIC BASIS OF THE ANTHOCYANIN SERIES IN ASIATIC COTTONS

The color of the petal in Asiatic cottons is controlled by two independent genetic systems. One of these determines the color background of the petal, which may be yellow (*Y*), pale (Y^p), or white (*y*) (HUTCHINSON 1931, SILOW 1941). The other (anthocyanin) system determines both the *pattern* in which red pigment is developed and also the *intensity* of that pigment throughout the vegetative and floral parts of the plant. At least twenty distinct genotypes are known which produce a series of phenotypically distinguishable types ranging from a type in which the whole of the vegetative and floral parts is intensely pigmented to a type in which the red pigment is completely absent. All the anthocyanin genes responsible for this series are located in a short section of one chromosome, and until recently were thought to be members of a single multiple allelic series. Actually, in their general breeding behavior, the alternative types do behave as alleles, with the additional feature that their heterozygotes exhibit supplementary phenotypic effects, cf. the *A* series in maize (EMERSON and ANDERSON 1932), the scutes in Drosophila (SEREBROWSKY 1938), color pattern in Harmonia (TAN 1946) etc. Thus a type with red vegetative parts and red margined petal, on crossing with a type having green plant body and red spotted petal will give an F_1 with red plant body and red spotted, red margined petal (SILOW and YU 1942). However, exceptions to true allelic behavior occur, which indicate that more than a single locus is involved. HUTCHINSON (1932) described a complementary action in the case of two "alleles" whose heterozygote resembled phenotypically a third "allele" in the series. The phenotypic appearance of the three types concerned –as they appear when the independent yellow (*Y*) gene is also present—is illustrated on the left of figure 1, (a), (b) and (c). "Red Spot" (a) is by far the commonest

pattern of anthocyanin pigmentation in the cotton flower. In "Ghost Spot" (b) there is no red pigment but a white spot in place of a red spot can be distinguished against the yellow background of the petal. In "Spotless" (c) no spot is present and the petal appears to be a homogeneous yellow. The same relationships hold in white flowered types (*y*) except, of course, that a white "Ghost Spot" on a white background cannot be distinguished phenotypically from "Spotless."

HUTCHINSON found that on crossing Ghost Spot with Spotless the F_1 was phenotypically Red Spot, but was distinguishable by breeding behavior as on selfing it segregated 1 Ghost Spot:2 Red Spot (heterozygous):1 Spotless. In order to explain this complementary interaction HUTCHINSON (1934) adopted a formal interpretation in which the anthocyanin locus was supposed to be divided into two "gene centers" each of which could carry one or more "episomes." It was supposed that in the recessive types episomes would be missing, and for the expression of Red Spot they would be present on both centers. However, the situation is so similar to MCCLINTOCK'S yellow-green/pale yellow interaction in maize, which is known to be the result of pseudo-allelism, that HUTCHINSON'S formal interpretation is no longer necessary. Instead it may be supposed that Red Spot has dominant genes at two adjacent loci, *G S*, Ghost Spot is deficient (or recessive) at one locus, *G O*, Spotless deficient (or recessive) at the other, *O S*, and in addition a fourth type, deficient (or recessive) at both loci, *O O*, is to be expected. This fourth type, "Basic Spotless," has recently been found by YU and CHANG (in press). Phenotypically it is indistinguishable from Spotless (see figure 1d) but, as expected, it behaves as a simple recessive to the other three types, and gives no complementary interaction with Ghost Spot. The four possible combinations of genes (on this basis responsible for the four phenotypes, Red Spot, Ghost Spot, Spotless and Basic Spotless) are illustrated on the right of figure 1.

Critical evidence for the pseudo-allelic nature of the anthocyanin series has recently been provided by YU and CHANG (in press) who have produced convincing evidence that the anthocyanin "locus" consists of a series of sub-units, probably arranged in a definite linear order, and certainly separable by the normal mechanism of crossing over. (The possibility that certain new "mutant" types had arisen by a form of intra-allelic exchange in heterozygotes had previously been considered by SILOW and YU (1942) but at that time critical evidence was lacking.) At least three sub-units (loci), and probably more, are necessary to explain the twenty pseudo-allelic types at present known.

In this paper attention will be confined to the two loci which control presence or absence of petal spot, and its pigmentation when present; that is the four combinations of genes illustrated in figure 1. Solely from the physiological viewpoint there is nothing unusual in a situation where two acyanic flower types give a hybrid with cyanic flowers. Similar cases have been observed in Lathyrus (BATESON, SAUNDERS and PUNNETT 1905), Antirrhinum (WHELDALE 1916), and Cheiranthus (GAIRDNER, cited by SCOTT-MONCRIEFF 1936) among others. These show merely that at least two chemically independent steps are involved in a chain of reactions leading to the expression of red pig-

ment in the flower. In the present case in Gossypium, however, there is the additional problem of why the genes controlling these two steps are located adjacently in the chromosome. One possibility is that the association is a chance one; that the loci are intrinsically unrelated and govern different pro-

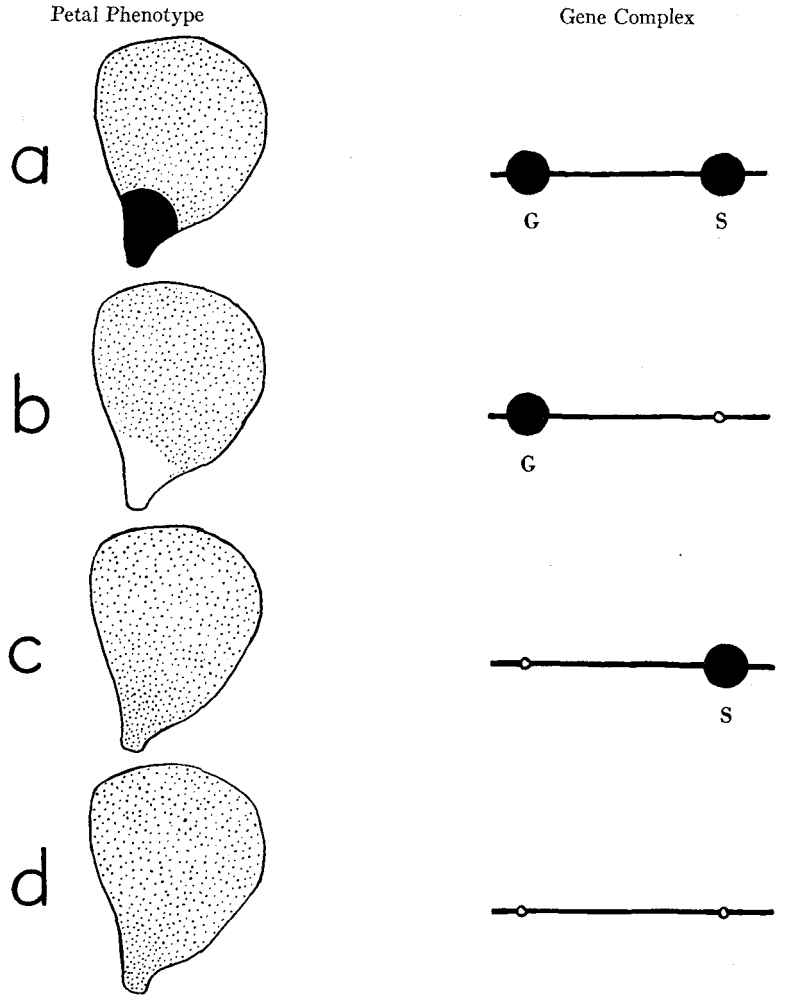


FIGURE 1.—Diagram illustrating the relation between four pseudo-alleles in the anthocyanin series and their effect on the petal phenotype in Asiatic cottons.

cesses which happen to be successive steps necessary for pigment expression. The other possibility is that the loci have arisen by duplication from a single "ancestral" locus, and that associated with (or subsequent to) that duplication, the "daughter" loci have differentiated—the differentiation, of course, is necessary to explain the complementary interaction observed between genes at the two loci. Now if the adjacent position of the loci is a matter of chance, there is clearly no reason to suppose any close relationship between the processes they

govern. If, alternatively, the loci are duplicates, then they must initially have been homologous and have governed homologous chemical processes. The nature of the chemical reactions governed by genes at the two loci should provide valuable evidence in discriminating between the above alternatives.

COMPARATIVE STUDIES OF PIGMENT DEVELOPMENT IN THE FLOWERS OF RED SPOT, GHOST SPOT, SPOTLESS AND BASIC SPOTLESS TYPES

The diagrams in figure 1 illustrate the appearance of the petals in newly opened flowers. Flowers of all Gossypium species fade on the afternoon of the day on which they open. Fading behavior differs in the four flower types under consideration. In Ghost Spot and Basic Spotless flowers, the petals wither and eventually become dry and brown before they are shed. In Red Spot and Spotless flowers, on the contrary, a red flush suffuses the petal at the onset of fading —the formerly acyanic parts become cyanic. Reference to figure 1 shows that the types which produce a red flush on fading, Red Spot and Spotless, both carry a gene at the S locus ,while Ghost Spot and Basic Spotless types do not.

Again referring to figure 1, it can be seen that the spotted types, Red Spot and Ghost Spot, carry a gene at the *G* locus, while the spotless types, Spotless, and Basic Spotless, do not. In formal genetic terms, then, the presence of a spot on the petal is dependent on *G*, and the pigmentation of that spot when present is determined by *S*.

In order to examine the phenotypic differences in more detail, petals of the four types under consideration were examined at successive stages of development, beginning at the time when the petals first protrude through the calyx sheath, that is at approximately the stage when the pollen mother cells are undergoing meiosis. The petals were flattened between two glass slides and examined by reflected light under the low power magnification of a dissection microscope. Blue light was found to be the best source for distinguishing between yellow pigmented and unpigmented tissues. The results of this comparative study are illustrated diagrammatically in figure 2. At the earliest stage the petals in all four types were unpigmented, but at subsequent stages all the cells in surface view became filled with the yellow pigment. The course of development of the yellow pigment was to some extent obscured by the fact that chlorophyll was also present in these early stages, so that the petals appeared yellow-green instead of yellow. The impression was gained, however, that the yellow pigment develops first in the cells towards the outer edges of the young petal, and later the pigmented region extends towards the petal-base. *There was no doubt however that there is a stage, common to all four types of petal examined, in which all the cells visible in surface view contain yellow pigment.*

From this stage onwards, in Basic Spotless and Spotless petals no further changes in pigmentation occurred, except that as growth continued, chlorophyll disappeared from the tissues leaving them bright yellow in color. In Ghost Spot petals from seven to ten days before flowering, the yellow pigment began to disappear from the future spot area of the petal, leaving white

streaks or patches of unpigmented cells. With continued disappearance of the yellow pigment the white patches coalesced, finally producing a homogeneous white spot characteristic of the mature Ghost Spot petal. In Red Spot petals at approximately the same time, a similar disappearance of the yellow pigment in the spot area occurred, but in addition, groups of cells in the resulting white streaks began to develop *red* pigment. These red areas then increased

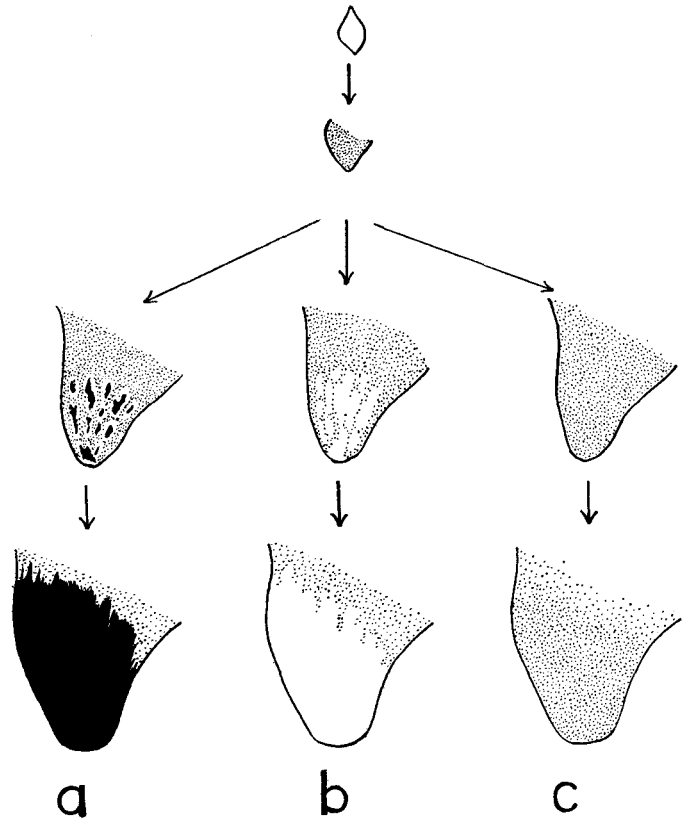


FIGURE 2.—Diagram illustrating distribution of pigments in the "spot area" of the petal at successive stages of development in the four types (a) Red Spot, (b) Ghost Spot, (c) Spotless and Basic Spotless. Anthocyanin indicated by black, leucosubstance by white, and anthoxanthin by stippled areas.

in size as neighboring unpigmented cells developed red pigment, and eventually they, too, coalesced giving rise to the homogeneous red spot of the mature petal. It is clear from these observations that the production of a red spot depends on two successive steps: first the disappearance of the yellow pigment from the cells situated towards the base of the petal, and second, the development of red pigment in these yellow-free cells. The first step is obviously under the control of the gene, *G*, and the second step under the control of the gene, *S*. Furthermore, these observations suggest that red pigment is not developed in cells of the potential spot area until the yellow pigment has disappeared from

them, which furnishes an explanation of the complementary interaction between *G* and *S* in the formation of a red spot.

It will be convenient at this point to anticipate certain results of the chemical findings which will be considered in the following sections. ROBINSON and ROBINSON (1935) and other workers have found that certain plant tissues, including acyanic flower petals, contain colorless (leuco) substances which are readily converted into red (anthocyanin) pigments on boiling with acids. Accordingly, extracts were made from petals of the three acyanic flower types Ghost Spot, Spotless and Basic Spotless. Equal numbers of petals from each type were ground in equivalent volumes of one percent hydrochloric acid. To each filtered extract was added an equal quantity of concentrated hydrochloric acid and the solutions were then boiled for a few minutes. Extracts from Ghost Spot petals developed a deep red color under this treatment, which was later shown to be due to an anthocyanin pigment. By contrast, only faint pink colors were developed by the Spotless and Basic Spotless extracts. Subsequently it was found by extracting the base of the petal, that is the portion roughly corresponding to the spot area in spotted forms, separately, that the bulk of the leuco-substance was present in the spot area.

Having found that Ghost Spot petals differ from Spotless and Basic Spotless petals, not only phenotypically but also quantitatively in respect of their leuco-substance content, it seemed necessary to trace the origin of the latter difference by testing extracts of developing petals at equivalent stages. The tests showed that petals from the youngest buds of the three acyanic, and also of Red Spot flower types, were rich in leuco-substance. At this stage no yellow pigment could be detected, though it may of course have been present in small amounts. Increasingly older flower buds of the three acyanic types continued to give positive tests for leuco-substance until about two days before flowering. At this stage the leuco-substance rapidly disappeared from Spotless and Basic Spotless petals, and at maturity, as described earlier, very little remained. In Ghost Spot petals, on the contrary, a high content of leuco-substance was maintained.

In summary, the results of these observations would appear to justify the following conclusions:—

(1) In Spotless and Basic Spotless, which lack the gene *G*, the petals from the earliest stage onwards contain a leuco-substance. This substance disappears rapidly shortly before flowering. During the same developmental period, a yellow pigment accumulates in all the visible cells of the petal.

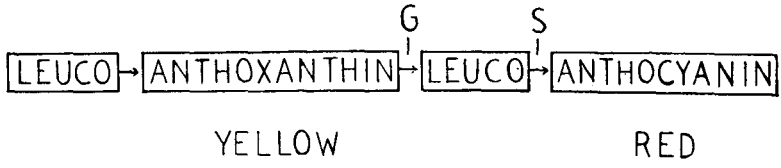
(2) In Ghost Spot, where the gene *G*, is present, the petals also contain a leuco-substance from the earliest stages onwards. This substance does not disappear at maturity, and is particularly abundant in the spot area. During the same developmental period, the yellow pigment disappears from the spot area.

(3) Differences between (1) and (2) above may be ascribed to the action of the gene *G* whose presence is therefore associated both with the disappearance of the yellow pigment from the spot area and also the maintenance of the leuco-substance content in the same area when the petal matures. This apparently

dual role could be interpreted as a single process if one assumption were made, *namely that the gene G converts the yellow pigment to leuco-substance.* In that case the disappearance of the yellow pigment would be due to its conversion into leuco-substance, which in turn would account for the accumulation of the latter in the mature Ghost Spot petal. Independent evidence which suggests that the yellow pigment can be converted into leuco-substance is provided by the fading behavior of Ghost Spot petals. Tests showed that two days after flowering, the faded petals contained very little yellow pigment as compared with fresh petals, but that this fall in yellow pigment content was associated with a rise in quantity of leuco-substance. Similarly, in Red Spot petals, the amount of yellow pigment diminishes during fading, while at the same time, the parts of the petal which are acyanic at flowering, develop a red flush.

(4) Differences between Ghost Spot (*GO*) and Red Spot (*GS*) may be accribed to the effect of the gene *S*. It is known that Ghost Spot contains leuco-substance in the spot area, while Red Spot contains red (anthocyanin) pigment in the same region. Since the leuco-substance can be readily converted into an anthocyanin pigment *in vitro*, since Red Spot contains the gene *G* responsible for the accumulation of leuco-substance in the spot area, and since the developmental evidence shows that a white spot precedes the formation of a red spot in the Red Spot petal, it is reasonable to suppose that *the gene S converts the leuco-substance to an anthocyanin.*

(5) If the interpretations offered in (3) and (4) above are correct, then it seems highly probable that the leuco-substance and the yellow and red pigments may be closely related chemically. Further, since the leuco-substance is present in the petals of all four types at a very early stage—before either the yellow or red pigments can be detected—it is possible that it may be a common precursor of both the yellow and red pigments. In short, the following relationship between the three substances may be proposed *as a working hypothesis* on which to base further tests:—



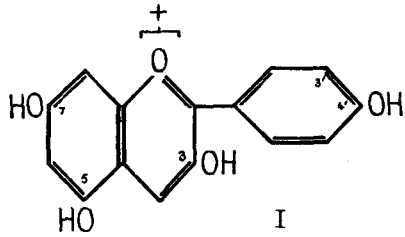
Pigments in the spot area of the petal

The red pigment did not appear until *after* the yellow pigment had appeared and again disappeared from the spot area of the petal. It is reasonable to conclude that Spotless petals which contain both the *S* gene, and, at an early stage, leuco-substance, do not produce red pigment because the yellow pigment has already been formed and the *S* gene does not work in cells containing yellow pigment. In order to test the validity of the hypothesis it was necessary to investigate the chemical nature of the red and yellow pigments, and their interrelations with the leuco-substance. These will be considered in the following section of this paper.

THE CHEMICAL BASIS OF FLOWER PIGMENTATION IN GOSSYPIUM

General

The sap soluble pigments in flowers fall into two main classes (1) the red or purple pigments (anthocyanins) and (2) the yellow pigments (anthoxanthins). Their chemical structures and interrelationships have been reviewed from the genetic point of view by SCOTT-MONCRIEFF (1936) and LAWRENCE and PRICE (1940). The basic structures of the two classes of pigments are closely similar as indicated below:—



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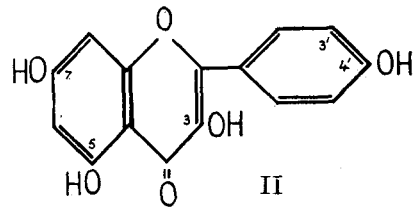


II

The anthoxanthins may be further divided into two sub-groups, the *flavonols* (as in II above) and the *flavones* (in which no substituent hydroxyl group is present at position 3).

The numerous anthocyanin and anthoxanthin pigments which have been isolated, differ in the number and character of substituent groups (chiefly—OH, and —OCH_3) which are present in the lateral benzene ring at positions, 3′ and 5′. Thus the formula of the anthocyanin pigment, *cyanidin*, is derived from the basic structure, I, by substitution with a hydroxyl group at position 3′. The analogous flavonol pigment, *quercetin*, is derived by exactly similar substitution in the basic structure, II. The anthocyanin pigment, *malvidin*, is derived from the basic structure I by substitution with —OCH_3 groups at positions 3′ and 5′ and so on.

In the plant the pigments apparently do not occur in the free state but are combined with various sugars to form *glycosides—monoglycosides* when a sugar molecule replaces the hydroxyl at position 3, and *diglycosides* when hydroxyls at both positions 3 and 5 are replaced. In strict terminology, *anthocyanin* refers to the glycosidal form, and when the sugars are removed by hydrolysis the resulting aglycone is known as an *anthocyanidin*. The considerable work which has been carried out on the anthocyanin pigments shows that the substitution with hydroxyl and methyl groups in the nucleus, and the nature of the sugar attachments is under the control of specific genes. Almost certainly the same situation occurs in the case of the anthoxanthin pigments, but less information is available at the present time.

During the early researches in flower pigment chemistry, the similar structures of anthocyanins and anthoxanthins suggested that their syntheses were interrelated (WHELDALE 1916, 1920). When EVEREST showed (1914) that the anthocyanin pigment, cyanidin, could be prepared *in vitro* by strong reduction of its analogous anthoxanthin quercetin, it was thought probable that the anthoxanthins were actually precursors of the anthocyanins during synthesis

of the latter in the plant. In recent years this view has fallen into disfavor, and the view more generally held is that the two classes of pigment are synthesized in parallel from a common precursor (see SCOTT-MONCRIEFF 1936, LAWRENCE and PRICE 1940). One of the chief arguments that has been advanced in support of parallel synthesis, is that if anthocyanins were synthesized sequentially from anthoxanthins, it would be expected that analogous types of the two pigments would be found in the same flower. Actually studies have shown that when anthocyanins and anthoxanthins occur together, they are frequently not analogous but differ in the nature of their substituent groups. From the genetic point of view, however, this argument scarcely constitutes a valid criticism of the alternative, sequential hypothesis (SANDO *et al.* 1922, 1935). Parallel syntheses of these structurally similar pigments would surely require that the genes controlling these syntheses should be extremely specific in their actions. Otherwise a gene responsible for a particular substitution in an anthocyanin molecule might be expected to bring about a similar substitution in the accompanying anthoxanthin. However if this situation is avoided by postulating extreme specificity of the genes, the whole argument in favor of parallel syntheses is much weakened. A situation involving sequential synthesis might be equally probable. For example, a certain genotype might contain specific genes for converting quercetin into cyanidin, and in addition a specific gene for methylating the cyanidin nucleus. In that case one would not necessarily expect quercetin to be accompanied by cyanidin in the same flower, since the cyanidin might easily be converted into a methylated form (that is, peonidin) as fast as it was formed. It seems therefore that a critical discrimination between the alternative hypotheses of parallel or sequential syntheses must await more information on the specificity of the genes concerned.

Rapid methods for the isolation and identification of the anthocyanin pigments have been developed by ROBINSON and ROBINSON (1934 and earlier papers) and have been summarized by SCOTT-MONCRIEFF (1936). As far as the writer is aware no equivalent methods have been developed for anthoxanthins. Both pigments are readily extracted by grinding the petals with dilute hydrochloric acid. The mixture of pigments so obtained can be separated by exhaustive extraction with ethyl acetate, in which the anthoxanthins are soluble, anthocyanins insoluble. The anthocyanins present in the anthoxanthin-free extracts can then be identified by their characteristic color reactions with certain reagents; sodium hydroxide, sodium carbonate, sodium acetate and ferric chloride. Determination of their glycosidal structure can be made by examining the partition of the pigment between two solvents, water and amyl alcohol. More detailed confirmatory studies depend on colorimetric comparison with standard pigments, and on spectrophotometric methods.

The anthoxanthins as a class may be detected by the yellow color produced with alkalies, their yellow or orange-yellow precipitates with lead acetate, and the olive or brownish colorations produced with ferric chloride. Some anthoxanthins can be converted to anthocyanins by reduction with nascent hydrogen. Discrimination between different anthoxanthins, however, depends on purification, determinations of melting point, and spectrophotometric methods.

The anthocyanin pigments in flowers of Gossypium species

As far as the writer is aware no chemical studies of the anthocyanin pigments in Gossypium have been made previously, although they have received considerable attention from the genetic aspect (HARLAND 1929, HUTCHINSON 1932, SILOW 1941, SILOW and YU 1942, YU and CHANG, in press). In the work to be described, the chemistry of the anthocyanin pigments in flowers of the following species was examined.

G. arboreum, *G. herbaceum*} Asiatic diploid species (cultivated)
G. barbadense—New World amphidiploid species (cultivated)
G. klotzschianum—American diploid species (wild)
G. anomalum—African diploid species (wild)

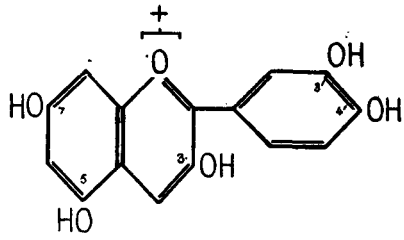
All of these, except *G. anomalum*, were similar phenotypically in having yellow flowers (*Y*), with a red, or more properly a magenta, spot at the base of the petals (as in figure 1a). The flowers of *G. anomalum* contain a pale yellow anthoxanthin pigment. In addition they have a spot at the base of the petal which is purple in color. The purple pigment is not confined to the spot area but is present in small amounts throughout the petal, so that the interaction between pale yellow and purple pigments produces a pale lilac flush on the main body of the petal. On placing the petals of *G. anomalum* in dilute acid, however, the purple color is immediately reddened, and the extract obtained by grinding the petals in one percent hydrochloric acid has the same, magenta, color as the extracts of the petals of the other four species listed above, prepared in the same way.

The crude acid extracts were made anthoxanthin-free by shaking repeatedly with ethyl acetate. The purified solutions of anthocyanin were next subjected to the qualitative color tests listed by SCOTT-MONCRIEFF (1936, p. 121). Identical color reactions were obtained with the anthocyanin extracts from the petals of all five species. They are summarized below:—

Reagent:	Sodium hydroxide	Sodium carbonate	Sodium acetate
Color:	Pure blue	Violet blue	Red-violet

Alcoholic solutions of the anthocyanins were prepared by grinding the petals with ethyl acetate, followed by repeated washing with the same solvent to remove the last traces of anthoxanthin, the anthocyanin pigment then being taken up in ethyl or amyl alcohol. In all cases the alcoholic solution gave a deep blue color on adding a few drops of ferric chloride. This combination of reactions is characteristic of monoglycosides of *cyanidin*. The glycosidal nature of the pigment was confirmed by examining its partition between aqueous and amyl alcohol solvents. In the strengths employed, the pigment was about equally distributed between the two solvents after shaking. On dilution with water, the distribution in the alcoholic layer was greatly decreased, but on saturating with sodium chloride the pigment was almost completely taken up by the amyl alcohol. This reaction is characteristic of pentose glycosides, and forms a ready means of distinguishing them from monoglycosides and diglycosides. It seems probable therefore, in view of the fact that the reactions of the

anthocyanin extracts were identical in the case of all five species examined, that cyanidin-3 pentose glycoside is the commonest, if not the only anthocyanin pigment present in the genus. The hydrolysed form has the following structure:



and in the glycosidal form the hydroxyl at position 3 would be replaced by a pentose molecule.

Subsequent examination of a white flowered (*y*) type of *G. arboreum* with red spotted petals showed that its anthocyanin pigment was also cyanidin 3-pentose glycoside. It may therefore be concluded—since yellow, pale yellow, and white flowers, containing different anthoxanthins, all contain the same anthocyanin pigment—that the genes governing the form of anthoxanthin pigment do not directly affect the form of anthocyanin produced in the petal, that is, they are highly specific.

The anthoxanthin pigments in flowers of Gossypium species

The early work of PERKIN (1899, 1909, 1916), later extended by VIEHOVER, CHERNOFF, and JOHNS (1918) showed that three anthoxanthin pigments occur in the petals of cotton flowers. The distribution of these pigments in the different species is summarized in table 1.

TABLE I

Distribution of three anthoxanthin pigments in five species of Gossypium.

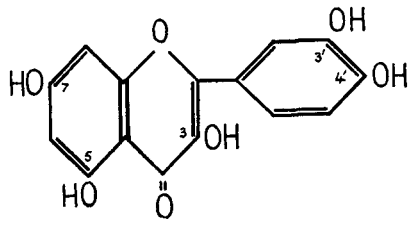
SPECIES	PETAL COLOR	ANTHOXANTHIN CONTENT		
		Gossypitrin	*Quercimeritrin*	*Isoquercitrin*
G. arboreum	Yellow	++	−	+
G. herbaceum†	Yellow	++	−	+
G. arboreum	White*	−	−	−
G. barbadense	Yellow	+	++	+
G. hirsutum	White	−	++	+

* Traces of unidentified anthoxanthin found.

† A fourth anthoxanthin, *herbacitrin*, was later found in this species by NEELAKANTAM and SESHADRI (1937).

Isoquercitrin and *quercimeritrin* are isomeric glucosides (that is, glycosides involving the sugar glucose) of the same anthoxanthin, *quercetin*. They differ only in the position of attachment of the glucose molecule on the quercetin

nucleus. *Gossypitrin* is a glucoside of *gossypetin*. Its empiric formula was found by PERKIN to contain an additional oxygen atom, as compared with that of the other two pigments. Later, BAKER, NODZU, and ROBINSON (1929) showed by synthesis that its structural formula consists of a quercetin nucleus in which an additional hydroxyl (—OH) group has been substituted. Reference to table 1 shows that isoquercitrin is common to all flower types examined, with the possible exception of white flowered types of *G. arboreum*, which contained only traces of anthoxanthin pigment. The amount of pigment obtained in these flowers, PERKIN found, was insufficient to carry out critical chemical analysis with the methods employed. With this reservation however, the evidence indicates that quercetin is the primary anthoxanthin to be synthesized in the flower. It has the following structural formula:—



The yellow flowered types (genetically, *Y*) differ from the others in the fact that they contain gossypitrin, which is also their principal pigment. It is clear that *Y* is associated with the presence of gossypitrin, and its probable genetic action is the conversion of quercetin to gossypetin by hydroxylation.

A further point of interest is that New World cotton flowers contain quercimeritrin, which is absent from the flowers of the Asiatic species which have been studied chemically. In current genetic terminology, both white flowered New World and white flowered Asiatic types are symbolized *y*, which is obviously inconsistent with the chemical evidence. Neither is it consistent with the fact that hybrids between white flowered New World cottons and white flowered Asiatic cottons have *yellow* flowers. However, SILOW (1941) has shown that a certain pale yellow flowered type of *G. arboreum* known as "Chinese pale," and the pale yellow flowered *G. anomalum*, also give complementary yellow flowered hybrids on crossing with standard pale yellow (Y^p) and white (*y*) flowered types of *G. arboreum*. It is clear that the *Y* series and its 'duplicates" need reinvestigation on a chemical basis.

In the present study, further investigations of a preliminary nature were carried out on two flower types in which chemical evidence is still lacking, namely *G. anomalum* (pale yellow flower) and *G. arboreum* (white flower, *y*). The anthoxanthin pigments were extracted with ethyl acetate, filtered, evaporated to dryness and taken up in alcohol. The alcoholic solutions were pale yellowish-green in color. Routine tests were then carried out on both extracts, using as a control a similarly prepared extract of white flowered *G. hirsutum*, ("*y*"), in which the anthoxanthin pigment is known to consist principally of quercimeritrin (see table 1). The results of the tests are summarized below:—

Reagent:—	Basic lead acetate	Sodium carbonate	Ferric chloride	Strong hydrochloric	Nascent hydrogen
G. hirsutum("*y*")	Orange ppc.	Golden yellow	Olive green	Golden yellow	Magenta
G. anomalum	Orange ppc.	Golden yellow	Olive green	Golden yellow	Magenta
G. arboreum(*y*)	Lemon yellow ppc.	Lemon yellow	Pale olive	Yellow-green	Magenta

No difference was apparent between the anthoxanthin pigments of the *G. hirsutum* and *G. anomalum* flowers. On evaporating their solutions to dryness and recrystallizing from hot water, both were found to deposit mixed crystals of plate and needle-like forms. According to PERKIN quercimeritrin differs from isoquercitrin and gossypitrin in the fact that it crystallizes in glistening plates; the other two pigments have needle-like crystals. It seems highly probable therefore that the petals of *G. anomalum*, like those of *G. hirsutum*, contain quercimeritrin. Similarity in form of anthoxanthin pigment, in addition to the previous genetic evidence that both species give yellow flowered hybrids on crossing with white flowered *G. arboreum*, suggests that both contain the same genotype in respect of anthoxanthin pigmentation and that therefore the present symbol, *y*, for *G. hirsutum* is incorrect.

The quantity of anthoxanthin pigment present in the white flowered *G. arboreum* petals was much less than in the *G. hirsutum* and *G. anomalum* petals. Furthermore, the tests tabulated above show that a qualitative as well as a quantitative difference is involved.

The leuco-substance in Ghost Spot flowers of Gossypium

Evidence for the presence of a leuco-substance

Since anthocyanins and anthoxanthins give blue and yellow colorations respectively in alkaline solution it is to be expected that crude extracts of petals in which both pigments occur will give a green color on treatment with alkalies. This was found to be the case in all the cyanic (Red Spot) flowers tested. Crude extracts of acyanic flowers of the Spotless and Basic Spotless types, since they contained no anthocyanin gave yellow colors with alkalies. Crude extracts of both yellow flowered (*Y*) and white flowered (*y*) Ghost Spot petals of *G. arboreum*, however, gave unexpected results. Although the petals of Ghost Spot flowers are acyanic, and although the crude extracts were pale yellow or colorless in acid solution, yet, on treating with alkalies the extracts usually gave a transient *green* color which changed into the expected yellow color on standing.

If the green solution was immediately made acid again with a few drops of hydrochloric acid, the green color was converted to pink. In short, the crude extracts of Ghost Spot petals, after being made alkaline, behaved as if traces of anthocyanin were present.

When the crude extracts in dilute acid solution were rendered free from anthoxanthin by repeated shaking with ethyl acetate, it was found that they no longer gave any color reaction with dilute alkalies, though tests of the ethyl acetate washings showed that no "anthocyanin-like" substance had been removed. On adding a few drops of concentrated hydrochloric acid to the anthoxanthin-free extracts and then boiling, a magenta color was rapidly developed. The solution was concentrated, and the precipitate separating out was removed by filtration, washed, and taken up in alcohol. On adding a few drops of ferric chloride, a deep blue color was developed, which suggested that the synthesized pigment might be cyanidin, and hence the same as the pigment occurring naturally in cyanic flowers. These results would seem to indicate that large quantities of a leuco-substance are present in Ghost Spot petals, that this substance, like the anthocyanidins, is soluble in alcohol and insoluble in ethyl acetate, and is rather easily converted into a true anthocyanidin pigment.

The nature of the conversion of leuco-substance to anthocyanidin

The ROBINSONS (1935) considered that the production of the anthocyanidin, peltogynidin, from its leuco-form, peltogynol, by boiling with hydrochloric acid probably depended on an oxidation process, although attempts to exclude oxygen during the conversion did not inhibit the reaction. In the present case, independent lines of evidence have been obtained which show that the leuco-substance in Gossypium petals on the contrary, is reduced (hydrogenated) during its conversion to an anthocyanidin. Preliminary experiments showed that while anthocyanin solutions were readily decolorized on shaking with hydrogen peroxide in acid solution, they were remarkably stable in the presence of reducing agents. The color was not discharged on boiling with formalin, and only with difficulty when the boiling solution was treated with nascent hydrogen. Finally two methods were found which made it possible to bring about a reversible reaction, leuco-substance$\rightleftarrows$anthocyanidin readily *in vitro*. It is well known that methylene blue may act as a hydrogen acceptor in certain reactions, being reduced to a colorless form in the process:—

$$\underset{\text{(blue)}}{MeB} + 2H \longrightarrow \underset{\text{(colorless)}}{MeBH_2}$$

Methylene blue is stable on boiling with hydrochloric acid, and the color is only slowly discharged (presumably due to disintegration of the methylene blue nucleus) when hydrogen peroxide is added to the boiling acidic solution. The leuco-substance extracted from Ghost Spot petals is converted into an anthocyanidin on boiling with hydrochloric acid, and the color of the latter is immediately discharged on adding hydrogen peroxide. It was found that when methylene blue was boiled with the leuco-substance in dilute acid solution, no color change took place, but on the addition of a few drops of hydrochloric acid to the boiling solution the color was rapidly discharged. As the last trace of the blue color disappeared, the magenta anthocyanidin color began to develop. On adding a few more drops of methylene blue the magenta color was discharged, but again appeared on continued boiling. In this manner the

reversible color change, blue⇄magenta could be continued indefinitely until the acid medium became very dilute, at which stage the blue color remained and no further anthocyanidin was produced. The reactions involved may be represented as follows:

$$\underset{\text{leuco-substance}}{A} \xrightarrow{+HCl} \underset{\text{anthocyanidin}}{AH} \qquad (1)$$

The anthocyanidin yields hydrogen to the methylene blue until all the latter is converted into its colorless (reduced) form:

$$2AH + MeB \longrightarrow 2A + MeBH_2 \qquad (2)$$

at which stage equation (1) goes to completion. That the decolorization of the methylene blue is due to its accepting hydrogen from the anthocyanidin is confirmed by the fact that i. hydrogen peroxide is added to the system when the anthocyanidin is present, the magenta color is immediately discharged, and simultaneously the methylene blue color is restored.

Additional evidence of the nature of the reaction was obtained by mixing anthocyanin extracts from Red Spot petals with methylene blue. When both original solutions were concentrated, a deep violet color due to a mixture of the blue and magenta pigments was produced. With more dilute solutions however it was noticed that there was a considerable reduction in the densities of the pigments after mixing. It seems possible that at this stage a partial dissociation had taken place, giving an equilibrium mixture of the four components:—

$$MeB + 2AH \rightleftarrows MeBH_2 + 2A$$

On boiling with strong hydrochloric acid the magenta anthocyanidin color was rapidly recovered, and the methylene blue was consequently all converted into its colorless form as shown by equations (1) and (2) above. Their similarity in reaction with methylene blue provides additional evidence that the natural and synthesized anthocyanidins are structurally very similar.

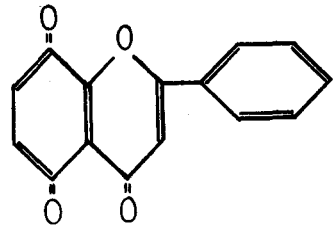
A parallel reaction was obtained by warming a three percent hydrochloric acid solution of anthocyanin from Red Spot petals, with a mild oxidizing agent, potassium ferricyanide. To the solution containing both substances, an equal volume of amyl alcohol was added. The mixture was shaken and then allowed to settle out into two layers; the upper, alcoholic layer (pink) containing the anthocyanin, and the lower, aqueous layer (yellow) containing the potassium ferricyanide. On warming, with frequent shaking, the potassium ferricyanide was slowly reduced as shown by the production of a fine blue suspension of the characteristic ferro-ferricyanide salt at the interface, and a change from yellow to green color in the lower layer. At the same time the pink color of the upper layer disappeared. Apparently the potassium ferricyanide had oxidized the anthocyanin to a colorless leuco-substance. This was confirmed by reversing the reaction. A few particles of granulated zinc were dropped into the solution and the nascent hydrogen thus generated slowly completed the reduction of the unchanged ferricyanide and ferro-ferricyanide remaining in the lower

layer, and at the same time restored the pink color of the upper layer. This reaction and the reaction with methylene blue leave no room for doubt that, the change from leuco-substance to anthocyanin *in vitro* involves chemical reduction. (Since the above was written, it has been found that anthoxanthin-free alcoholic solutions of leuco-substance extracted from Ghost Spot petals can be reduced directly to anthocyanin with nascent hydrogen. The reaction does not take place in aqueous solution.)

The relation between leuco-substance and anthoxanthin

A one percent hydrochloric acid solution of the leuco-substance, from which the anthoxanthin had previously been separated with ethyl acetate was boiled with a few drops of one percent hydrogen peroxide. A yellow color developed in the solution. With excess hydrogen peroxide a dull yellowish brown suspension also appeared and separated out on standing. The dull yellowish brown precipitate was easily removed by filtration and the clear pale-yellow filtrate was found to possess some of the general properties of an anthoxanthin, namely it was soluble in ethyl acetate, gave a golden yellow color with alkalies and a pale yellow precipitate with basic lead acetate.

It seems probable that the dull yellowish brown precipitate also obtained on oxidation was a further oxidation product, since PERKIN (1916) found that the anthoxanthins could be readily oxidized to corresponding quinones which have the following basic skeletal structure:—



Although there can be no critical information on this point without detailed chemical analysis, two lines of evidence make it probable. In the first place, similar dull yellowish brown precipitates settle out when acid solutions of natural anthoxanthins from Gossypium petals are boiled with hydrogen peroxide, which suggests that the anthoxanthin and leuco-substance can be oxidized to similar substances. Secondly, PERKIN found that the anthoxanthins can be oxidized, either in the hydrolyzed or glycosidal states, to the corresponding quinones. Thus the hydrolyzed anthoxanthin pigment, gossypetin, could be oxidized to the quinone, *gossypitone*, and similarly in the corresponding glycosidal state, gossypitrin could be oxidized to *gossypitrone*. Gossypitrone is red in color and can be converted to its corresponding hydrolyzed form, gossypitone, which has a pale brown color. In the present studies it was frequently found that oxidation of the anthoxanthins from yellow petalled flowers (containing gossypitrin) gave a transient red color (gossypitrone?) which rapidly changed to the dull yellowish brown color (gossypitone?) due presumably to hydrolysis in acid solution.

It is clear that if it could be shown that the *in vitro* conversion of quercetin

to cyanidin was accomplished *via* an intermediate leuco-substance, then the analogy with the naturally occurring plant pigments would be complete, and no reasonable doubt could be entertained that the change, anthoxanthin→ leuco-substance→anthocyanin actually occurs in the plant. In order to investigate this point, anthoxanthin solutions from Basic Spotless petals (containing gossypitrin and isoquercitrin) were reduced with (1) zinc and *dilute* (one percent) hydrochloric acid and (2) sulfur dioxide.

(1) The anthoxanthin solution in one percent hydrochloric acid was stirred with a little granulated zinc until the yellow color faded to a pale straw color, at which stage the reaction was stopped, the unconverted zinc being removed by filtration. The unconverted anthoxanthin was next removed by exhaustive extraction with ethyl acetate. The remaining colorless solution was then boiled with strong hydrochloric acid, and a magenta color rapidly developed showing that a leuco-substance had been produced by reduction of the anthoxanthin, and subsequently converted to an anthocyanin in strong acid solution.

(2) A similar anthoxanthin solution to that used in (1) above was boiled with excess sodium bisulfite until sulfur dioxide was no longer evolved. The sodium chloride and unchanged sodium bisulfite was removed by adding a larger bulk of 95 percent alcohol and filtering; adding a few drops of dilute hydrochloric acid to the filtrate and filtering again. The final filtrate had a clear pale yellow color. It was diluted to twice its bulk with water and then concentrated to its original volume, in order to remove most of the alcohol. On shaking with ethyl acetate, the yellow color was removed, showing that it was due to unconverted anthoxanthin. The remaining almost colorless solution yielded large quantities of anthocyanin on boiling with strong hydrochloric acid.

Although the above experiments only refer to the anthoxanthin solutions obtained from Basic Spotless petals, quite parallel reactions were obtained with the anthoxanthins from petals of *G. anomalum* and white flowered *G. arboreum*, the only observable difference being that in the case of the last two types very little unconverted anthoxanthin remained in the solution after reduction. It is not known whether this difference is attributable to the qualitative differences between the anthoxanthins concerned, or merely to quantitative differences in anthoxanthin content of the respective solutions. However since the anthocyanin pigment obtained in each case appeared to be cyanidin, since it gave a blue coloration with ferric chloride in alcoholic solution, it is likely that any gossypitrin present in the original anthoxanthin solutions was first reduced to quercetin, and subsequently converted to cyanidin *via* the leuco-substance. The main point is that the leuco-substance represents an intermediate stage in the reduction of an anthoxanthin to an anthocyanin—a situation which is in complete accordance with the genetic evidence and evidence from studies of pigment development presented earlier in this paper.

DISCUSSION

The last two steps in the production of a red spot at the base of the petal in flowers of Asiatic cottons are (1) the replacement of a yellow pigment (anthox-

anthin) by a leuco-substance (2) the replacement of the leuco-substance by a red pigment (anthocyanin) in the spot area. Genetic evidence has shown that (1) and (2) are controlled by the *G* and *S* genes respectively, which are located in adjacent positions on the chromosome. Comparative morphological studies of the interrelations of the three pigment substances concerned, during the development of the petal, suggested that the changes observed might be due to the actual conversion of the anthoxanthin to the anthocyanin pigment in two successive steps, the leuco-substance being an intermediate stage in the conversion. The chemical evidence shows that the primary anthoxanthin pigment in the petal is quercetin, and the corresponding anthocyanidin pigment, cyanidin. Therefore, since in the petal the change, quercetin→leuco-substance→cyanidin, is associated with two simple successive genetic steps, while a similar change can be performed *in vitro* by two simple successive chemical steps, it is a reasonable conclusion that the *in vitro* changes correspond to the gene controlled processes in the petal.

The chemical evidence is consistent with the hypothetical relationship between anthoxanthin, leuco-substance and anthocyanin which was deduced from the genetic and developmental evidence and summarized diagrammatically on page 198. Critical confirmation, however, is required to show that the leuco-substance present in the young buds of all cotton flowers is identical with the leuco-substance present in mature Ghost Spot petals and also with the intermediate colorless substance which can be obtained by reduction of quercetin in aqueous solution *in vitro*. Recently it has been found possible to demonstrate this identity by spectrophotometric methods and the results will be published in detail elsewhere. It would seem, therefore, that the leuco-substance is a common precursor of both anthoxanthin and anthocyanin pigments and chemically intermediate between the two. In the absence of the gene *G* it is oxidized to an anthoxanthin pigment; when *G* is present the anthoxanthin in the spot area is reconverted at a later stage to leuco-substance, and when *S* is also present the latter is further reduced to an anthocyanin pigment. Why Spotless petals, which lack *G* but carry *S* should not produce anthocyanin is difficult to understand, since in the bud stage they, in common with the flowers of the other genetic types, contain the leuco-substance. However, petals of Spotless types, unlike those of the phenotypically identical Basic Spotless types, do produce anthocyanin on fading. Furthermore, Spotless types are known which have considerable quantities of anthocyanin in the petal margins. In such "Red Margin Spotless" types the anthocyanin (but no anthoxanthin) appears at a much earlier stage in the petal margins of the young flower buds that is approximately the same time as the anthoxanthin (but no anthocyanin) appears in the main body of the petals. Presumably the marginal anthocyanin is produced directly from the leuco-substance and does not result from the extended sequence: leuco-substance→anthoxanthin→leuco-substance→anthocyanin which occurs in the spot area of Red Spot types. In short, it appears that there must be some form of competition for the common precursor which inhibits the simultaneous production of anthoxanthin and anthocyanin pigment in the spot area of the petal.

The main purpose of this paper has been to attempt to decide between the two possibilities (1) that the adjacent *G* and *S* loci control successive and similar, but not identical, mechanisms (2) that they control successive but chemically unrelated steps in a common chain reaction. It is clear that the first alternative is the more likely, since the genes control chemically similar reactions, involving chemical reduction of structurally very similar though not identical substrates. *This would suggest that they are daughter genes originating from a common ancestral gene by duplication, and that in the process they have developed specificity.* Such an interpretation requires no new concept, since studies of salivary chromosomes in Drosophila and Sciara (BRIDGES 1936; METZ 1937, 1938; and LEWIS 1945), have demonstrated the existence of serial duplications ("repeats"), and in two cases, Bar and Star/Asteroid, have shown that the cytological repeat is associated with a change in phenotypic expression (a position effect). Other pseudo-allelic series, e.g. scute in Drosophila (SEREBROWSKY 1938, RAFFEL and MULLER 1940) and the pale yellow/yellow-green series in maize (MCCLINTOCK 1944) may have had a similar origin. Evidence from salivary chromosomes does not suggest that extensive serial duplication is common, though this would be necessary to explain the more complex pseudo-allelic series. On the other hand, MCCLINTOCK (1941) has shown that by a specialized mechanism in maize, long series of duplications may sometimes be accumulated. In cotton it may be significant that most of the anthocyanin "mutants" have been obtained in closely related stocks of *G. arboreum*, while in other species fewer variants are known. Possibly in these stocks a single repeat followed by unequal crossing over with the original unmodified chromosome may have given rise to a further duplication (cf Double Bar in Drosophila) and so have initiated a cumulative process. As a result it is conceivable that a series of duplications could have been established as readily as those obtained by MCCLINTOCK with a totally different mechanism.

If serial duplication were a universal mechanism underlying the development of pseudo-allelic systems (which is not proven), it would be difficult to detect it in all but the most favorable material. It would be necessary to show (1) that more than one locus were involved (2) that the adjacent loci had a basic similarity. Cytological demonstration of the association of a supposedly "allelic" series with repeats, or, alternatively, genetic demonstration of rare crossovers within a series whose members controlled similar but not identical processes, would seem to satisfy both conditions. But the former demonstration can only as yet be provided with the use of salivary chromosomes, and the latter requires the identification of specific gene-controlled chemical reactions. However, the existence of complementary interaction between members of a series, with or without supporting cytological evidence, is perhaps indicative of a similar underlying mechanism. For example the *A* series in maize (EMERSON and ANDERSON 1932), has two features in common with the anthocyanin series in Gossypium. The same pigments, quercetin and cyanidin, are involved (SANDO *et al.* 1922, 1935) and the *A* and a^p "alleles" give a complementary heterozygote phenotypically resembling a third "allele," A^t. On the other hand, the *A* series in maize differs from the analogous case in Gossypium in that the basic

recessive "allele," *a*, cannot be fitted readily into a "two loci" interpretation, and no evidence of crossing over has yet been reported. Similarly in a second (*R*) anthocyanin series in maize, the hybrid $R^g r^r$ phenotypically resembles homozygous $R^r R^r$ which at first sight would suggest by analogy with cotton that R^r is bipartite ($R^g r^r$). However STADLER (1946) has shown that homozygous R^r mutates spontaneously to R^g and r^r which apparently rules out equal crossing over as a source of these mutants. Nevertheless this evidence does not negate the possibility that the *R* locus is bipartite, or that crossing over may provide an additional source of "mutation." It would seem that observation of such mutants in stocks heterozygous for R^r and for other markers in the same chromosome might provide a more stringent test. In the case of the Brachy series in mice, (DUNN and CASPARI 1945), the opposite difficulty is encountered, that is evidence of crossing over has been established but the nature of the material prohibits comparison of gene action in the neighboring loci at a sufficiently critical level. Provisionally it may be permissible to regard all these cases as representing a graded series of the same underlying phenomenon, ranging from very small repeats between which the chances of crossing over are infinitely small to large and complex repeats in which the basic similarity between the duplicates has been lost. This would be consistent with the cytological observations of RAFFEL and MULLER (1940) in the scute series of Drosophila that a quantitative series exists between cytologically undetectable changes (point mutations?) and relatively gross changes in pattern of the salivary chromosomes.

Of particular interest in the present case is the suggestion that repetition of an original ancestral locus has been accompanied by divergence in the functions of the daughter loci. Further the change involved would seem to fulfil the primary requisite for the initiation of *new* loci, and to differ radically from the change involved in a point mutation. Multiple substitution of genes by their mutant alleles should lead to no loss in cytological homology, which would also imply that genes and their mutants, in spite of considerable differences in phenotypic expression, retain considerable biochemical similarity. Otherwise one would have to admit the possibility of complementary interaction between a gene and its mutant allele when combined in a heterozygote, whereas in fact, dominance or intermediacy is the rule. When it has been possible to compare the actions of genes and their mutants at the biochemical level, as in the numerous cases reported in Neurospora, it appears that the change from normal to mutant allele involves a loss or inactivation of the power to control a specific chemical reaction. In contrast to this situation, the production of pseudo-alleles by duplication implies a loss of cytological homology *per se*, and, in the present case at least, a loss of biochemical homology as well. The complementary interaction observed when pseudo-alleles are combined in a heterozygote would be expected to result from such a loss in biochemical homology. Finally, a mechanism leading to divergence in genic function would seem capable of providing more significant variability in the evolutionary sense, than a mechanism in which the major proportion of the variability is attributable to specific inactivations or losses.

It must be admitted that these speculations offer no clue as to the mechanism by which serial duplication *per se* could result in differentiation of the daughter loci at a biochemical level. Although a repeat necessarily involves a local change in chromosomal pattern which could presumably modify genic expression, it would be expected that this "position effect" would be removed when the loci were separated by crossing over. This is apparently only found in the Drosophila cases which have been considered in this paper. Thus in the present case there is no evidence of any phenotypic difference between *G S/O O*, and *G O/O S* types, and similarly in maize the hybrid, yellow-green/pale yellow, is phenotypically green like the homozygous normal type.

SUMMARY

1. The pseudo-allelic anthocyanin series in Asiatic cottons depends on the interaction of at least three neighboring loci two of which (*G* and *S*) govern the presence or absence of a spot at the base of the petal, and its pigmentation when present. When both *G* and *S* genes are present, there is a red spot at the base of the petal; when *G* alone is present the spot is white; and when *G* is absent the petal is spotless.

2. Studies of pigment development in the petal favor the hypothesis that *G* converts a yellow pigment to a leuco-substance, and that *S* further converts the leuco-substance to a red pigment.

3. Chemical studies of the flower pigments show that the primary yellow pigment in the flower is the anthoxanthin, quercetin, and the primary red pigment is the structurally analogous anthocyanidin, cyanidin. The leuco-substance can be converted *in vitro* by *reduction* to cyanidin, and furthermore the naturally occurring anthoxanthin can be reduced *in vitro* to cyanidin via an intermediate leuco-substance.

4. Genetic and chemical evidence combined therefore suggest that the leuco-substance is an intermediate stage and probable common precursor in the natural syntheses of both anthoxanthin and anthocyanin in the petal.

5. If this interpretation is correct the following situation is indicated:— Two genes, *G* and *S*, situated in neighboring loci control similar (reduction) processes and act on very similar though not identical substrates (leuco-substance and anthoxanthin). This would suggest that the loci arose by duplication from a common ancestral locus, and that in the process of duplication, or as a direct result of it, specificity was developed, that is the daughter loci lost both their cytological and biochemical homology and became qualitatively *new* loci.

6. The possible evolutionary significance of these studies is briefly discussed.

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