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Spectral Methods of Characterizing Anthocyanins

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Because of the close relationship that exists between anthocyanins and genetic factors controlling flower colour in higher plants (Lawrence, 1950), considerable efforts have been made to devise rapid and simple methods of characterizing this group of glycosidic pigments. Existing methods, e.g. the well-known colour and distribution tests (Robinson & Robinson, 1932), although of value, provide only limited information about the glycosidic nature of the anthocyanins. More recently paper-partition chromatography has been widely used as an alternative to the older tests (Bate-Smith, 1948, 1949; Hayashi & Abe, 1956), and it provides an excellent method of obtaining pure pigments on a small scale. A method of identifying the sugars of anthocyanins with this technique has recently been described (Harborne & Sherratt, 1957a, b). In a study of the genetic control of anthocyanin production in a number of different plants (Harborne, 1958a), the absorption spectra of a large number of chromatographically pure pigments were measured. As a result, new ways in which spectral measurement can be used for characterizing anthocyanins became apparent.

Although the spectra of many anthocyanins have been examined in the past (Schou, 1927; Hayashi, 1936; Robinson, 1954), little attempt was made to apply these data to the characterization of these

pigments. More recently, spectral measurements have been used for identifying anthocyanidins, mainly in connexion with studies of plant leucoanthocyanins (Bate-Smith, 1954; Roux, 1957a, b). In addition, the effect of aluminium chloride on the spectra of anthocyanins has been described as distinguishing pigments containing *o*-dihydroxyl groups (Geissman, Jorgensen & Harborne, 1953; Geissman & Jurd, 1955).

In the present work complete spectral data are given for all those anthocyanidins which occur naturally as glycosides, as distinct from those which are obtained by treatment of leucoanthocyanins with acid. A method of distinguishing the two main classes of anthocyanin, the 3- and the 3:5-glycosides, is described. Some anthocyanins occur naturally in association with hydroxy aromatic acids in acylated form and the nature and amount of acyl components have now been determined by spectral means.

MATERIALS AND METHODS

Sources of pigments

Anthocyanidins. Small crystalline samples of authentic delphinidin, cyanidin, petunidin, malvidin and hirsutidin were available. Larger amounts of these pigments were prepared from suitable plant material. Pelargonidin was

obtained by acid hydrolysis under standard conditions (Geissman, 1955) of petal extracts of *Dianthus caryophyllus* var. William Sim. Similarly, peonidin was obtained from *Peonia* spp., and rosinidin from *Primula rosea* and *P. polyanthus*. Luteolinidin, apigeninidin and peonidin, and their 5-*o*-benzoyl derivatives, were synthesized by known methods (Murakami & Robinson, 1928; León & Robinson, 1931; Robinson, Robinson & Todd, 1934).

Simple anthocyanins. The following pigments were either available in crystalline form, or isolated in crystalline form, from authentic plant sources (Wawzonek, 1951): pelargonidin 3-monoglucoside and 3:5-diglucoside, cyanidin 3-rhamnoglucoside, 3-gentiobioside and 3:5-diglucoside, peonidin 3-monoglucoside and 3:5-diglucoside, delphinidin 3-monoglucoside and 3:5-diglucoside, petunidin 3-monoglucoside, malvidin 3-monoglucoside and 3:5-diglucoside.

The following pigments, which have not been fully characterized before, were obtained from the plant sources given in parentheses. Pelargonidin derivatives: the 3-rhamnoglucoside (orange-yellow and pink flowers of *Antirrhinum majus*, dark-red tubers of *Solanum phureja*), the 3-gentiobioside (flowers of *Papaver rhoeas*, *P. orientale*, nasturtium), the 3-diglucosido-7 (or -4')-glucoside (flowers of *Papaver orientale*), the 3-rhamnoglucosido-5-glucoside (red tubers of *Solanum phureja*) and the 3-diglucosido-5-glucoside (red skin of *Raphanus sativum*). Cyanidin derivatives: the 3-monoglucoside and the 3-xyloglucoside (berries of *Sambucus nigra*, stems and leaves of *Streptocarpus* spp.). Peonidin 3-rhamnoglucosido-5-glucoside (red-and-blue tubers of *S. phureja*). Delphinidin 3-rhamnoglucoside (deep-blue tubers of *S. phureja*). Petunidin and malvidin 3-rhamnoglucosido-5-glucoside (purple tubers of *S. andigenum* var. Congo). When the pigments occur in acylated form (see below), the simple anthocyanins were obtained by deacylation of the complex with alkali (Robinson & Robinson, 1932).

The following pigments were obtained by partial acid hydrolysis of certain anthocyanins (Harborne & Sherratt, 1957b), and were separated from the other products of partial hydrolysis by paper chromatography. The 5-glucosides of pelargonidin and peonidin were obtained from the corresponding 3:5-diglucosides. Petunidin 3:5-diglucoside was similarly obtained from the corresponding 3-rhamnoglucosido-5-glucoside.

Acylated anthocyanins. Monardein and salvanin were isolated from flowers of *Monarda didyma* and *Salvia splendens* respectively (Karrer & Widmer, 1928; Willstätter & Bolton, 1917). Raphanin was isolated from the skin of the radish (*Raphanus sativum*). Pelanin, petanin and negretein were obtained from the tubers of cultivated potatoes (*S. phureja* and *S. andigenum* var. Congo).

Methods

Isolation. Well-characterized pigments were isolated by the usual methods (Wawzonek, 1951). Details of the isolation of the previously uncharacterized pigments will be given in later papers. The general method of isolating anthocyanins by paper chromatography is a modification of that used by other authors (e.g. Nordström, 1956).

Freshly collected plant material was extracted by maceration with methanol-conc. HCl (97:3, v/v) at room temperature. The extract was centrifuged, and filtered through Celite, and concentrated to a small volume *in vacuo* at 50–60° and refiltered. The concentrated extract was applied as a thin band near one edge of several sheets of

Whatman no. 3 filter paper. When dry, the papers were equilibrated for 24 hr. in a tank with the lower phase of a mixture of butan-1-ol-2*N*-HCl (1:1, v/v) and then developed with the upper phase of the same mixture.

The anthocyanin bands were cut out from the dried chromatograms and eluted separately with water-methanol-acetic acid (25:70:5, by vol.). The individual bands were then separated from the polyphenolic impurities by application on Whatman no. 3 paper and development with butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase) and by repeating the process with water-acetic acid (85:15, v/v) as the solvent.

Purification. Anthocyanidins were purified by chromatography on Whatman no. 1 paper by development with acetic acid-conc. HCl-water (30:3:10, by vol.) or formic acid-conc. HCl-water (5:2:3, by vol.).

Anthocyanin specimens were purified by chromatography on Whatman no. 3 paper by development with one or more of the solvent systems: butan-1-ol-acetic acid-water (4:1:5, by vol., upper phase), butan-1-ol-2*N*-HCl (1:1, v/v, upper phase), acetic acid-water-conc. HCl (15:82:3, by vol.) and conc. HCl-water (3:97, v/v). The anthocyanins were eluted from the paper with water-methanol-acetic acid (25:70:5, by vol.) and the eluates were concentrated *in vacuo* at 50°. After the final purification, the eluate containing the anthocyanin was evaporated to dryness *in vacuo*. The anthocyanin thus obtained was assumed to be pure if it (a) gave a single anthocyanidin after acid hydrolysis; (b) appeared as a single spot on paper chromatograms developed with different solvent mixtures; (c) had a characteristic absorption spectrum in the region 250–600 mμ.

Spectrophotometry. All measurements were made with a Unicam SP. 500 spectrophotometer. The pure dry pigments were dissolved in methanol containing 0.01% of conc. HCl and the solutions diluted to give an optical-density reading in the range 0.800–1.300 at the visible maxima. For measurements in the ultraviolet region, the solutions of those anthocyanins obtained from eluting chromatograms were measured against a solution obtained from an appropriate blank area of the chromatogram, prepared at the same time as the corresponding pigment solution.

For the purpose of measuring spectral shifts in the presence of aluminium chloride, three drops of a solution of the anhydrous salt in ethanol (5%, w/v) were added to the cell solution. Measurements of the shift were made as quickly as possible, since in some cases the colour of the resulting solution faded on standing.

For measuring the spectra of mixtures of pelargonidin 3:5-diglucoside and *p*-coumaric, caffeic and ferulic acids, 1 m-mole of each compound in pure, anhydrous form was dissolved in 50 ml. of methanol containing 0.01% of conc. HCl. Portions (1 ml.) of these stock solutions were mixed together in varying proportions and the solution was made up to 10 ml. The compounds were previously purified by recrystallization and then dried *in vacuo* at 100° over phosphorus pentoxide.

RESULTS

The absorption maxima of some 50 anthocyanidins and anthocyanins in the visible region have been measured and the results are collected in Tables 1–3.

No attempt has been made to record values of the extinction coefficients, since many compounds were not available in sufficient amounts for accurate weighing. All pigments were carefully purified by paper chromatography, as described in the Materials and Methods section.

The same solvent (methanol containing 0.01 % of hydrochloric acid) was always used, in order to avoid shifts in the position of the visible maxima due to pH changes (Sondheimer & Kertesz, 1948) or solvent effects. Many spectra were also recorded in ethanol and, less frequently, in water, both containing 0.01 % of hydrochloric acid. In general, the values for $\lambda_{\max}$ in ethanol are about 10 m μ towards longer wavelengths and in water about 15 m μ towards shorter wavelengths than those given in methanol. For example, the values for cyanidin 3-rhamnoglucoside are 507 m μ in water, 523 m μ in methanol and 533 m μ in ethanol.

Spectra were also measured in the presence of traces of aluminium chloride at a suitable pH (Geissman & Jurd, 1955) and the resulting spectral shifts of pigments containing two or more adjacent hydroxyl groups (Geissman *et al.* 1953) noted. The results obtained here with a much larger number of anthocyanins confirm the use of this reagent for detecting the presence of *o*-dihydroxyl groups in these pigments.

Spectra of the naturally occurring anthocyanidins

The visible spectra of all the anthocyanidins known to occur in nature as glycosides are collected in Table 1. Their structures are given in Fig. 1. Six of these pigments are of frequent occurrence, namely pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Two others, hirsutidin (7-methylmalvidin) and rosinidin (probably 7-methylpeonidin) have been found only

in a few plants (Karrer & Widmer, 1927; Harborne, 1958*b*; Forsyth & Simmonds, 1957). The other two, which lack a 3-hydroxyl group, namely apigeninidin (Robinson *et al.* 1934) and luteolinidin (Harborne, 1957) also occur rarely. The value of spectral measurements for distinguishing them is clear, and the method fails only with delphinidin and petunidin. Fortunately, these two anthocyanidins have quite different chromatographic properties (Bate-Smith, 1954). Apigeninidin and luteolinidin are exceptional in also giving stable spectral maxima in methanolic alkali, with bathochromic shifts of 56 and 70 m μ respectively.

As a result of using two different solvents, methanol and ethanol, it is apparent that there are small but detectable differences between the visible maxima of cyanidin and peonidin, and between delphinidin, petunidin and malvidin. This point has not been noted previously (*cf.* Bate-Smith, 1954). Whereas methylation of the 3'- and 5'-hydroxyl groups causes only small hypsochromic

Table 1. *Spectra and characteristics of the naturally occurring anthocyanidins in the visible region*

Pigment	Spectral maxima $\lambda_{\max}$ (m μ)		AlCl ₃ shift $\Delta\lambda$ (m μ)
	Methanol-HCl	Ethanol-HCl	
Hirsutidin	536	545	0
Malvidin	542	554	0
Petunidin	543	558	24
Delphinidin	546	557	23
Rosinidin	524	534	0
Peonidin	532	542	0
Cyanidin	535	545	18
Pelargonidin	520	530	0
Luteolinidin	493	503	52
Apigeninidin	476	483	0

Fig. 1. Naturally occurring anthocyanidins.

shifts, a more substantial shift of 8–10 $m\mu$ is produced when the 7-hydroxyl group is methylated (rosinidin, hirsutidin).

Characterization of the anthocyanins

The spectra of some 30 anthocyanins have been measured in the range 240–600 $m\mu$. All the simple anthocyanins show similar absorption in the ultra-violet region and it is only in the visible spectra that differences in absorption are apparent. The position of the visible maxima, and the percentage intensity of absorption at 440 $m\mu$ compared with that at the maximum wavelength (i.e. between 500 and 550 $m\mu$), have been measured and the results are collected in Table 2, together with similar data for the related anthocyanidins.

There are two ways in which these measurements can be used to characterize anthocyanins. First,

Fig. 2. Absorption spectra: curve *A*, pelargonidin 3:5-diglucoside; curve *B*, pelargonidin 3:5 diglucoside acylated with *p*-coumaric acid (monardein); curve *C*, pelargonidin 3-monoglucoside. The concentrations of the pigments were about 50 μM , in methanolic 0.01% (w/v) HCl.

Table 2. *Spectral maxima of anthocyanins in the visible region*

	Pigment*	$\lambda_{max.}^{\dagger}$ ($m\mu$)	$E_{440}/E_{max.}^{\dagger}$ (as %)	Mean (%)
Pelargonidin and derivatives	With 5-hydroxyl group free			
	Pelargonidin	520	39	39
	Pelargonidin 3-monoglucoside	506	38	
	Pelargonidin 3-rhamnoglucoside	508	40	
	Pelargonidin 3-gentiobioside	506	36	
	Pelargonidin 3-diglucosido-7 (or 4')-glucoside	498	42	
Cyanidin and derivatives	Cyanidin	535§	19	23
	Cyanidin 3-monoglucoside	525§	22	
	Cyanidin 3-rhamnoglucoside	523§	23	
	Cyanidin 3-gentiobioside	523§	25	
	Cyanidin 3-xyloglucoside	523§	22	
	Peonidin	532	25	
	Peonidin 3-monoglucoside	523	26	
Delphinidin and derivatives	Delphinidin	544§	16	18
	Delphinidin 3-monoglucoside	535§	18	
	Delphinidin 3-rhamnoglucoside	537§	17	
	Petunidin	543§	17	
	Petunidin 3-monoglucoside	535§	18	
	Malvidin	542	19	
	Malvidin 3-monoglucoside	535	18	
Pelargonidin and derivatives	With 5- <i>O</i> -substituent			
	Pelargonidin 5-glucoside	513	15	20
	Pelargonidin 3:5-diglucoside	504	21	
	Pelargonidin 3-rhamnoglucosido-5-glucoside	505	19	
	Pelargonidin 3-diglucosido-5-glucoside	503	21	
	Monardein	505	21	
	Salvianin	505	20	
Cyanidin and derivatives	Cyanidin 3:5-diglucoside	522§	13	12
	Peonidin 3:5-diglucoside	523	13	
	Peonidin 3-rhamnoglucosido-5-glucoside	523	12	
	Peonidin 5-glucoside	528	12	
	Peonidin 5-benzoate	528	11	
Delphinidin and derivatives	Delphinidin 3:5-diglucoside	534§	11	10
	Petunidin 3:5-diglucoside	533§	10	
	Petunidin 3-rhamnoglucosido-5-glucoside	535§	10	
	Malvidin 3:5-diglucoside	533	12	
	Malvidin 3-rhamnoglucosido-5-glucoside	534	9	
	Negretein	536	9	

* As the chloride.

† Measured in methanolic 0.01% HCl; values are correct to $\pm 1 m\mu$.

‡ Average of five determinations, mean error $\pm 2\%$.

§ Only these pigments had their spectra shifted to the blue region by the addition of ethanolic $AlCl_3$.

the spectral characteristics of a particular anthocyanin are obviously related to the anthocyanidin from which it is derived. The three main groups, those based on pelargonidin, cyanidin and delphinidin, are well differentiated spectrally, a reflexion of their characteristically different colours in solution and on chromatograms. Furthermore, cyanidin, delphinidin and petunidin glycosides can be distinguished from the other anthocyanins by the use of aluminium chloride (see above).

Secondly, spectral measurements are of value in determining the position of attachment of the sugars in the anthocyanin molecule. The general effect of glycosidation on the visible spectrum of the anthocyanidin molecule (represented by pelargonidin-3:5:7:4'-tetrahydroxyflavylium) is to shift it towards shorter wavelengths. The amount of this hypsochromic shift depends upon which and how many hydroxyl groups are glycosidated. The largest shift is caused by the introduction of a sugar residue in the 3-position. Thus pelargonidin has $\lambda_{\max}$ at 520 m μ , its 3-glycosides at 505 m μ ($\Delta\lambda = 15$ m μ); cyanidin has $\lambda_{\max}$ 535 m μ , its 3-glycosides at 523 m μ ($\Delta\lambda = 12$ m μ); and delphinidin has $\lambda_{\max}$ 544 m μ , its 3-glycosides at 534 m μ ($\Delta\lambda = 10$ m μ). The hypsochromic shift caused by the introduction of a sugar residue in the 5-position is 7 m μ (cf. pelargonidin, peonidin and their 5-glucosides, Table 2). Only very small differences in the spectral maxima can be detected between anthocyanins containing sugars in both the 3- and 5-positions and 3-glycosides. However, it has now been observed that the absorption spectra in the 400–570 m μ region of all antho-

cyanidins with sugar or benzoyl residues on the 5-hydroxyl group show characteristic differences from those of anthocyanidins in which the 5-hydroxyl group is free. The spectra of the latter group all show a distinct shoulder to the main absorption peak in the 410–450 m μ region (compare curves *A* and *C*, Fig. 2). The best way of recording the differences is to compare the ratio of the optical density at an arbitrarily chosen wavelength (i.e. 440 m μ) with that at the wavelength of maximal absorption. It will be seen (Table 2) that within the three main groups of pigment, the anthocyanins fall into two classes depending on whether or not they have a free 5-hydroxyl group. In effect, the percentage intensity at 440 m μ of 5-substituted anthocyanidin is approximately half that of the corresponding anthocyanidin in which the 5-hydroxyl group is free. This relationship also holds true in the apigeninidin and luteolinidin series (J. B. Harborne, unpublished work). This provides a valuable new means of distinguishing between the two main classes of anthocyanin, the 3- and the 3:5-glycosides.

Acylated anthocyanins

The absorption spectra of some acylated anthocyanins are given in Table 3. The pigments examined are typical of the majority of acylated anthocyanins that have been examined to date, as they contain a hydroxy aromatic acid as their acyl component. They include several pigments of the cultivated potato, all of which contain *p*-coumaric acid (*p*-hydroxycinnamic acid) (Chmielewska, 1936; Harborne, 1956). Salvianin and

Table 3. *Absorption spectra of acylated anthocyanins and pelargonidin 3:5-diglucoside-cinnamic acid mixtures*

Acylated anthocyanins			$\lambda_{\max}$ in methanol-HCl (m μ)	$E_{\max}$ acid/ $E_{\max}$ pigment (as %)
Pigment	Aglycone	Acyl group		
Pelanin	Pelargonidin	<i>p</i> -Coumaric acid	289, 313, 505	67
Monardein	Pelargonidin	<i>p</i> -Coumaric acid	286, 313, 507	60
Salvianin	Pelargonidin	Caffeic acid	285, 329, 507	48
Raphanin A	Pelargonidin	<i>p</i> -Coumaric acid	286, 313, 505	60
Raphanin B	Pelargonidin	Ferulic acid	282, 328, 505	52
Petanin	Petunidin	<i>p</i> -Coumaric acid	282, 308, 536	66
Negretein	Malvidin	<i>p</i> -Coumaric acid	282, 308, 535	71
Cinnamic acids with pelargonidin 3:5-diglucoside			$\lambda_{\max}$ in methanol-HCl (m μ)	$E_{\max}$ acid/ $E_{\max}$ pigment (as %)
Alone	—	Ratio	269, —, 505	6
<i>p</i> -Coumaric acid	1:1			57
	1:2		288, 312, 505	107
	2:1			31
Caffeic acid	1:1			47
	1:2		287, 328, 505	83
Ferulic acid	1:1			49
	1:2		287, 326, 505	91

monardein, two well-characterized pigments, have also been examined. In addition, a new acylated pelargonidin glycoside from the radish has been looked at.

Confirmation of the presence (or absence) of a hydroxy aromatic acid as an acyl component can be made by spectral means, as has already been indicated (Harborne, 1956). The spectra of anthocyanins acylated with *p*-coumaric acid (curve B, Fig. 2) show two peaks in the ultraviolet at 289 and 310 $m\mu$, due to the superimposition of the absorption of the cinnamic acid ($\lambda_{\max}$ 310 $m\mu$) upon that of the pigment absorption. The spectra of simple anthocyanins (curve A, Fig. 2) exhibit a simple peak at about 270 $m\mu$ and only very weak absorption at 310 $m\mu$. In addition, the ratio of the intensity at 310 $m\mu$ to that at the colour maximum (say, 505 $m\mu$) is a measure of the molar ratio of cinnamic acid to anthocyanin in the complex pigment. The results of this estimation are in Table 3, which also includes values for synthetic mixtures, in varying proportions, of pelargonidin 3:5-diglucoside with *p*-coumaric, caffeic (3:4-dihydroxycinnamic) and ferulic (3-methoxy-4-hydroxycinnamic) acid. The value for monardein, known to be pelargonidin 3:5-diglucoside acylated with one molecule of *p*-coumaric acid (Karrer & Widmer, 1928), agrees well with the value for a synthetic mixture. In addition, the results show that the potato pigments (pelanin, petanin and negretein) all have the same molar ratio of anthocyanin to *p*-coumaric acid of 1:1. In addition, the spectral ratio appears to be independent of the nature of the anthocyanin, since pelargonidin and malvidin derivatives (e.g. pelanin and negretein) give values of the same order.

This ratio has been measured in anthocyanins acylated with other cinnamic acids, and the results agree with synthetic mixtures of pelargonidin 3:5-diglucoside with the respective acid. For example, salvianin, thought to contain *p*-coumaric acid by Karrer & Widmer (1929), is a derivative of caffeic acid and has its spectral maximum in the ultraviolet at 329 $m\mu$ (caffeic acid $\lambda_{\max}$ 330 $m\mu$) and not at 310 $m\mu$. A preliminary examination of raphanin, the pigment of the radish, showed it to be an acylated pelargonidin 3-diglucoside-5-glucoside (Harborne & Sherratt, 1957*b*). It has now been found that the pigment contains two components, raphanin A, which is acylated with one molecule of *p*-coumaric acid, and raphanin B, which is acylated with one molecule of ferulic acid (see Table 3).

In all cases, the cinnamic acids present in the above pigments and obtained from them by alkaline hydrolysis have been identified by spectral and chromatographic comparison with authentic compounds.

DISCUSSION

In searching for new methods of characterizing anthocyanins, it should be remembered that they are difficult compounds to deal with by the usual techniques of organic chemistry. In solution, they are unstable to light and pH changes. They are difficult to isolate in a pure state as a general rule. Many of them do not have sharp melting points and do not give meaningful results on elementary analysis. No suitable derivatives are known for characterizing them. The procedure of methylation and hydrolysis, commonly used with flavones for determining the position of sugar residues, is of limited value in the anthocyanin series (Nordström, 1956).

The present study provides additional means for characterizing anthocyanins, which avoid the above difficulties. Spectral methods require only small amounts of pigment and are therefore ideally suited for biochemical studies. The examination of the spectra and chromatographic behaviour of anthocyanins provides the basic information obtainable by the Robinson tests and, in addition, certain other structural conclusions can be drawn. With the naturally occurring anthocyanidins, spectral and chromatographic methods have already been used in a number of investigations (e.g. Dodds & Long, 1955) for identification purposes. Data for the less-common anthocyanidins have not generally been available until now. That these pigments have quite different spectral properties from the commonly occurring compounds strengthens the value of this approach to identification of anthocyanidins.

The most valuable feature of the present study is that the position of the sugar residues in the anthocyanin molecule may be deduced from the spectral data. This is particularly clear in comparing 3- with 3:5-glycosides. It is also likely that 3:7-glycosides are spectrally distinguishable from 3- or 3:5-glycosides. That this is true, at least in the pelargonidin series, is borne out by the discovery of a novel pelargonidin glycoside in the petals of *Papaver orientale*, which has $\lambda_{\max}$ 498 $m\mu$, as compared with $\lambda_{\max}$ 505 $m\mu$ of pelargonidin 3- and 3:5-glycosides. A preliminary investigation of this pigment (Harborne & Sherratt, 1957*b*) indicates that it has two sugar residues in the 3-position, none in the 5-position and one in the 7- or 4'-position. Of the two alternative structures, that in which the third sugar is in the 7-position is the more likely for the following reason. 4'-Glycosides are quite different from other glycosides in their colour reactions in alkaline media (León, Robertson, Robinson & Seshadri, 1931), and no such difference has been noted with this pigment.

Finally, it should be pointed out that these methods have successfully been applied to studies of the anthocyanins present in colour mutants of *Solanum phureja* and *Primula sinensis*, and in a variety of other plant material. Provisional structures for a number of newly discovered pigments have been advanced on the basis of spectral and chromatographic data, of identifying the sugars present (Harborne & Sherratt, 1957a) and of examining the simpler glycosides produced by partial acid hydrolysis. Further details of these results will appear in later papers.

SUMMARY

1. Spectral data for both the common and rare naturally occurring anthocyanidins are given.

2. Spectral measurements have been made of a large number of anthocyanins. It is shown that 3- and 3:5-glycosides have characteristically different absorption properties in the 400–450 mμ region.

3. Measurements of the absorption spectra in the ultraviolet region of anthocyanins acylated with hydroxy aromatic acids can be used to determine the hydroxy aromatic acid:pigment ratio in these complex pigments.

4. The value of these measurements for characterizing anthocyanins is discussed.

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A Sensitive Method for Estimation of Oxaloacetate

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The coupling of acetoacetate with an excess of diazotized *p*-nitroaniline has been utilized by Walker (1954) to develop a sensitive colorimetric

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method for the estimation of acetoacetate. With defined conditions at pH 4.9–5.0, the reaction proceeds as in equation 1 ($R = p\text{-NO}_2\cdot\text{C}_6\text{H}_4$) with the formation of *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan.