XXI. OBSERVATIONS ON ANTHOCYANINS. I. THE ANTHOCYANINS OF THE YOUNG LEAVES OF THE GRAPE VINE.

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THESE investigations were undertaken with the object of obtaining experimental evidence of a biochemical nature with regard to the formation of anthocyanin pigments by the plant. Until recently two working hypotheses had been advanced in this connection. They both owe their origin to the views of Palladin [1909], who looked upon anthocyanin as a respiratory pigment. Miss Wheldale [1911, 1916] suggested that Palladin's respiratory prochromogens were flavone (or xanthone) derivatives, and expressed their change into anthocyanins in general terms as follows:

Glucosides + water $\rightleftarrows$ chromogen (flavone) + sugar

x chromogen + oxygen $\rightarrow$ anthocyanin.

The first reaction was assumed to be controlled by a glucosidase, and the second by an oxidase. Keeble, Armstrong and Jones [1913] proposed a very similar hypothesis and assumed a preliminary reduction (not necessarily of an enzymatic nature), followed by an oxidation due to the interaction of a peroxidase and an organic peroxide. Both these theories have this in common; they require the presence of oxidases and they assume oxidation as the essential condition for anthocyanin formation. The evidence for the simultaneous occurrence in plants of oxidases and anthocyanins was, however, by no means satisfactory [see Atkins, 1915], and necessitated the further assumption of "inhibitors" of oxidases, if the theory was to be retained. The two theories became finally untenable when it was shown by Combes [1913] and confirmed by Everest [1914], by Willstätter and Mallison [1914], and by Shibata [1919] that the conversion of flavonol derivatives into anthocyanins is not an oxidative process at all, but is due to reduction.

As any hypothesis on the biochemical anthocyanin formation must be based on the chemistry of these substances, a short résumé of our present knowledge of the anthocyanin chemistry is necessary here. A detailed account, based on the work of Willstätter and his co-workers, is given by Perkin and Everest [1918].

It has now been established that the anthocyanin pigments of flowers, fruits and leaves are glucosides and they are the first representatives of a

new class of non-nitrogenous vegetable bases [Willstätter and Everest, 1913; Willstätter, 1914]. They are derived from a reduced β-phenyl-γ-pyrone (*i.e.* 2-phenyl-pyrylium) nucleus (see Formula III). The characteristic of this nucleus is a quadrivalent oxygen atom, which conveys basic properties to its derivatives and enables them to form salts with mineral and organic acids. The methods for the isolation of anthocyanins are based mainly on the formation of these well-crystallised oxonium salts. The differences in constitution of the various anthocyanins are due (1) to the number, nature and position of the carbohydrate radicles attached to the nucleus, (2) to the number and position of phenolic OH groups introduced, whilst (3) methylation of one or more of the OH groups gives rise to further variation. One and the same anthocyanin may occur in three different modifications in the plant, and these modifications may artificially be produced from the isolated crystallised pigment, *viz.* (1) the oxonium salts with mineral or organic acids are red, (2) the phenolic alkali salts are blue and (3) the neutral form, the free oxonium base, is violet. According to Shibata organo-metallic complex compounds of calcium and magnesium are also important factors in the production of flower colour. A fourth colourless modification will be discussed later.

On heating with 20 % hydrochloric acid the anthocyanins are hydrolysed into carbohydrates (glucose, galactose or rhamnose) and their chromogenic components, for which the general name of *anthocyanidins* was introduced by Willstätter and Everest. A very simple reaction makes it possible to decide whether the glucoside, anthocyanin, or the sugar-free pigment, anthocyanidin, occurs in any plant. The reaction depends on the difference which these pigments show in their distribution between amyl alcohol and aqueous acid. The diglucoside anthocyanins are insoluble in amyl alcohol, and are therefore quantitatively retained as oxonium salts in the aqueous layer on shaking their faintly acid solution with amyl alcohol. The sugar-free anthocyanidins on the other hand are soluble in amyl alcohol and are quantitatively removed from their aqueous solution by shaking with amyl alcohol. There are a few exceptions to this general rule, *e.g.* some monoglucosides and rhamnoglucosides are partially soluble in amyl alcohol, but they can be quantitatively removed again by shaking the coloured amyl alcohol solution with fresh aqueous acid.

By means of this reaction it has been established by Willstätter and Everest that all the anthocyanins of flowers, fruits and leaves occur as glucosides, and only in one single instance, the fruit of the grape vine, has it been found that a variable but small percentage of free anthocyanidin is present in the ripe fruit [Willstätter and Zollinger, 1915, 1916].

It was this exception which led me in the first instance, apart from other considerations, to select the grape vine as a suitable subject in which to investigate the relationship of the pigments of the leaves to those of the fruit. The fact that one of the constituents of the pigment exists in the free condition in the fruit in measurable amounts, suggested that such an investigation might throw some light on the mechanism of the synthesis by which

the living plant builds up its anthocyanins. Further, in plants bearing fruits rich in anthocyanin, such as the grape vine, the chances of following the gradual pigment formation during the slow ripening process seemed to be more hopeful than in the case of the flowering plants, in which the pigments are rapidly formed and the pigment carriers themselves are relatively short-lived.

The material for this study was obtained from two established vines, growing in the open, covering several square yards of wall space on my house and producing yearly a large crop of fully matured grapes. On examining the red pigment of the young leaves of these vines, I found the rather remarkable fact that the whole of it was soluble in amyl alcohol and consisted of an anthocyanidin, most probably oenidin, which occurs in combination with glucose as the chief pigment of the purple grape. The pigment itself was obtained in well-formed crystals by a relatively simple method. It was further found that the leaves contained, in an amount equal to that of the preformed red pigment, a colourless modification of the pigment for which the term *leuco-anthocyanin* is proposed. This is convertible into the anthocyanidin by means of hydrochloric acid. The occurrence of anthocyanidin in young leaves appears to be limited to the species *Vitis vinifera*, and presents a Mendelian character of possible value in genetic investigations.

Experimental.

The red pigment of the young leaves of Vitis vinifera. The young red leaves of two varieties ("Black Hamburgh" and "Esperione") were used. They were at first examined separately, but as no qualitative or quantitative difference with regard to their pigments could be detected, the leaves of both varieties were used indiscriminately. The young leaves are produced during the whole of the growing season, and the nature and distribution of their pigments is practically the same in spring and in autumn. Identical results were obtained with the freshly gathered leaves and with air dried material. As the latter has the advantage of smaller bulk and makes its investigation independent of the season, a large quantity of the leaves was collected and dried at ordinary temperature in air. The powdered material was kept for examination in a desiccator over sulphuric acid.

(*a*) *Oenidin.* In some preliminary experiments 5 g. of the fresh leaves (or 1 g. of the dry powder) were ground up finely with 15 g. of clean sand and treated with 40 cc. of 1 % hydrochloric or sulphuric acid. The extract filtered easily through a folded filter and gave a bright wine red filtrate. Of this 10 cc. were shaken gently with an equal volume of amyl alcohol in a separating funnel. A large amount of the pigment present passed into the amyl alcoholic layer and a second extraction removed practically the whole of the pigment. The extracted fluid has a slightly yellowish tint, which appears to be largely due to the presence of flavone derivatives (see later).

The amyl alcohol solution of the pigment is of a fine bluish-red colour and none of the pigment is removed by shaking it with fresh dilute acid (absence of mono- and rhamno-glucosides). On shaking with a dilute solution of sodium acetate the tint becomes more purple, but the pigment remains in the amyl alcoholic layer. On the other hand, dilute sodium carbonate solution removes the whole of the pigment from amyl alcohol, its alkaline solution turning rapidly brown and yellow. On neutralisation with hydrochloric acid in the early stages, the soda solution again turns red and gives up its pigment once more to amyl alcohol. These reactions clearly characterise the pigment as an anthocyanidin.

Preparation. The pigment crystallises remarkably easily, and extracts of leaves made with 1 % hydrochloric acid at a temperature of about 30° gradually deposit the pigment as a microcrystalline sediment on standing in an open flask for some days. The yield of the crude pigment from two extracts is about 2·5 % of the dry material or 0·5 % of the fresh leaves. It is contaminated by a relatively small amount of colourless substances, from which it can easily be freed by repeated recrystallisation from 3 % hydrochloric acid. The pigment is insoluble in this solvent in the cold, but dissolves easily on slight warming. On allowing its solution to cool gradually, the dark red pigment crystallises out in advance of the colourless admixtures. It is filtered under suction, washed with cold 3 % hydrochloric acid and dried in a desiccator over soda lime.

The pigment appears to be pure and consists of uniform crystals when recrystallised several times in the above way. It may be further purified by making use of its solubility in amyl alcohol and by converting it into its well-crystallised picrate. For this purpose the hydrochloride of the pigment is dissolved in slightly warmed 1 % hydrochloric acid and shaken out with amyl alcohol. From its amyl alcoholic solution it is precipitated by adding $2\frac{1}{2}$ volumes of light petrol (B.P. 40°–60°). A bright carmine red watery layer settles out, from which the hydrochloride soon begins to crystallise. The crystals are redissolved by slightly warming the separated watery layer and removing at the same time the dissolved petrol by a current of air. A saturated solution of picric acid in water is added. On cooling, the picrate crystallises in fine deep red prisms, from which the hydrochloride may be easily obtained by the general method described by Willstätter.

This method does not yield the whole of the pigment present in the young leaves, but its simplicity and the relative purity of the product obtained in the first instance make it on the whole preferable to the method subsequently worked out. The latter need therefore only be described in its outlines. It was found that whilst butyl alcohol is an excellent solvent for anthocyanins [see Rosenheim, 1920] it does not dissolve any red pigment from the dry leaf powder in the absence of mineral acid. It removes, however, the whole of the yellow and green pigments (flavones, chlorophyll, etc.). The dry powder is

therefore extracted at ordinary temperature[1] with butyl alcohol until the solvent remains colourless. Extraction is continued with moist butyl alcohol containing 3–5 % hydrochloric acid. The dark red extract is filtered through a layer of sand on a Buchner funnel, and the pigment precipitated with a mixture of ether and light petrol. It is taken up again in amyl alcohol, re-precipitated by light petrol and finally recrystallised several times from warm 3 % hydrochloric acid.

Properties of the crystallised pigment. The hydrochloride of the pigment is obtained on recrystallisation from 3 % hydrochloric acid in well-formed uniform needles, often arranged in rosettes (see Fig. 1). In transmitted light

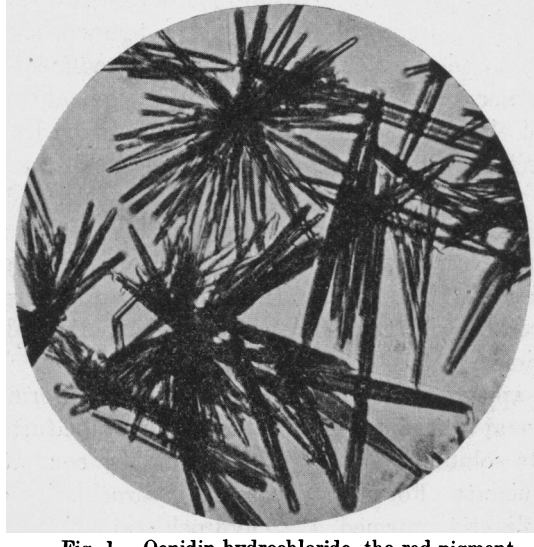


Fig. 1. Oenidin hydrochloride, the red pigment of young vine leaves.

the crystals, seen singly, are transparent with a brownish red tint; where the crystals are superimposed or cross each other their colour is carmine red. They show a red bronze appearance in reflected light, and are anisotropic under the polarisation microscope. Macroscopically they form a reddish brown non-hygroscopic powder. The purest product so far obtained melts on rapid heating at 200°–202° (uncorr.), on slow heating between 195°–197° (uncorr.). The melting point may possibly be raised on further purification. The crystals dissolve with a brownish red colour in warm water, but the solution becomes rapidly colourless on dilution with water, owing to isomerisation into the pseudo-base. This isomerisation also takes place quickly when the alcoholic solution is diluted with hot water, without the intermediate precipitation of the violet colour base, as in the case of cyanidin. The colour is immediately restored by hydrochloric acid. The salt is readily

[1] Extraction in a Soxhlet apparatus is not to be recommended since the pigment appears to become partially isomerised at the high temperature obtaining during the extraction.

soluble in methyl, ethyl, amyl and butyl alcohol, giving fine violet red solutions. The hydrochloride is easily soluble in 0·1 % hydrochloric acid, very easily if warmed; in cold 1 % hydrochloric acid it is only soluble with difficulty and still more so in 3 % hydrochloric acid, from which it may be recrystallised by allowing its hot solution to cool. The salt is also soluble, especially on warming, in dilute sulphuric acid, even up to 5 % sulphuric acid. With picric acid it forms a characteristic picrate, crystallising in deep carmine red prisms. Ferric chloride gives no colour reaction, and an excess quickly destroys the pigment.

The solutions of the pigment show a single dark band in the green, when examined spectroscopically. The following measurements were taken, when a solution of 20 mg. in 10 cc. butyl alcohol was examined:

5 mm. layer: 530—570; blue absorbed from 420 onwards.
10 mm. layer: 515—585; „ „ „ 430 „

The substance is laevorotatory. The observations were made in white light, and as monochromatic red light was not available no measurements were recorded.

In all its properties and reactions the crystallised pigment isolated from young vine leaves agrees with oenidin, the colour component of the pigment of the purple grape. According to Willstätter and Zollinger, oenidin represents a dimethyl ether of delphinidin, the exact positions of the methyl groups in the molecule being still doubtful. Theoretically, eleven dimethyl ethers are possible, of which only two are known (oenidin and malvidin). It will be necessary therefore to determine not only the number of methoxy groups, but also the products of alkaline decomposition in order to settle the identity of the pigment. Material is being collected for this purpose.

(*b*) *Leuco-anthocyanin.* The presence of a colourless modification of the pigment was first observed during the course of some experiments in which the quantitative distribution of the pigments contained in an acid extract of leaves was investigated. For this purpose 1 g. of the dry leaf powder was extracted with 30 cc. of 1 % hydrochloric acid. 10 cc. of the extract were shaken with an equal volume of amyl alcohol (saturated with 1 % hydrochloric acid) which removed 70 % of the red pigment. Two more extracts with amyl alcohol were made, the third showing only a faint pink tint. The completely extracted residual fluid was compared in a Hellige-Autenrieth colorimeter with the original extract, and the result showed that more than 99 % of the pigment had been removed by amyl alcohol. A more exact estimation of the small fraction left in the solution could not be made, owing to the slightly yellowish tint of the fluid. An attempt was therefore made to determine the minute residual anthocyanin fraction indirectly, by converting it into free anthocyanidin through hydrolysis with hydrochloric acid. On boiling the practically colourless solution with 20 % hydrochloric acid, the surprising fact was noticed that the solution assumed immediately a deep

wine red tint, of apparently the same colour intensity as the original extract. The pigment formed was easily soluble in amyl alcohol with a violet red colour, which could be matched with the original extract in the colorimeter. Its strength was determined and found to be exactly the same as that of the original pigment present.

The experiment was repeated in a modified form with another sample of leaves. In this case the extraction was made with acid alcohol in order to exclude the possibility of the formation of any colourless pseudo-base during the extraction, which was rapidly carried out. From the acid-alcoholic extract the pigment was precipitated by a mixture of ether and light petrol and dissolved subsequently in 0·5 % hydrochloric acid. The solution was divided into two equal parts, of which (1) was hydrolysed with hydrochloric acid and the whole of the oenidin present transferred into amyl alcohol (= total anthocyanidin). From (2) the free oenidin was removed by means of amyl alcohol (saturated with 0·5 % hydrochloric acid) and the colourless residual fluid hydrolysed with 20 % hydrochloric acid (= combined anthocyanidin). This was subsequently extracted with the same volume of amyl alcohol as (1) and the two extracts compared in the colorimeter. The following results were obtained:

(1) Total anthocyanidin = 100.

(2) Combined anthocyanidin = 52.

(3) Free anthocyanidin = 48.

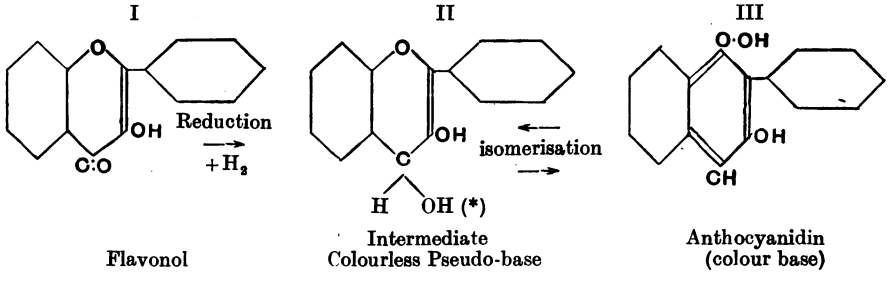
It will be seen that this result agrees well with the previous one and shows that the amount of anthocyanidin present in the colourless modification is about equal to that of free anthocyanidin. This result was confirmed by several other experiments in which various modifications of the procedure were adopted.

The pigment possessed all the characteristics of an anthocyanidin; it remained in the amyl alcoholic layer on shaking with fresh dilute acid, assumed a bluish-violet tint on shaking with dilute sodium acetate solution, and descended quantitatively into dilute sodium carbonate. On rapid acidification of the latter the colour changed into red and the pigment was again given up to amyl alcohol.

On investigating the conditions under which the change from the colourless stage to the pigment takes place, it was found to be independent of the presence of oxygen, for the identical colour intensity is developed with equal rapidity whether the process is carried out in a current of carbonic acid or in air. The chromogenic substance is not extracted by ethyl acetate, after neutralisation of its solution with sodium bicarbonate, thus showing that it does not belong to the rather indefinite group of substances called catechol- or phloba-tannins. These two negative observations are mentioned, since they disprove an assumption, current in the botanical literature of the subject,

according to which the pigments of the grape are formed by oxidation of a colourless oenotannin[1].

In the light of the recent work on the constitution of the anthocyanins, the following suggestion is advanced, as affording an easy and natural explanation of the observed facts. According to Willstätter and his co-workers, the oxonium salts of all anthocyanins and anthocyanidins are hydrolytically dissociated in dilute solutions, giving rise to the formation of the free oxonium base (III) which more or less easily isomerises into the colourless pseudo-base (II). This isomerisation takes place owing to the migration of the basic hydroxyl group from oxygen to carbon. The sugar-free pseudo-bases have been obtained crystallised. They are again transformed into the coloured oxonium salts by acids. The process is therefore a reversible one and according to my observations it is the reverse of this reaction which plays an important rôle in the synthesis of anthocyanins by the plant. It is suggested that the colourless pseudo-bases are the primary products formed by the plant during the reduction of flavonols to anthocyanidins. A consideration of the formulae given below demands, indeed, their formation as a theoretical necessity.



Flavonol — Intermediate Colourless Pseudo-base — Anthocyanidin (colour base)

This reduction has been successfully carried out in the laboratory by Willstätter and Mallison [1914] and, since I have now shown that the free anthocyanidin occurs in young vine leaves, it is not surprising to find also the intermediary product. In the young leaf, however, the pseudo-base does not occur in the free state, but in combination with either a carbohydrate or possibly another complex. For this combination the general name *leuco-anthocyanin* is proposed. If we assume that the carbohydrate (or similar complex) is attached to the pseudo-base by means of the hydroxyl group marked * in II, one can easily understand that the change from the colourless modification into the basic pigment cannot take place until the carbohydrate radicle is split off. This hydrolysis is rapidly effected by strong acids, but only very slowly if at all by 1 % hydrochloric acid. In the presence of the strong

[1] On the other hand, however, I have obtained certain experimental evidence which points to a relationship of a different character between anthocyanins and phlobatannins and which suggests the existence of reduced flavonol rings in the latter substances. Additional support for this suggestion is afforded by the correlation in the distribution of tannins, flavonols and anthocyanins, and by the fact that they all three furnish identical cleavage products on alkaline decomposition (phloroglucinol and various hydroxy-phenolic acids).

acid, the liberated pseudo-base immediately isomerises into anthocyanidin. An attempt to effect hydrolysis by means of emulsin has so far not yielded any decisive results. The chromogenic substance is not extracted by ether even after saturation of its solution with ammonium sulphate. As the free pseudo-bases are soluble in ether, under these conditions, their absence is proved, and this fact also shows incidentally that the colourless modification is not artificially produced, during the preparation of the extract, by the isomerisation of preformed anthocyanidin.

I have so far been unable to isolate the leuco-anthocyanin itself from the young leaves of the grape vine. I found, however, that the unripe berries of the purple grape and the ripe berries of white grapes contain leuco-anthocyanin in much larger amounts and from this source it may be easily obtained, so far only in an amorphous condition. (Further details will be given in a future communication.)

It is interesting to note that Willstätter and Nolan [1915] recorded an unexplained observation in their work on the pigment of *Rosa gallica*, which points to the existence of leuco-anthocyanins in this flower. They noticed that an acid methyl alcoholic extract of the flower showed, on standing, the remarkable phenomenon of gradually increasing its colour to twice its original intensity. They were unable to explain this phenomenon as being due to the occurrence of the pseudo-base of cyanin, since this rapidly changes into the coloured salt under these conditions. It was also not due to oxidation since it occurred equally in the absence of oxygen. They assume the presence of an unknown anthocyanin, the colourless isomer of which is slowly changed into the coloured salt.

Oenidin as a Mendelian Factor. The occurrence of free anthocyanidin as the only red pigment of young leaves in *Vitis vinifera* naturally suggested an investigation of the pigments of young leaves of other plants, in order to find out whether this occurrence represents a general stage in the formation of anthocyanins by the plant organism. For this purpose I examined the pigmented leaves of 85 different species of various families, the enumeration of which would occupy too much space. For a large selection of suitable material I am indebted to the authorities of the Royal Botanic Gardens at Kew, and especially to Mr Dallimore, who also kindly supplied the botanical names. In most cases the young as well as the autumnal leaves were examined. The fresh material was ground with clean sand and extracted with 1 % sulphuric acid. A clear filtrate was obtained in most cases, 5 cc. of which was shaken out with amyl alcohol. The result showed that in all the varieties of *Vitis vinifera* examined, the pigment of the young leaves was soluble in amyl alcohol, whilst all the other plants contained only anthocyanins, insoluble in amyl alcohol, in both their young and autumnal leaves. In a few cases (several species of *Euonymus* and *Geranium*) the amyl alcohol extract was slightly pigmented. The pigment, however, was easily removed by fresh dilute acid, thus proving the absence of anthocyanidins.

It would appear therefore that the occurrence of free anthocyanidins is limited to *Vitis vinifera*, and it became of interest to examine other species of the *Vitis* family. As is well known, *Vitis vinifera* is the only European species of this family, whilst at least 18 species are known in America and about eight in Asia. I was able to examine six Asiatic species (*V. pulchra, Thomsonii, Wilsonii, amurensis, Baileyana* and *Coignetiae*) and three American species (*V. arborea, aestivalis* and *Bourquiniana*). They all gave negative results with the single exception of *Vitis Bourquiniana*. In this case the pigment of the young leaves was to a large extent soluble in amyl alcohol and consisted of anthocyanidin.

This result is rather suggestive in so far as the origin of *V. Bourquiniana* and its recognition as a species has given rise to a great deal of discussion in viticultural journals[1]. According to the standard work on American grapes by Hedrick [1907], the name *V. aestivalis Bourquiniana* was given by Munson, who ranks a group of several similar varieties as a species, in honour of the Bourquin family of Savannah, whose ancestors were supposed to have brought them from France to America over 150 years ago. Munson's derivation of the origin of this vine has not been accepted by either French or American botanists, and the general opinion appears to be that *V. Bourquiniana* is a hybrid between *V. aestivalis* and some form of *V. vinifera*.

This conclusion seems to receive an experimental confirmation by the above described reaction, which clearly demonstrates the influence of *V. vinifera* on the nature of the pigment of the supposed hybrid. If further investigation of other species of *Vitis*, which I hope to carry out, should confirm the observation that the occurrence of free anthocyanidin is limited to *Vitis vinifera*, this biochemical test might prove useful for investigations of genetic problems.

Summary.

(1) The red pigment of the young leaves of the grape vine has been isolated in a crystalline form. It is most probably identical with oenidin, the non-glucosidic component of the pigment of the purple grape. This is the first known, and so far the only instance, in which the red pigment of leaves consists of free anthocyanidin.

(2) The occurrence of a colourless modification of the pigment has been demonstrated, for which the general name *leuco-anthocyanin* is proposed. It is present in combination possibly with a carbohydrate or other complex, and is converted into anthocyanidin by strong acids.

(3) The European species *Vitis vinifera* appears to be the only representative of the family *Vitis*, which is characterised by the production of free anthocyanidin, and the bearing of this observation on genetic problems is discussed.

[1] I am greatly indebted to Mr E. A. Bunyard, of Maidstone, for kindly referring me to the extensive literature of this subject.

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