Phytochemistry, 1966, Vol. 5, pp. 1263 to 1271. Pergamon Press Ltd. Printed in England

THE FORMATION OF METAL AND "CO-PIGMENT" COMPLEXES OF CYANIDIN 3-GLUCOSIDE

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(*Received* 2 *March* 1966)

Abstract—Quercitrin, chlorogenic acid, and methyl gallate have no measurable effect on the color, spectra, or stability of cyanidin 3-glucoside in aq. solutions at pH 3–6·5. In acetate buffer solutions (pH 5·45) containing aluminum salts, however, quercitrin and chlorogenic acid form highly colored co-ordination complexes with the anthocyanin (anhydro base). The chlorogenic acid complex is blue and insoluble in water. In these properties it distinctly differs from the cerise aluminum chelate of cyanidin 3-glucoside which forms in the absence of chlorogenic acid or other co-pigments. The formation of these co-pigment-aluminum-anthocyanin complexes depends not only on pH but also on the type of organic acids which constitute the buffering system. Thus, complexes do not form in citrate buffers at pH 5·45, since citric acid itself preferentially complexes with the metal and thus makes it unavailable for reaction with the co-pigment and anthocyanin.

RED and blue fruit and flower pigments are chiefly derived from pelargonidin, cyanidin and delphinidin glycosides and their partial methyl ethers. These anthocyanins exist as the red flavylium salts, e.g. (I), only in fairly acidic media. Differences in the shade of color of red fruits and flowers that are acidic, therefore, may be explained simply by variations in the relative and total concentrations of these types of flavylium salts. The extensive occurrence of mauve, violet, and blue flowers and fruits, however, indicates that in many plants red anthocyanins undergo structural modifications to yield relatively stable violet and blue pigments. Factors which may be involved in the formation of blue pigments have been extensively described in reviews by Harborne,[1] Hayashi[2], Blank,[3,4] and Paech.[5] Because of obvious experimental difficulties, however, some of the original literature in this field is not unequivocal and it is clear that the precise nature of these modifications, as well as the factors that induce them, are still largely obscure and speculative. Willstatter's early suggestion that in blue varieties the cell sap is alkaline and thus results in the conversion of the red flavylium salts into blue, ionized anhydro bases has generally been discarded since Hayashi and Isaka[6] and others reported that the pH of flowers, fruits and leaves, irrespective of their colors, is always acidic (usually about pH 5·5, but within the range 2·8–6·2). G. M. and R. Robinson[7]

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[1] J. B. HARBORNE, In *The Chemistry and Biochemistry of Plant Pigments* (Edited by T. W. GOODWIN), p. 225. Academic Press, New York (1965).

[2] K. HAYASHI, In *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 248. Pergamon Press, Oxford (1962).

[3] F. BLANK, *Botan. Rev.* **13**, 241 (1947).

[4] F. BLANK, *Handb. Pflanzenphysiol.* **10**, 300 (1958).

[5] K. PAECH, *Ann. Rev. Plant Physiol.* **6**, 273 (1955).

[6] K. HAYASHI and K. ISAKA, *Proc. Japan Acad.* **22**, 256 (1946).

[7] G. M. ROBINSON and R. ROBINSON, *Biochem. J.* **25B**, 1687 (1931); **26B**, 1647 (1932).

introduced the general concept of co-pigmentation when they proposed that great changes in color may be due to the formation of anthocyanin complexes with organic substances (co-pigments) and possibly with metals. Notwithstanding that each of the natural blue pigments so far isolated, e.g. Bayer,[8] Hayashi, Abe and Mitsui,[9, 10] contain an anthocyanin combined with both metals *and* an organic substance(s). co-pigmentation and metal chelation are apparently considered to be generally distinct, unrelated processes. e.g. "chelation of anthocyanins with metal ions is, *besides* co-pigmentation, the *other* major factor responsible for blueing the flower pigments of higher plants.... Metal complexing *rather than* co-pigmentation is presumably responsible for most of the blue colors in plant berries".[1] As evidence of co-pigment-anthocyanin complex formation, it has been reported that numerous flavonol glycosides, tannins, and other naturally occurring substances have a blueing effect *per se* on anthocyanins in strongly acid solutions. We have now measured quantitatively the effects of quercitrin, chlorogenic acid, and methyl gallate on the spectra (color) and stability of cyanidin 3-glucoside. In aq. solutions under pH conditions similar to those of plant cells, these compounds have no measurable effect on either the color or the stability of the anthocyanin pigment. In the presence of metals (aluminum), however, they form stable, co-ordination complexes with the anthocyanin.

EXPERIMENTAL

The following standard solutions were prepared: cyanidin 3-glucoside (0·212 g/l. in 0·1% aq. HCl); quercitrin (0·212 g/l. in water); chlorogenic acid (0·155 g/l. in water); methyl gallate (1% in water); aluminum chloride ($AlCl_3 . 6H_2O$, 4·23 g/l. in acetate buffer, pH 5·45); ferrous sulfate and ferric chloride solutions (0·5% in water).

To determine the influence of pH on the color and stability of the anthocyanin, 2·0 ml of the standard cyanidin 3-glucoside solution were diluted to 25·0 ml with an aq. buffer solution, the pH was determined, and the spectrum measured immediately and at timed intervals on a Cary 15 recording spectrophotometer, 1 cm silica cells.

To determine effects of added reagents on the color and stability of the anthocyanin, aliquots (usually 1·0 ml) of the appropriate standard solutions were added to an aq. buffer solution, 2·0 ml of the cyanidin 3-glucoside solution were *then* added, the volume made up to 25·0 ml, and the pH and spectrum determined as before.

Acetic acid–sodium acetate, citric acid–disodium hydrogen phosphate, and citric acid–sodium citrate buffer solutions were employed as discussed in the text.

RESULTS AND DISCUSSION

Influence of pH on the Color (Spectra) and Stability of Cyanidin 3-Glucoside

In Figs. 1 and 2 the visible spectra of cyanidin 3-glucoside (I) in the pH range 1–8 are shown. These spectra were determined within 1 or 2 min after mixing. The intensity of absorption markedly decreases as the pH is raised and above pH 4 the λ_{max} shifts progressively from 510 mμ (the λ_{max} of the flavylium salt) to 538 mμ (the λ_{max} of the anhydro base). In the pH range 4–5 the solutions are virtually colorless, and it is apparent that at these pH the anthocyanin is almost quantitatively and immediately converted via the unstable anhydro

[8] E. BAYER, *Chem. Ber.* **91**, 1115 (1958).
[9] K. HAYASHI, Y. ABE and I. MITSUI, *Proc. Japan Acad.* **34**, 373 (1958).
[10] S. MITSUI, K. HAYASHI and S. HATTORI, *Botan. Mag. Tokyo* **72**, 325 (1959).

base (II*a* or *b*) into its colorless carbinol base (III). At pH above 5 the stability of the anhydro base progressively increases. The *unionized* anhydro base is most stable at about pH 6·10.

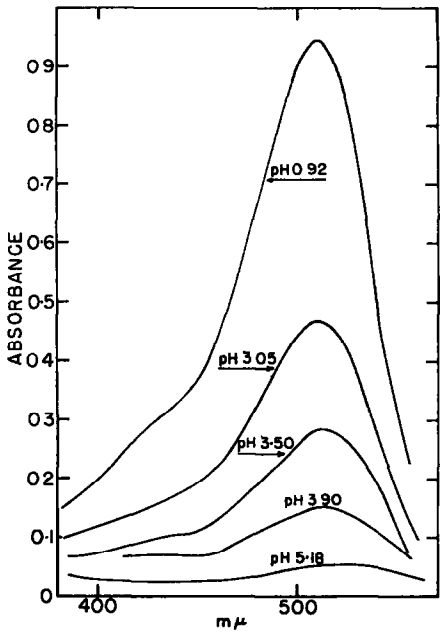


FIG. 1. VISIBLE SPECTRA OF CYANIDIN 3-GLUCOSIDE (CONC. $= 3{\cdot}5 \times 10^{-5}$ MOLE/L.) AT pH 0·92–5·18. Spectra measured in aq. buffer solutions 1 min after mixing.

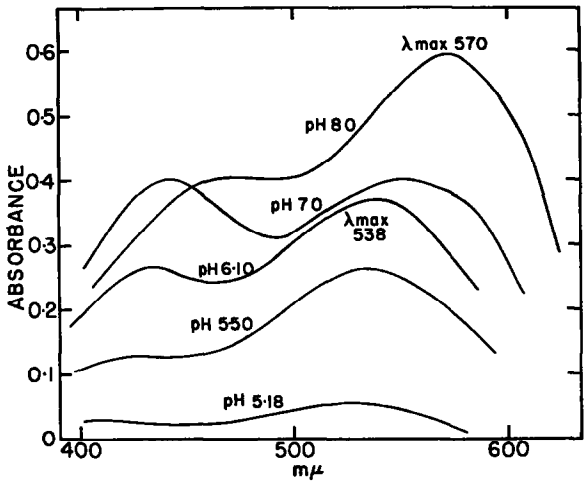


FIG. 2. VISIBLE SPECTRA OF CYANIDIN 3-GLUCOSIDE (CONC. $= 3{\cdot}5 \times 10^{-5}$ MOLE/L.) AT pH 5·18–8·0. Spectra measured in aq. buffer solutions 1 min after mixing.

The rate of decoloration of the anhydro base at this pH is indicated in Fig. 3, which shows that even at this pH the anhydro base is highly unstable and loses almost all color in an hour.

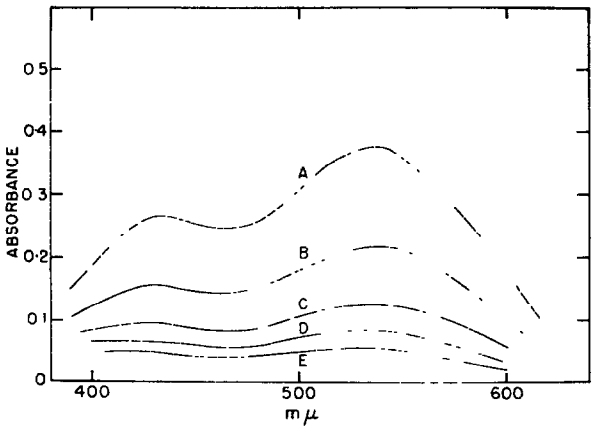
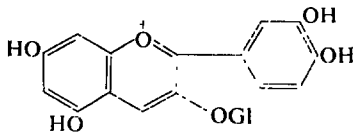
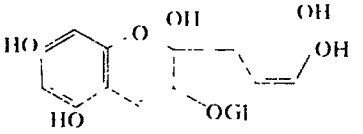


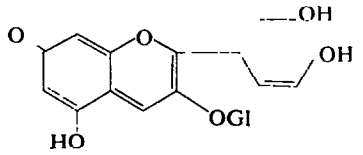
FIG. 3. VISIBLE SPECTRA OF CYANIDIN 3-GLUCOSIDE (CONC. = $3{\cdot}5 \times 10^{-5}$ MOLE L^{-1}) IN AQ. BUFFER, pH 6·10. Spectra measured after: (A) 1 min, (B) 10 min, (C) 20 min, (D) 30 min, and (E) 60 min



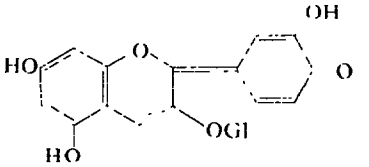
(I) λ_{max} 510 mμ



(III) Colorless

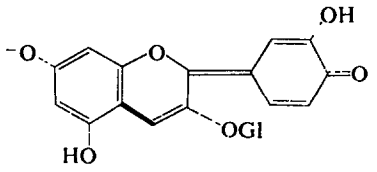


(IIa)

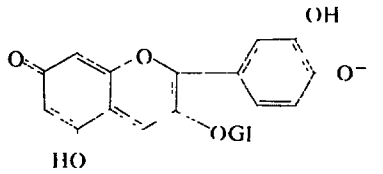


(IIb)

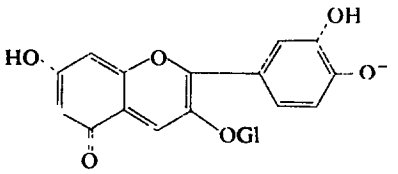
λ_{max} 538 mμ



(IV)



(V)



(VI)

At pH 7·0 the spectral solutions become deep blue, the absorption band broadens considerably, and the stability of color increases (complete decoloration takes about 24 hr). These effects indicate that the anhydro base begins to partially ionize between pH 6·10 and 7·0, and that the increased stability of the ionized anhydro base is due to the possibility of resonance between forms of type (IV)–(VI). On the basis of these observations it would seem that *free* cyanidin 3-glucoside could contribute color (red) only to those plant tissues with a pH of about 4 or less. Because of the pronounced instability of both colored forms (flavylium salt and anhydro base) of this anthocyanin at higher pH, it would appear most improbable that the anthocyanin *per se* could contribute significant color to those flowers and fruits whose pH lies in the range 4–6·2.*

Influence of "Co-pigments" on the Color and Stability of Cyanidin 3-Glucoside

G. M. and R. Robinson[7] observed that acid extracts of flowers are generally bluer than solutions of pure anthocyanins. They obtained the same blueing effects, however, by adding a variety of co-pigments to strongly acid anthocyanin solutions. They found that flavonol glycosides, e.g. quercitrin, and gallates were among the most effective co-pigments in this respect. It was also recognized that chlorogenic acid, a ubiquitous, natural polyphenol, may have some significance in relation to flower color, when it was found that blue hydrangeas do *not* contain any chlorogenic acid whereas red forms have substantial amounts.[11] Blue flowers of *Ipomea laurii* similarly are free of chlorogenic acid but fading, accompanied by reddening, causes development of the acid. In more recent years co-pigmentation of anthocyanins with co-occurring flavonol glycosides and, in one instance, with the xanthone, mangiferin, has been reported in blue flowers of a number of species.[1, 12, 13] Although few quantitative spectral measurements have been published, it has been reported that quercetin and kaempferol glycosides interact with cyanidin and malvidin glycosides *in acid solutions* to produce 1–5 mμ bathochromic shifts in the λ_{max} of these anthocyanins.[14, 15]

We have now examined the effects of one to ten molecular equivalents of quercitrin, chlorogenic acid and methyl gallate on cyanidin 3-glucoside in aq. solutions at pH 3–6. As illustrated by the spectra of quercitrin–anthocyanin mixtures at pH 3·50 (Fig. 4) and pH 5·50 (Fig. 5) none of these compounds showed any measurable effect on either the position or the stability of the λ_{max} of the anthocyanin. The spectral curves of the anthocyanin-co-pigment mixtures were simply the sum of the curves of the individual components.

Metal Chelates of Cyanidin 3-Glucoside

It is well known that anthocyanins that contain an *ortho*-dihydroxyl system form colored metal complexes.[16, 17] The influence of pH on chelation of cyanidin 3-glucoside with aluminum is shown in Fig. 6. Maximum complex formation occurs at about pH 5·5 and above and the cerise complex that forms at pH 5·5 is remarkably stable on long standing in

* Pigmented cells often occur only in a small and specific portion of a plant tissue, e.g. the epidermis of a flower or fruit. The pH of the pigmented cells, therefore, may actually vary considerably from that determined for the whole flower or fruit.

[11] G. M. Robinson, *J. Am. Chem. Soc.* **61**, 1606 (1939).
[12] R. C. Peckett and A. R. A. A. Selim, *Nature* **195**, 620 (1962).
[13] E. C. Bate-Smith and J. B. Harborne, *Nature* **198**, 1307 (1963).
[14] J. Harborne and H. S. A. Sherratt, *Biochem. J.* **78**, 298 (1961).
[15] J. B. Harborne, *Experientia* **17**, 72 (1961).
[16] E. Bayer, *Chem. Ber.* **92**, 1062 (1959).
[17] E. Bayer, K. Nether and H. Egetter, *Chem. Ber.* **93**, 2871 (1960).

diffused light. Ferrous and ferric salts similarly form stable blue complexes (λ_{max} 600 and 560 mμ, respectively), maximum complex formation also occurring in these cases above pH 5·5.

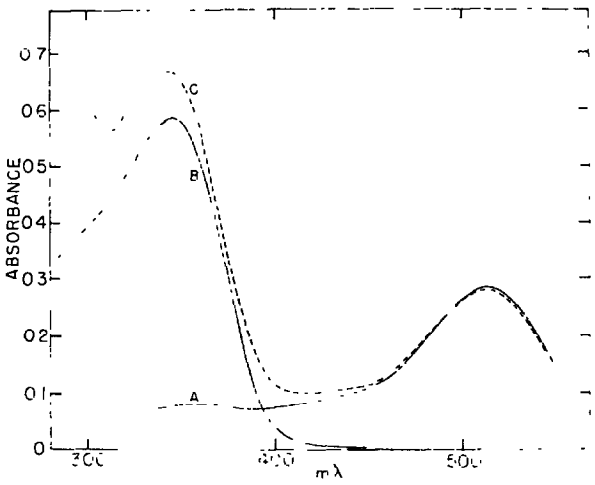


FIG. 4. SPECTRA IN AQ. SOLUTIONS (pH 3·50) OF (A) CYANIDIN 3-GLUCOSIDE; (B) QUERCITRIN AND (C) (A)+QUERCITRIN.

Spectra measured immediately after mixing.

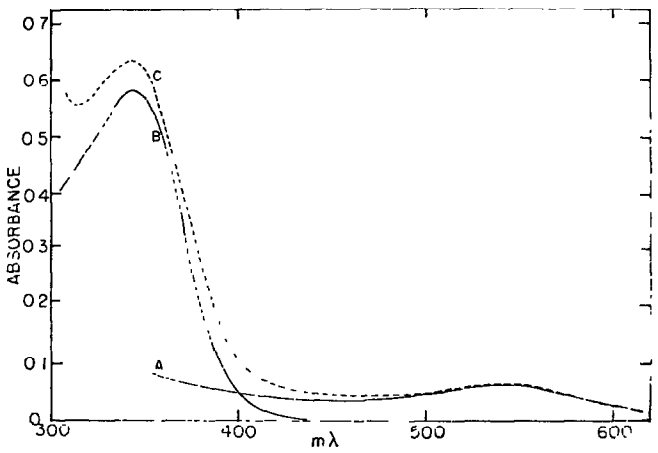


FIG. 5. SPECTRA IN AQ. SOLUTIONS (pH 5·50) OF (A) CYANIDIN 3-GLUCOSIDE; (B) QUERCITRIN AND (C) (A)+QUERCITRIN.

Spectra measured 1 hr after mixing.

It is noteworthy that the absorbance at the λ_{max} (555 mμ) of the chelate rapidly decreases during the first hour (to about 70 per cent of the initial value) and then the rate of decrease levels off (~60 per cent after 220 hr). Since the colored chelate is presumably an aluminum-anhydro base derivative, the initial rapid decomposition may be due to establishment of an equilibrium with the colorless carbinol base (or aluminum–carbinol base chelate).

The aluminum chelate described above formed only in acetate buffers. In citrate buffers

aluminum did not chelate with cyanidin 3-glucoside. Thus, citrates clearly react preferentially with the metal and render it unavailable for complex formation with the anthocyanin. This observation is in accord with that of Bate-Smith *et al.*[18] who noted that citric acid inhibits the formation of a colored complex between iron and chlorogenic acid in potatoes. In plant cells, therefore, it would seem that complex formation involving metals must depend not only on the pH of the cell, but also on the nature of the organic acids that constitute the buffering system of the cell.

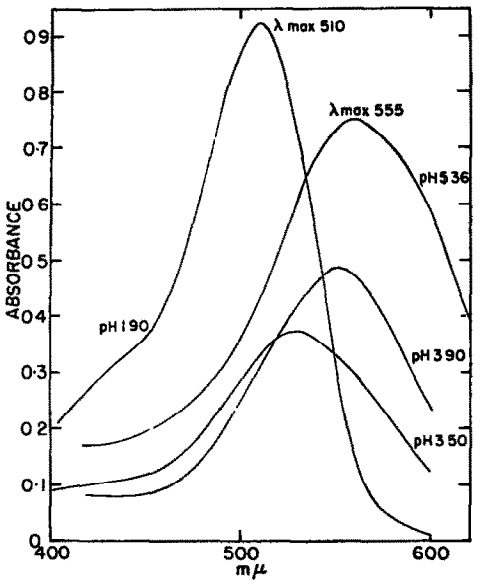


FIG. 6. EFFECTS OF pH ON VISIBLE SPECTRA OF CYANIDIN 3-GLUCOSIDE (CONC. $= 3{\cdot}5 \times 10^{-5}$ MOLE/L.) $+ AlCl_3$ ($8{\cdot}3 \times 10^{-4}$ MOLE/L.).
Spectra measured in aq. buffer solutions after 1 min.

Aluminum-co-pigment Complexes of Cyanidin 3-Glucoside

As previously shown in Figs. 5 and 6, quercitrin has no effect on the spectrum of cyanidin 3-glucoside at pH 5·5, but aluminum ions form a stable, cerise aluminum–anhydro base chelate (λ_{max} 555 mμ). The effect of quercitrin on the spectrum of cyanidin 3-glucoside at pH 5·45 in the presence of excess of aluminum is shown in Fig. 7. Compared to the aluminum–anthocyanin chelate, the λ_{max} undergoes a marked increase (45%) in intensity and a pronounced bathochromic shift to 580 mμ. It is apparent that, under these conditions in the presence of metal ions, quercitrin is involved in complex formation with the anthocyanin.*

As shown in Fig. 8, addition of chlorogenic acid to solutions of cyanidin 3-glucoside and aluminum chloride in an acetate buffer (pH 5·45) results in a marked increase in intensity and a bathochromic shift of the λ_{max} (from 555 mμ to 570 mμ). These effects progressively increase as the concentration of chlorogenic acid is increased (Fig. 8, spectra B and C). These spectra indicate that chlorogenic acid, aluminum, and cyanidin 3-glucoside form a complex which,

[18] E. C. BATE-SMITH, J. C. HUGHES and T. SWAIN, *Chem. & Ind. (London)* 627 (1958).

* An aluminum–quercitrin complex (yellow) is also present in this solution. It shows no absorption above 500 mμ, however, and is not involved, therefore, in the observed shift of the λ_{max} of the anthocyanin complex.

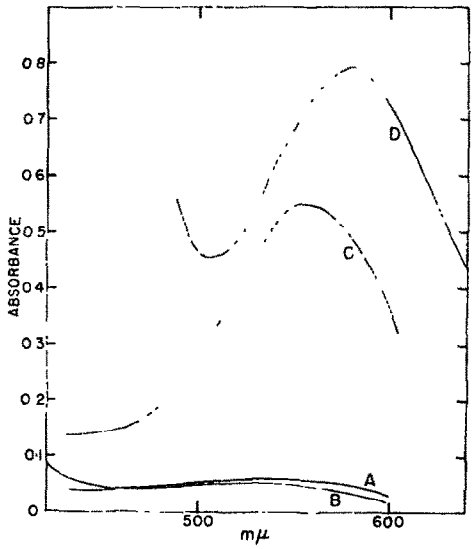


FIG. 7. SPECTRA IN AQ. SOLUTIONS AT pH 5·45 OF (A) CYANIDIN 3-GLUCOSIDE ($3{\cdot}5 \times 10^{-5}$ MOLE/L.); (B) (A)+QUERCITRIN (5 MOLE EQUIV.); (C) (A)+$AlCl_3$ ($8{\cdot}3 \times 10^{-4}$ MOLE/L.) AND (D) (B)+$AlCl_3$ ($8{\cdot}3 \times 10^{-4}$ MOLE/L.).

Spectra measured 1 hr after mixing.

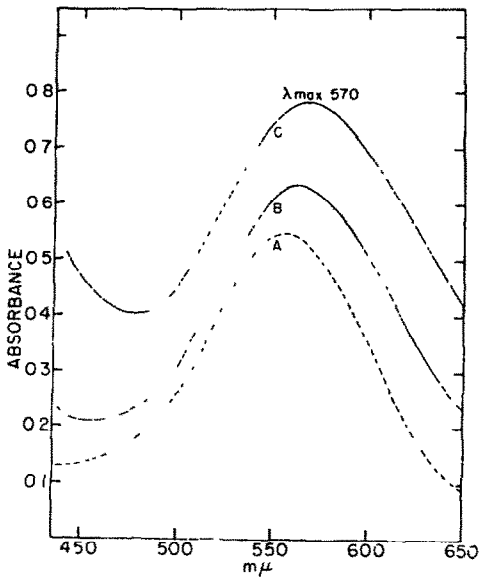


FIG. 8. SPECTRA IN AQ. SOLUTIONS AT pH 5·45 OF (A) CYANIDIN 3-GLUCOSIDE ($3{\cdot}5 \times 10^{-5}$ MOLE/L.) +$AlCl_3$ ($8{\cdot}3 \times 10^{-4}$ MOLE/L.); (B) (A)+CHLOROGENIC ACID (2·5 MOLE EQUIV.) AND (C) (A)+CHLOROGENIC ACID (5·0 MOLE EQUIV.).

Spectra measured 1 hr after mixing.

unlike the cerise aluminum–anthocyanin chelate, is almost "pure" blue (due to the intense absorption in the 600–660 mμ region). This complex is extremely insoluble and, when the spectral solutions stand for a few hours it separates completely as a blue, flocculent precipitate from the colorless, supernatant solution. Prolonged acid hydrolysis of the precipitate has shown the presence of both the anthocyanin and chlorogenic acid.* As in the case of quercitrin, the chlorogenic acid–aluminum–anthocyanin complex formed only in an acetate buffer. In citrate buffers at the same pH (5·45) no complex formation occurred. Furthermore, addition of citrate buffer (pH 5·45) to a suspension of the solid, blue complex in acetate buffer (pH 5·45) resulted in the rapid solution and decoloration of the complex. This is due to abstraction of aluminum from the complex by the citrate. These observations demonstrate that in order to function as a co-pigment with cyanidin 3-glucoside in the physiological pH range, quercitrin and chlorogenic acid require the presence of a metal. A co-ordinate complex with the anthocyanin (anhydro base) can then form. In this connexion note that Harborne and Sherratt[14] suggested (without further elaboration) that the effectiveness of flavonol glycosides as co-pigments "may involve other factors (e.g. metal ion concentration)".

On the basis of the above results it would appear reasonable to speculate that chlorogenic acid occurs in both red and blue hydrangea flowers, but that in blue varieties it is present as a water-insoluble blue complex with aluminum and delphinidin. Fading of blue flowers of *Ipomea laurii*, accompanied by simultaneous reddening and formation of chlorogenic acid, may be due to the formation of acids, such as citric acid, which, by abstracting metals, liberate chlorogenic acid from a blue complex with the anthocyanin.

Acknowledgement—The authors are indebted to Dr. A. Neubert for the gift of cyanidin 3-glucoside.

* Tentative, preliminary work indicates a caffeic acid–cyanidin ratio of 3:1 or 4:1.