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REVIEW

THE *IN VIVO* EXPRESSION OF ANTHOCYANIN COLOUR IN PLANTS

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Key Word Index—Anthocyanins; monomeric species; structural transformations; pH effect; copigmentation; self-association; UV-visible spectroscopy; Raman spectroscopy.

Abstract—During the last two decades both model experiments and *in vivo* investigations have given a deeper insight into our understanding of the organization of anthocyanins in living tissues. The main factors known to affect the colour of anthocyanin containing media are: the chemical nature of an anthocyanin, its concentration, the co-occurrence of several pigments, the pH and the presence of copigments. Under conditions as similar as possible to the conditions in nature, low concentrated aqueous solutions of the most common anthocyanins are characterized by the existence of several chemical equilibria: a fast acid and base catalysed prototropic tautomerism; a hydration of the pyrylium ring and a ring-chain prototropic tautomerism leading to opening of the pyranol ring. In their natural states, anthocyanins must be protected against nucleophilic reagents, especially water which is the main constituent of the vacuole. Nucleophilic addition of water to the flavylium cation results in colour loss. Copigmentation is probably the most efficient mean of protecting the flavylium cation against these compounds. Until now, the only suitable spectroscopic technique for anthocyanins *in vivo* studies was UV-visible absorption spectroscopy. Very recently, resonance Raman spectroscopy was successfully applied to both aqueous anthocyanin solutions and to living tissues containing these pigments.

INTRODUCTION

Flowers and fruits offer an infinite variety of colours; anthocyanins are responsible for most of their orange, red and blue colours [1]. Like other phenolic compounds, they are located in the cell vacuole [2]. These pigments are water-soluble and, though they accumulate essentially in the epidermal cells of flowers and fruits, they are frequently found also in other parts of a plant, e.g. roots and leaves [1, 3–5]. They are synthesized by almost all the higher plants and their biosynthetic pathways are now well-established [6]. Their main function seems to be to attract insects and birds for pollination purposes (flowers) and animals for seed dissemination (fruits). Complete identification of the anthocyanins occurring in the plant world is far from being accomplished, and more and more accurate and detailed investigations of the anthocyanins present in plant species are regularly reported [5, 7–15].

From a chemical point of view anthocyanins belong to the flavonoid series. Whereas flavonoids are generally colourless, it is noteworthy that anthocyanins occur in the cell sap in chemical states strongly absorbing visible light. Model experiments have shown, however, that under conditions as similar as possible to natural conditions (room temperature and slightly acidic aqueous solutions), most of the anthocyanins found in nature are stable but in a colourless form. Therefore, there must exist *in vivo* mechanisms strongly stabilizing the coloured at the expense of the colourless structures. Since many substances coexist with anthocyanins in the vacuoles (other phenolic compounds, proteins, peptides, waste products of nucleic acids, sugars, amino acids, organic acids, minerals such as calcium, potassium and magnesium ions), there is no doubt that some may interact with anthocyanins resulting in the prevention of colour loss or even in colour enhancement.

Twenty years ago Dean [16] in his outstanding review *Naturally Occurring Oxygen Ring Compounds* recognized that: "Attempts to discover the precise form in which anthocyanins exist in plant material have absorbed much effort without producing any result that can be stated succinctly. For most studies the plant material has been crushed or otherwise damaged severely enough to upset the complex equilibria existing in both cells and sap and to alter the conditions in which the pigments originally existed." Today the situation is no longer so bad, and during the last two decades, results obtained by many research workers have laid the foundations of our present understanding of the organization of anthocyanins in the plant itself.

In vitro experiments, conducted under physico-chemical conditions which prevail in nature, have given a deeper insight into the chemical processes likely to take place *in vivo*. For such studies the most popular tool is UV-visible absorption spectroscopy. This is because water and alcoholic solvents are transparent in the visible region, and also because some anthocyanin chromophores strongly absorb part of the visible light. UV-visible absorption spectroscopy was mainly used for identification purposes [1, 17] and, more recently, for investigating the kinetics and the thermodynamics of anthocyanins and structurally related compounds [18–21]. Circular

dichroism was shown to be a sensitive probe to demonstrate the existence of association processes in the case of anthocyanins [22–24]. ^{1}H NMR spectroscopy, never successfully applied previously to natural anthocyanins, has now been shown to be an invaluable tool in the hands of Goto and his collaborators [25–30]. In 1967, Saito [31] applied UV-visible absorption spectroscopy to intact plant tissues. In the early 1970s Asen *et al.* [32] developed a microspectrophotometric method of anthocyanin analysis by coupling a microscope to an UV-visible spectrophotometer; spectra from areas as small as a single cell were recorded. Very recently, resonance Raman spectra of anthocyanins, either in pure water or in the living cells, have been obtained [33]. Fast atom bombardment mass spectrometry of anthocyanins was achieved very recently and it was possible to get good molecular ions of the simple anthocyanins as well as more complex acylated pigments and also some structural information from the fragmentation patterns [N. Saito and C. F. Timberlake, personal communication]. Thus, the molecular mass of violanin as 919 was confirmed. Platyconin is now known to possess two additional molecules of glucose [N. Saito and K. Abe, unpublished results] and this was confirmed by measuring the accurate mass of platyconin as 1421.3740, i.e. within 6 ppm error of the calculated mass (1421.3830) for $C_{63}H_{73}O_{37}$ [N. Saito and C. F. Timberlake, personal communication].

The method, used in studies of anthocyanin pigmentation of flowers and fruits, consists firstly in extracting and isolating the anthocyanins and then mixing them with substances also found in the vacuoles. The agreement between the absorption spectrum of an intact cell and the absorption spectrum of a suitable model experiment is taken as good evidence for the existence of a similar phenomenon in the living cell [34, 35]. Another approach is to isolate so-called genuine anthocyanins. This is achieved by mild extraction techniques. This method, however, has frequently led to conflicting results for the *in vivo* anthocyanin structures, as far as metalloanthocyanins are concerned [22, 36–39].

In this review, attention will be focused first upon the transformations undergone by anthocyanins when left in pure water at low concentrations (10^{-5} M or less) and room temperature. Under such conditions association processes usually do not take place, and the observed reactions characterize the monomeric anthocyanin species. As previously stated, and as long as one deals with simple anthocyanins and slightly aqueous solutions, almost colourless solutions result from these conditions. However, in nature the concentrations of anthocyanins are frequently much larger (10^{-2} M or even more). Later, we shall see whether anthocyanins confer colour to the cells by themselves or whether other factors are involved in the pigmentation phenomenon. Emphasis will be given to the most important factors stabilizing the anthocyanin chromophores. Finally, *in vivo* microspectroscopic investigations will be considered. A complete survey of the parameters controlling flower colour in higher plants has been given [3]. Chemical structures and biosynthesis of anthocyanins as well as their relation to the food industry have been discussed very recently [40].

COMPETITION BETWEEN WATER ADDITION AND PROTON TRANSFER REACTIONS

Anthocyanins are generally isolated as flavylium cations, the counter-ion being the chloride or perchlorate anion. In the plant itself, the counter-ion is probably an organic anion [1]. In the case of anthocyanins, the flavylium heterocycle bears hydroxyl and methoxyl groups as well as glycosyl groups. The flavylium cation of gentiodelphin, an anthocyanin very recently extracted from *Gentiana makinoi*, is shown in Fig. 1 [29]. Complete elucidation of its stereostructure was made possible by use of ^{1}H NMR spectroscopy. The position of attachment of each glucosyl residue was determined by the nuclear Overhauser effect and they were established to be in the β-pyranosyl form. The ^{1}H NMR signals of the methylene groups of the glucosyl groups at C-5 and C-3′ reveal that the glucose 6-hydroxyl groups are esterified by a caffeic acid molecule shown to be in the *trans* form. In phosphate buffer, pH 6.50 and room temperature, the colour of gentiodelphin is stable (colour retention is 85% after 15 hr), whereas *bis*-deacylgentiodelphin loses its colour in *ca* 1 hr [28]. The importance of the two acyl residues in stabilizing the colour will be considered later. Many anthocyanins possessing acyl residues linked to the sugars are now known. The acylating agents most frequently encountered belong to the hydroxylated cinnamic acid series. With a few exceptions—3-deoxyanthocyanins—there is always a glycosyl group at C-3. Classes of anthocyanin glycosides known at the present time have been reviewed [1, 4]. When more than one sugar is present in the molecule, the other sugar(s) can be attached to any one of the hydroxyls at C-5, C-7, C-3′, C-5′ and, perhaps, even at C-4′ [14]. Methoxyl substituents are found at the 3′ and 5′ positions and, less frequently, at positions 7 and 5. [1, 41]. It is remarkable that, until now, no natural anthocyanin where all the three hydroxyl groups at the 5, 7 and 4′ positions are substituted at the same time has been isolated [42]. This observation emphasizes the importance of the quinonoidal bases in flower and fruit pigmentation due to anthocyanins. Effectively, if a free hydroxyl is present at any of these three positions, a quinonoidal

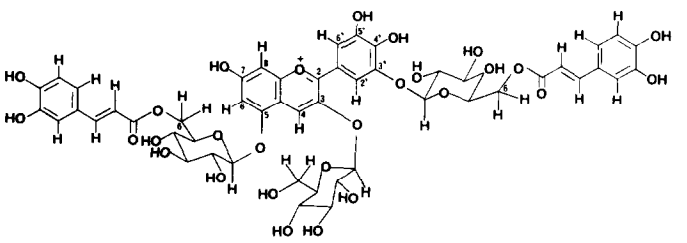

Fig. 1. Stereostructure of the flavylium cation of gentiodelphin (by courtesy of Goto *et al.* [29] and the permission of Pergamon Press).

base can be readily formed. In its absence the flavylium cation remains the only coloured species. It is generally believed that glycosylation confers solubility and stability to anthocyanins [43]. In the light of the most recent findings on acylated anthocyanins, the glycosyl groups may play a major chemical role by either bearing the acyl groups which can then interact with the pyrylium ring [29, 44], or by favoring association of the flavylium cation with another flavonoid molecule as exemplified by the commelinin pigment [22].

In the plant cell, anthocyanins are usually dissolved in the slightly acidic aqueous vacuolar sap. Almost all anthocyanins give rise to many elementary reactions when placed in slightly acidic aqueous solutions. Owing to the high sensitivity of UV-visible spectrophotometry, these reactions are easy to investigate at low concentrations (10^{-5} M or less). It is only during the past 5 years that they have been firmly established [19, 20, 42, 45, 46]. The main results are: (a) the discovery of a kinetic and a thermodynamic competition between the hydration reaction of the pyrylium ring and the transfer of a proton from a hydroxyl of the aglycone to a water molecule; and (b) the existence, in the case of the natural anthocyanins, of an open form, the chalcone pseudobase.

Proton transfer reactions

The hydroxyl groups at C-5, C-7 and C-4′ of the flavylium cation can lose a proton at pH values close to 4–5 giving rise to quinonoidal bases in fast acid–base equilibrium (Fig. 2). For natural anthocyanins and some synthetic structurally related compounds, the pK'_a values ($K'_a = ([A]/[AH^+])\ a_{H^+}$ and $pK'_a = -\log K'_a$) known today, range from 3.50 to 4.85 [42]. Such a high acidity of the phenolic groups is due to the delocalization of the positive charge over the whole flavylium system. If a second hydroxyl is present, further deprotonation leads to a resonance-stabilized quinonoidal anion. This occurs in the pH range 6–8, i.e. at neutrality.

Covalent hydration of the flavylium cation

3-*O*-Glycosylated flavylium cations are rapidly and completely hydrated to carbinol pseudobases at pH values ranging from 3 to 6. Water addition essentially takes place at the 2 position and it has been demonstrated that no measurable amount of the 4-adduct occurs, owing to unfavorable kinetic and thermodynamic conditions [42] (Fig. 3). In the case of some non-hydroxylated flavylium cations, carbinol 4-adducts have been detected [21]. In the absence of a 3-glycosyl substituent, the hydration process is less efficient and the carbinol only forms at pH values up to 4–5 [R. Brouillard, G. A. Iacobucci and J. C. Sweeny, unpublished]. The hydration–dehydration equilibrium leading to the carbinol 2-adduct B is characterized by a pK'_h value which is expressed in the same way as the pK'_a value ($pK'_h = -\log K'_h$ with $K'_h = ([B]/[AH^+])\ a_{H^+}$, where a_{H^+} is the hydronium ion activity that is $pH = -\log a_{H^+}$). Usually, pK'_h values are in the range 2–3 [18, 47]. The carbinol pseudobases are always colourless and their maximum of absorption in water is close to 275 nm. Therefore, whenever the covalent hydration of the flavylium cation occurs to an appreciable extent only poorly coloured media are observed. It is easy to follow the progress of the hydration process by recording the decrease in the visible absorption band as a function of time or as a function of the acidity [20]. Effectively, most of the natural flavylium salts have visible absorption maxima situated between 505 and 530 nm [48].

It is reasonable to believe that the carbinol 4-adduct constitutes an intermediate stage during the reduction of flavones and flavonols to anthocyanidins. Once the 4-adduct is formed, it can evolve either to the more stable 2-adduct or to the flavylium cation depending upon the acidity of the medium. Conversion of the 4-adduct to the 2-adduct should be sufficiently slow to permit its observation by choosing a suitable method of analysis. It does not seem, however, that anthocyanins have been frequently prepared by reduction of the corresponding flavonol glycosides [49–51]. Nevertheless, reduction of rutin (quercetin 3-rutinoside), for instance, conducted in a slightly acidic aqueous solution should give cyanidin 3-rutinoside in the form of the carbinol 2-adduct.

Opening of the pyranol ring of the carbinol pseudobase

Formerly, it was thought that natural anthocyanins do not form a chalcone pseudobase [52]. Recently, by

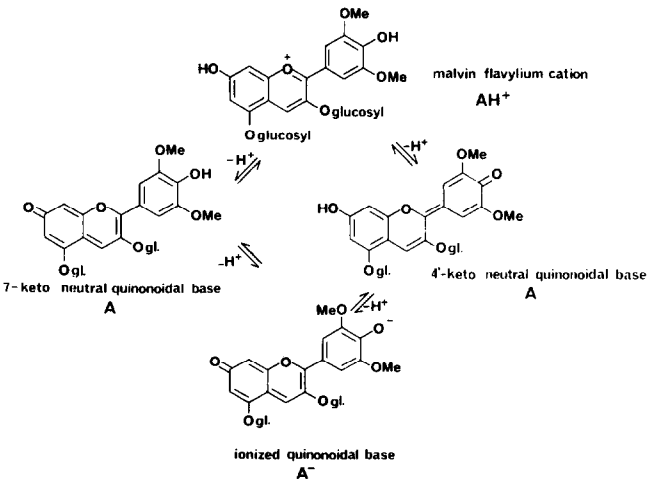

Fig. 2. Fast acid–base equilibria between the flavylium cation AH^+, the neutral quinonoidal bases A and the ionized quinonoidal base A^-.

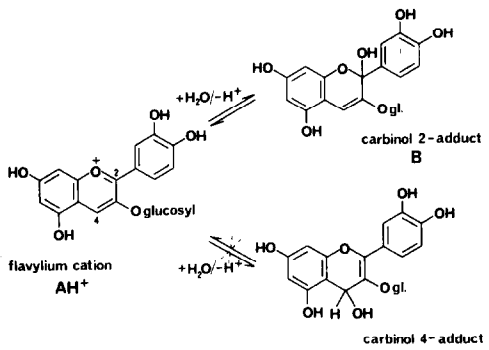

Fig. 3. Hydration reaction of the flavylium cation. The water 4-adduct does not form in appreciable quantity.

increasing the temperature, it has been possible to demonstrate the existence of a ring–chain prototropic tautomerism between the carbinol pseudobase and the chalcone pseudobase (Fig. 4). At room temperature and in slightly acidic aqueous solutions the establishment of the tautomeric equilibrium is slow, and for natural anthocyanins only small amounts of the chalcone pseudobase have been observed, indicating that the tautomeric equilibrium constant $K_T = [C]/[B]$ is always less than one [20]. These facts enabled Preston and Timberlake [53] to quantitatively prepare the chalcones of malvin and malvidin 3-glucoside by use of high performance liquid chromatography. Some synthetic 3-deoxyanthocyanidins have been demonstrated having K_T values much larger than one [42]. In this case, the pigment behaves as if the only existing reaction is an equilibrium between the flavylium cation and the chalcone pseudobase.

When dissolving a flavylium salt in slightly acidic or slightly alkaline solutions, the neutral and/or the ionized quinonoidal bases are formed at once. However, they generally slowly evolve to the much more stable carbinol pseudobase or its ionized form. As a consequence, slightly acidic or slightly alkaline solutions of the most common anthocyanins have feeble colour properties when their equilibrium state is attained. The main reason for this is that the hydration constant K'_h is *ca* 10–100 times larger than the acid–base constant K'_a. If these reactions were the only ones occurring in plants, the anthocyanins would not confer much colour to them. There is no doubt that there exist processes, not present in pure aqueous weakly-concentrated solutions, which strongly favor the chromophores, i.e. the flavylium cation and the neutral and ionized quinonoidal bases. One can assert that any process reducing the efficiency of the hydration reaction will enhance the stability of the chromophores. Such a process will result in an apparent decrease of K'_h. On the other hand, any effect increasing the acidity constant K'_a will also improve the stability of the colour. However, K'_a seems to be relatively insensitive to the structure of the anthocyanin [42]. Therefore, a good protection of the pyrylium ring against water attack is absolutely necessary in order to get intensely coloured media. It is rather surprising that anthocyanins, which in their natural state are dissolved in a medium essentially made of water, must be protected against the reactivity of the water molecule itself in order to manifest their maximal colouring power! It should be stressed, however, that reducing the K'_h values from 10^{-2}–10^{-3} to 10^{-5} M is quite sufficient for a good stabilization of the chromophores. In such case, the neutral and the ionized quinonoidal bases are, at the same time, the kinetic and the thermodynamic products.

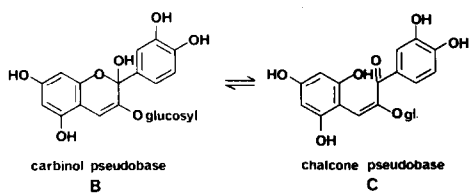

Fig. 4. The ring–chain prototropic tautomerism between the carbinol and chalcone pseudobases.

ORGANIZATION OF ANTHOCYANINS IN THE LIVING CELLS

Attempts to unravel the exact structure of anthocyanins in a flower or a fruit cell have received much attention. Earlier reviews dealing with the *in vivo* organization of anthocyanins are those of Hayashi [49], Dean [16], Timberlake and Bridle [4], Harborne [3], Asen [54], Timberlake [55] and Osawa [39].

Frequently, several anthocyanins coexist in a flower or a fruit and, in petals of some flowers, their concentrations have been shown to be as large as 10^{-2} M [32]. Reports have been made that, in a few cases, anthocyanins like pelargonin and cyanin are alone responsible for the colour [31, 56]. For instance, Asen *et al.* [57], were able to reproduce the absorption spectrum of a portion of a vacuole of the orange sport of Red Wing azalea by placing cyanin in a pH 2.8 aqueous solution. The pH of expressed sap is 2.8–3.0. This experiment strongly suggests that the flavylium structure of cyanin accounts solely for the orange colour, without any other chemical species being needed. One should admit, therefore, that large amounts of the colourless carbinol pseudobase exist in the orange sport. The natural occurrence of carbinol pseudobases has rarely been observed [1], but one can guess that they may constitute a reservoir for the coloured structures. Moreover, a carbinol pseudobase is not subject to the same instability as is the flavylium cation, and it could sometimes be useful to the plant to store an anthocyanin in such a form in order to protect it against damaging agents. We are confronted here with the difficult problem of evaluating the amount of the colourless pseudobase in the plant. No answer to the question is available at the moment. Reports have been made that anthocyanins can be extracted from some green leaves or white flowers of acyanic plants, a feature which demonstrates the *in vivo* existence of anthocyanins in colourless forms [1].

During the last 10 years several new anthocyanins have been discovered whose chromophores are stable in water whatever the pH. The first pigment identified in this series is platyconin from *Platycodon grandiflorum*, and was demonstrated by Saito *et al.* [58, 59] to be delphinidin 3-dicaffeoylrutinoside-5-glucoside. It is now known to contain two additional molecules of glucose [N. Saito and K. Abe, unpublished results]. This anthocyanin is remarkably stable in slightly acidic water. Anthocyanins with a colour stability similar to that of platyconin have been reviewed very recently [39, 55]. Among these new pigments, only the stereostructure of gentiodelphin (Fig. 1), previously proposed by Iino and Takeda [60, 61] to be a dicaffeoyl triglucoside of delphinidin, has been com-

pletely elucidated [29]. One should note that these anthocyanins are generally characterized by the existence of two or more acyl residues linked to the sugars. The chemical role played by the acyl groups will be considered later. It is assumed that polyacylated anthocyanins *per se* are responsible for the colours of the flowers in which they occur [62].

On the other hand, in many cases anthocyanins alone cannot account for the colours of flowers and fruits. Other substances are involved in the pigmentation process. Compounds bringing about an *A* increase in the visible range and/or a bathochromic shift in the visible absorption maximum of an anthocyanin have been called copigments. Many copigments are flavonoids. The copigment effect was originally discovered by Robinson and Robinson [63]. Since that time the number of copigments has been increasing regularly [39, 48, 64]. The simultaneous presence in the cell sap of anthocyanins and accompanying water-soluble copigments has frequently been reported [39, 54]. Since, in general, no pigment–copigment complex can be isolated, the two molecules are probably bound together by weak intermolecular forces, the nature of which has still to be firmly established.

Blue pigments have been investigated with the idea that metallic elements participate in their structures. According to the most recent findings of Takeda and Hayashi [38], commelinin, the blue pigment of the flower of *Commelina communis*, is composed of three moieties: awobanin (delphinidin 3-*p*-coumaroylglucoside-5-glucoside), flavocommelinin (swertisin 4′-glucoside) and magnesium in the molar ratio 2:2:1, respectively. Commelinin-like blue pigments were also prepared using other metals, manganese, cobalt, nickel, zinc and cadmium [65]. Among several anthocyanins structurally related to awobanin, only shisonin (cyanidin 3-*p*-coumaroyl-glucoside-5-glucoside) gives stable blue metallo-anthocyanins. It has been stressed that the nature of the 3-sugar substituent is a determining element in the formation of metallic anthocyanin complexes [66]. In contrast, Goto *et al.* [22], came to the conclusion that commelinin consists only of awobanin and its copigment flavocommelinin, magnesium not being essential for the formation of the complex. Another pigment reported to contain metal in its structure is the blue pigment of *Centaurea cyanus* isolated by Bayer [67]. It should be pointed out that further investigations are necessary in order to establish that metals are essential ingredients of these blue pigments.

Saito [31] and later Ishikura [68] classified the absorption spectra of intact pigmented tissues or cells into four groups. It was suggested that the colours of some flowers are due to the anthocyanins alone. In other cases, copigmentation was considered to be the cause of the blueing effect. Finally, some pigments were believed to fall within the metallo-anthocyanin group.

FACTORS INVOLVED IN THE EXPRESSION OF ANTHOCYANIN COLOUR

The chemical nature of an anthocyanin, its concentration, the presence of pigment mixtures, the pH, the presence of compounds known as copigments and sometimes the presence of certain metal ions seem to be the major factors influencing the colour of anthocyanin-containing media.

Concentration effect

The amount of anthocyanin in a plant may vary several fold. For instance, Asen *et al.* [35] found that the molarity of cyanin in epidermal cells of a petal of 'Better Times' rose was as large as 2.4×10^{-2} M. Yasuda [69] measured a large increase in cyanin content when comparing pale with deep pink rose petals. Yokoi *et al.* [70] correlated the visible *A*s of intact tissues of rose petals with their anthocyanin contents. These authors also observed that anthocyanin concentration is greater in the buds than in half- or fully-opened flowers. Akavia *et al.* [15] reported that the six common anthocyanidins (pelargonidin, peonidin, malvidin, cyanidin, delphinidin and petunidin) are present in *Gladiolus* petals, and that they occur in four different glycosylation states. With the exception of the 3-glucosides, which are always minor components, each of the 18 remaining anthocyanins can make the major contribution to the pigmentation of a given *Gladiolus* flower. For these flowers the colours range from pink (cv Rose Supreme) to violet purple (cv Dean Dixon). In general, several anthocyanins are found in a plant tissue, and it is not easy to disentangle the contribution of each pigment to the overall colouring effect.

New glycosylation and acylation patterns

Natural anthocyanins divide essentially into two large groups: the 3-glycosides and the 3,5-diglycosides [3]. However, anthocyanins with sugars attached to other positions of the aglycone are no longer rare. For instance, a few pigments bearing a sugar at C-7 have been identified [10, 71–76]. Another major structural feature is the discovery of anthocyanins possessing sugars linked to the B-ring 3′- and 5′-hydroxyl groups [10, 29, 74–78]. Among the acylating agents, the most frequent are *p*-coumaric, caffeic and ferulic acids. Very recently, caffeic acid 4-β-glucoside was identified as the acyl moiety of rubrocinerarin, the anthocyanin of red petals of *Cineraria* [79].

Ishikura and Shimizu [80] and Asen *et al.* [62], recently analysed the major anthocyanin of *Ipomoea* cv 'Heavenly Blue'. The former authors came to the conclusion that HBA* is peonidin 3-diglucoside-5-glucoside with two molecules of caffeic acid, whereas Asen and his group demonstrated it to be peonidin 3-dicaffeoylsophoroside-5-glucoside. By a careful examination of the ^{1}H NMR spectrum of tris-deacyl HBA, the anomeric configurations of the sugars were assigned the β configuration [27]. Alkaline hydrolysis of HBA afforded also unusual acyl groups, i.e. caffeate esters having glucose at C-3 and C-4 [26]. Finally, controlled alkaline hydrolysis gives *bis*-deacyl HBA (Fig. 5), and it was concluded that HBA consists of *bis*-deacyl HBA acylated by glucosylcaffeoyl-glucosylcaffeic acid [28]. Consequently, HBA is the first anthocyanin characterized by a side chain of alternating glucosyl and caffeoyl groups.

Physicochemical effects

It was established by X-ray analysis that the angle between the benzopyrylium part and the phenyl ring of apigeninidin is 4.1° [81]. For 6,7,4′-trihydroxyflavylium chloride and cyanidin bromide, the respective values are

*HBA, 'Heavenly Blue' anthocyanin.

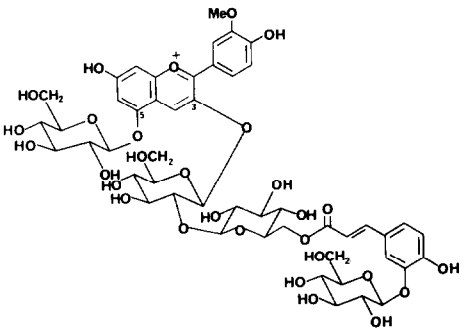

Fig. 5. Structure of bis-deacyl 'Heavenly Blue' anthocyanin (by courtesy of Goto *et al.* [28] and the permission of *Heterocycles*).

5.9 and 10.1° [82, 83]. Calculated spectra of some anthocyanidins predict that, in solution, this angle is as high as or even larger than 45°, the 3-hydroxyl group being responsible for the twisting of the molecule [84, 85]. Unfortunately, no results are available concerning the conformations adopted by the aglycones of natural anthocyanins.

The geometry of an anthocyanin can be determining in its interaction with a solid surface. Physical adsorption onto a suitable surface of either the flavylium cation and/or the neutral and anionic quinonoidal bases will provide an excellent means of preventing colour loss caused by the water reaction, by taking these chromophores out of the bulk of the solution. If only one layer is formed, it will not lead to much colour stabilization. On the contrary, if many layers of the pigment are adsorbed, then a good colour retention should take place. In nature, the adsorbing surface could be the vacuolar inner membrane or any solid material inserted into the vacuole. No evidence for the existence of such a phenomenon has been presented so far, but it is possible that the high MW complex of the larkspur pigment made of delphinidin associated to a pectin-like polysaccharide results from such an interaction [32, 36]. Another example may be the colouring of rice-cakes using the blue water extract of *Clitorea ternata* [86].

Another way of retaining anthocyanin colour is to remove water and so displace the hydration–dehydration equilibrium (Fig. 3) towards the chromophores. In this connection, very interestingly, Pecket and Small [87], on one hand, and Hemleben [88], on the other, indicated that anthocyanins accumulate in crystal-like structures called anthocyanoplasts. In these organelles anthocyanin synthesis is believed to be achieved, and storage of the pigments is sometimes made possible by the presence of an organic membrane surrounding the anthocyanoplasts, as evidenced by solubilization of a crystal in Triton X-100 and sodium dodecylsulfate solutions [88]. The presence of strong blue crystals in some vacuoles of aged flowers of larkspur has been previously reported by Asen *et al.* [89]. They concluded that formation of the crystals has a considerable effect on the blueing of cells.

Copigmentation at the molecular level

The copigment effect is now sufficiently well understood so that it is possible to distinguish between intramolecular copigmentation (the copigment is part of the anthocyanin molecule) and intermolecular copigmentation (the copigment is not covalently bound to the anthocyanin molecule). Generally, a copigment has no colour by itself but when present in a sufficient amount, it greatly enhances the stabilities of the coloured species. It has been pointed out that the intramolecular effect is more efficient than the intermolecular effect [42]. At the molecular level, however, the characteristics of both phenomena are certainly similar.

Intramolecular copigmentation

Intramolecular copigmentation is responsible for the extraordinary stability of the chromophores of polyacylated anthocyanins [44]. The first pigment in this series is platyconin which was extracted from *Platycodon grandiflorum* by Saito *et al.* [58]. It was also demonstrated that the blue-violet colour is expressed by platyconin alone, and that neither metal ions nor other compounds are necessary to produce it [59]. Yoshitama and Hayashi [77] isolated cinerarin from garden cineraria (*Senecio cruentus*), and established that its structure is dicaffeoyldelphinidin 3,7,3'-triglucoside [74]. They also made the useful observation that cinerarin retains its colour within the pH range 3.5–7, where anthocyanins are usually colourless. The hypothesis was put forward that some sort of interaction took place between the caffeoyl moieties and the *ortho*-dihydroxyl group in the B-ring. However, it was shown later by Asen *et al.* [62], that the main pigment of *Ipomoea tricolor* Cav. cv Heavenly Blue (peonidin 3-dicaffeoylsophoroside-5-glucoside) exhibits the same unusual colour stability in neutral solutions. It was concluded, therefore, that the catechol-like structure is not involved in the colour stabilization mechanism. The colour stability against pH of this pigment was sufficient for it to be recommended as a food colourant [90].

It is now believed that the aromatic residues of the acyl groups stack with the pyrylium ring of the flavylium cation and, consequently, greatly decrease the aptitude of water to add to the C-2 and C-4 positions [44]. The extent of the hydration reaction is, therefore, reduced, and since the proton transfer reactions apparently remain unaffected by the stacking process, the stability of the chromophores strongly increases. It was assumed that the driving force for the stacking process is of a hydrophobic type [44]. A similar explanation was given more recently for the colour stability of gentiodelphin [29]. In Fig. 6, the hypothetical stacking mechanism protecting the pyrylium ring from water attack is shown. The acyl residues are arbitrarily attached to the 3 and 6 positions of the 3-sugar

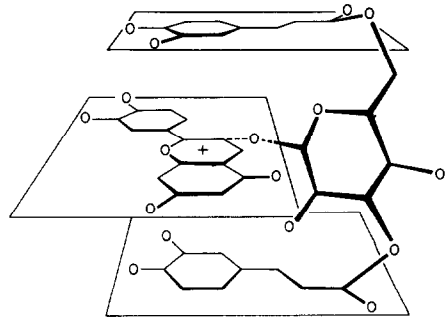

Fig. 6. Model for the stacking of two caffeoyl residues with the pyrylium nucleus [R. Brouillard, unpublished results].

and, for the sake of clarity, only the carbon skeleton and the oxygen atoms are represented. Owing to the flexibility of the acylated glycosyl substituents and the eventual rotation of the B-ring plane with respect to the benzopyrylium plane, interaction of an acyl group born by either a 3,5,7,3'- or 5'-sugar with the pyrylium nucleus is almost always possible. Nevertheless, its position of attachment to the sugar, its length and its structure, as well as the structure of the sugar, should be important factors in the stacking process. For instance, dissolving the larkspur pigment in an aqueous solution of pH 5.6 does not result in a stable colour [8]. The larkspur flower pigment (cv Dark Blue Supreme) is delphinidin 3-di-*p*-hydroxybenzoylglucosyl glucoside. Although this delphinidin derivative is a diacylated one, it appears that the two hydroxybenzoyl groups do not interact efficiently with the pyrylium nucleus. Whether in this particular case the length and/or the positions of attachment of the benzoyl groups are important remains to be demonstrated. Monoacylated anthocyanins do not show such a high stability of their neutral and ionized quinonoidal bases [77]. In this last case, one can assume that only one side of the pyrylium ring can actually be protected, and that water is free to attack on the other side. In this connection it is noteworthy that intermolecular copigmentation seems more efficient with monoacylated anthocyanins than with unacylated pigments [24]. In the former case, some weak intramolecular copigment effect may occur which is not possible with the more simple unacylated pigments.

Intermolecular copigmentation

It is now generally accepted that the intermolecular copigment effect produces a bathochromic shift of the maximum of absorption in the visible range and that an *A* increase also occurs in the least acidic media [3, 14, 35, 39, 54, 57, 64, 89, 91–94]. A detailed mechanism for the intermolecular copigment effect has just been established [42]. It is assumed that the anthocyanin–copigment complex forming reaction competes with the hydration reaction and that hydration of the complex does not occur at all. The stability constant of the cyanin–quercitrin complex is estimated to be close to $2 \times 10^3\ M^{-1}$ and the hydration constant K'_h is reduced from 10^{-2} to 7×10^{-4} M [42]. It was demonstrated also that the common anthocyanins all form complexes and that complex formation is strongly affected by pigment and copigment concentrations [64]. Many compounds structurally related to anthocyanins (flavonoids), as well as other compounds, have been shown to act as copigments [48, 63, 64, 91]. The interesting suggestion was made that anthocyanins can themselves serve as copigments [95]. Until now, the effectiveness of a copigment has always been related to the extent of the bathochromic shift of the visible maximum of absorption and to the increase of the visible absorption. These two features probably satisfactorily reflect the stability of an anthocyanin copigment complex. However, no measure of the stability constant associated with a given complex has been reported. The copigmentation constant, K_c introduced by Hoshino *et al.* [24], is not a true thermodynamic constant, since it not only depends on the anthocyanin and copigment structures, but also on their concentrations. K_c is defined as the ratio of the copigment concentration to the anthocyanin concentration which yields an *A* value 50% that of the maximum value attainable by use of large quantities of the copigment, with respect to the anthocyanin. The lower the K_c, the higher the stability of a complex. For instance, K_c values for awobanin (delphinidin 3-*p*-coumaroylglucoside-5-glucoside) and delphinin (delphinidin 3,5-diglucoside) at a 5×10^{-4} M concentration are 1 and 12, respectively, flavocommelinin being the copigment. It has been concluded that the *p*-coumaroyl moiety strongly enhances the stability of the complex. These results are in support of the remarkable stability of commelinin. Adding flavocommelinin to phosphate buffered solutions of malvin and tibouchinin (malvidin 3-*p*-coumaroylglucoside-5-glucoside) at pH 6 produces a drastic effect on the stabilities of their quinonoidal bases [24]. At pH 6, both malvin and tibouchinin solutions are virtually colourless after standing for 2 hr. Nevertheless, the tibouchinin quinonoidal bases are a little more stable than the malvin quinonoidal bases. Through a sufficient increase in the copigment–pigment ratio, the neutral quinonoidal bases of both pigments can be completely stabilized for a 2 hr period. Whether the stabilizing phenomenon is of kinetic or thermodynamic nature still remains to be demonstrated.

Copigmentation is affected by several factors among which pH is an important one. It has been demonstrated that the copigment effect occurs from pH values close to 1 to neutrality [32, 96, 97]. Spiraeoside, a quercetin glycoside, is found in *Fuchsia* petals in close association with malvin. The colour differences between young and old *Fuchsia* petals can be explained by both copigmentation and pH changes [96]. The *A* changes as a function of pH, observed in model experiments for malvin alone and malvin copigmented with spiraeoside [96], can be interpreted in the light of the recently proposed mechanism for the intermolecular copigment effect [42]. At pH 1, malvin exists essentially in the flavylium form, and the 15 nm shift in spectral maximum, observed in the presence of the copigment, is due to the interaction of the malvin flavylium cation with the copigment. The molecular extinction coefficient at the visible maximum of the complex is identical to the one of the uncopigmented cation. At pH 2–3, there is an important colour loss for malvin alone and a significant colour retention for the solutions containing the spiraeoside molecules. Thus, the copigment effect is to reduce the production of the carbinol pseudobase. At pH 4–6, the solutions containing only malvin are practically colourless, whereas the solutions with both malvin and copigment are strongly coloured. In this pH range quinonoidal bases are formed, and again colour retention is derived through a decrease in the amount of the carbinol pseudobase.

There is a controversy as to the structure of the anthocyanin–copigment complex [55, 98]. In order to clarify this problem, we have to answer two questions. Which anthocyanin species associate with a copigment? What is the nature of the driving force giving rise to a complex?

To our knowledge, Asen *et al.* [64] were the first to propose that interaction could occur with both the flavylium cation and the neutral quinonoidal bases. Sweeny *et al.* [98], demonstrated that pentamethylcyanidin chloride strongly associates with quercetin 5'-sulfonic acid in 0.01 M citric acid (pH 2.8). This supports the previously suggested proposition that the flavylium structure forms a complex with the copigment. One can also note that neither a glycosyl residue, nor a hydroxyl group is necessary for intermolecular copigmentation to

take place. On the other hand, commelinin is believed to be a complex made of awobanin neutral quinonoidal base and flavocommelinin [22]. One can conclude that a copigment can interact with both the flavylium ion and the neutral coloured bases.

Hydrogen bonding has been suggested as the driving force for the pigment–copigment association [48, 97, 99]. It is unlikely that in water the anthocyanin–copigment interaction is due solely to hydrogen bonds since water is an excellent hydrogen bond donor and acceptor. Furthermore, formation of an end-to-end complex does not prevent the pyrylium ring from suffering water attack. Association probably occurs by a stacking process related to hydrophobic forces [22]. This provides good protection against water nucleophilic addition and subsequent colour loss. At the moment, nothing is known about the formation rates of such complexes. This type of reaction probably enters the field of fast reactions.

Why intramolecular copigmentation is more efficient than intermolecular copigmentation can be explained in the following way. From a thermodynamic point of view the intramolecular effect has an important entropic advantage over the intermolecular effect, since the former effect does not need to bring together molecules initially separated in solution. One can now understand why the mixing of caffeic acid with deacylated cinerarin (cinerarin is acylated by caffeic acid), does not regenerate the blue colour of cinerarin [77]. It is also noteworthy that, when the intramolecular effect occurs, little or no intermolecular copigmentation can take place. Thus, Asen *et al.* [62], on one hand, and Ishikura and Yamamoto [100], on the other, observed that adding rutin, which is known as a good copigment, to HBA does not significantly alter the visible absorption spectrum of HBA, near neutrality. The intramolecular effect produces a large bathochromic shift of the visible maximum of absorption for HBA, with respect to its deacylated form, in the pH range 2.5–6 [100]. This indicates that intermolecular and intramolecular copigmentations are very similar phenomena. That intramolecular copigmentation also occurs *in vivo*, has been demonstrated by matching the visible absorption spectra of intact petals of *Ipomoea tricolor* with the absorption spectra of pure HBA aqueous solutions [62]. It is reasonable to believe that the pigments classified in group C by Saito [31] and Ishikura [68] all belong to the category showing a strong intramolecular copigment effect. Surely most of them are polyacylated anthocyanins.

pH effect

In 'Better Times' rose petals, Asen *et al.* [35] found that the vacuolar pH of epidermal cells varies from 3.70–4.15 in freshly harvested petals, to 4.40–4.50 in 3-day-old petals. A concomitant change of the colour from red to blue was observed. In the case of 'Heavenly Blue' flowers, when the reddish-purple buds turned to light blue opened flowers, the pH changed from 6.5 to 7.5 [62]. More generally, it was assumed that the bluing of flowers as they age is accompanied by a decrease in the free acidity [35, 89, 92, 101]. The colour change from blue-violet, in young petals of *Fuchsia*, to purple-red in old ones, is caused by the reverse effect, that is a pH shift from 4.8 to 4.2 [96].

In 1949, Shibata *et al.* [102] reported the pH values of numerous flower, fruit and leaf extracts to be between 2.8 and 6.2. Stewart *et al.* [101], using a microspectrophotometric technique, showed that the pH of petal epidermal cells in young flowers ranged from 2.5 to 7.5. Lowering the pH displaces the proton transfer equilibria between the numerous chromophores, from the ionized quinonoidal bases to the neutral quinonoidal bases and, finally, to the flavylium cation, and vice-versa. It is remarkable that the pK values governing these structural modifications, fall exactly within the range pH 2.5–7.5 (Fig. 7). In the most acidic media the pigment exists mainly in the flavylium form, copigmented or not. In the pH range 3.5–5.5, the flavylium cation and the neutral quinonoidal bases coexist. Then, up to pH 6.5 the quinonoidal bases largely predominate. At neutrality, the ionized quinonoidal bases also appear. Any pH increase will lead to a bathochromic shift; any pH decrease to a hypsochromic shift. The more important colour changes will occur for pHs close to the pK'_a values.

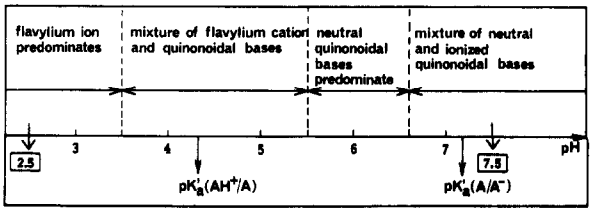

Fig. 7. Predominant anthocyanin chromophores in different pH ranges.

Self-association

It was first suggested by Asen *et al.* [64] that anthocyanins self-associate. At pH 3.16 increasing the concentration of cyanin from 10^{-4} to 10^{-2} M gives a 300-fold increase in the visible A and a 5 nm hypsochromic shift of the visible λ_{max}. A similar phenomenon was reported at pH 3.5, in the case of malvidin 3-glucoside [94]. Scheffeldt and Hrazdina [93], mentioned that competition can take place between the copigmentation and self-association reactions. For sufficiently acidified solutions (pH < 1), self-association (i.e. a 'positive' deviation from the Beer–Lambert law) is not observed; rather, the contrary (i.e. a 'negative' deviation) can be shown in 1 N hydrochloric acid [C. F. Timberlake, personal communication]. Since at pH 3–4 large quantities of the carbinol pseudobase are present, this species may take part in the abnormal A increase. The occurrence of such an effect in nature remains to be demonstrated.

Self-association also occurs in concentrated neutral aqueous solutions [89]. In a recent series of papers, Hoshino and coworkers, pointed out some of the major factors involved in self-association at neutrality. In a pH 7 phosphate buffered solution, cyanin quinonoidal bases slowly self-associate and, after 1 hr a precipitate, showing a large circular dichroism effect, comes out of solution [23]. A positive Cotton effect is exhibited by an ageing cyanin solution. On the other hand, under the same conditions, malvin quinonoidal bases also strongly self-associate, but the Cotton effect is negative and the optical activity is the largest at the beginning of the experiment, which indicates a much faster rate of self-association than in the case of the cyanin molecule [103]. Some malvin derivatives were also tested for self-association. Hirsutin (7-*O*-methylmalvin), shows a large optical effect and an hypsochromic shift of its visible λ_{max} with concentration

increase. On the contrary, the 4′-*O*-methylated derivative of malvin shows none of these effects. From these results and the similar colours of malvin and hirsutin solutions, it was concluded that malvin quinonoidal base has a 4′-keto structure. It is not clear whether self-association actually leads to a colour retention effect, since, at equilibrium, only 3–5% of the analytical pigment concentration remains in the coloured form [103], and this is not very different from the values observed in dilute solutions. It was also established that a 4′-hydroxyl and a 5-glucosyl are essential structural elements for the self-association process to occur. Urea and DMSO disrupt the aggregates, whereas sodium chloride and magnesium chloride promote self-association. It was demonstrated also that self-association, at pH 7, arises from a vertical stacking of the neutral quinonoidal bases leading to chiral aggregates, with a left- or right-handed screw axis, according to the anthocyanin structure [104]. Further evidence for the vertical stacking is given by the concentration dependence of the proton chemical shifts of malvin, the anthocyanin being assumed to be in the quinonoidal structure [30].

IN VIVO SPECTROSCOPIC INVESTIGATIONS

Only a very limited number of spectroscopic techniques are suitable for the *in vivo* investigation of anthocyanins. ^{1}H NMR spectroscopy, which is so useful for their structural elucidation in model experiments, is of little help when living tissues are concerned. On the contrary, it is possible today to get a visible absorption spectrum of even a portion of a pigmented vacuole. However, due to the small number of bands present in a visible spectrum, structural determination of the *in vivo* absorbing species is rather uncertain. Very recently, Raman spectra of anthocyanins in the living cells have been recorded [33].

UV-visible absorption spectroscopy

The method consists of reproducing, in a model experiment, the absorption spectrum of an intact flower tissue or an intact flower cell. This is possible by a good choice of the anthocyanin–copigment ratio and of the pH. In particular Asen *et al.* [32] were able to reconstitute the absorption spectrum of the Professor Blaauw iris intact cells. The spectrophotometer used to record absorption spectra of samples as small as a flower cell (20–100 μm) is equipped with a light microscope [57]. The method was successfully applied to many flowers and the spectra of their intact cells were analysed [89]. The microspectrophotometric technique permits the measurement of the acidity of the vacuolar content of as few as 5–10 epidermal cells [101]. The absorption spectra made on fresh flower petals were classified into four groups according to their main features—number and position of maxima in the visible range [31, 68].

Resonance Raman spectroscopy

Atomic motions in a polyatomic molecule give rise to vibrational and rotational energy levels. IR spectroscopy measures the absorption of photons from one of these levels to another. Water strongly absorbs in the IR region and, therefore, IR spectroscopy is of little help for the study of aqueous solutions. Raman spectroscopy relates to the scattering of light. A simplified energy level diagram for IR absorption, ordinary Raman effect, resonance Raman effect and fluorescence is shown in Fig. 8. Among other processes, when the incident radiation of energy $h\nu_0$ hits the sample, scattering essentially occurs with no change in energy (elastic or Rayleigh scattering). A very small fraction of the incident radiation is inelastically scattered, giving rise to the Stokes lines ($\nu_0 - \nu_i$ frequencies) when the molecule is in the vibrational ground state, and the anti-Stokes lines ($\nu_0 + \nu_i$ frequencies) when it is in a vibrationally excited state. The frequency shifts, $\nu_0 - \nu_i$ and $\nu_0 + \nu_i$, relate to the scattering medium and do not depend on the ν_0 frequency of the excitation light. Generally, one reports the Stokes lines only, since they are much more intense than the anti-Stokes lines, due to the fact that most of the molecules are in their lowest

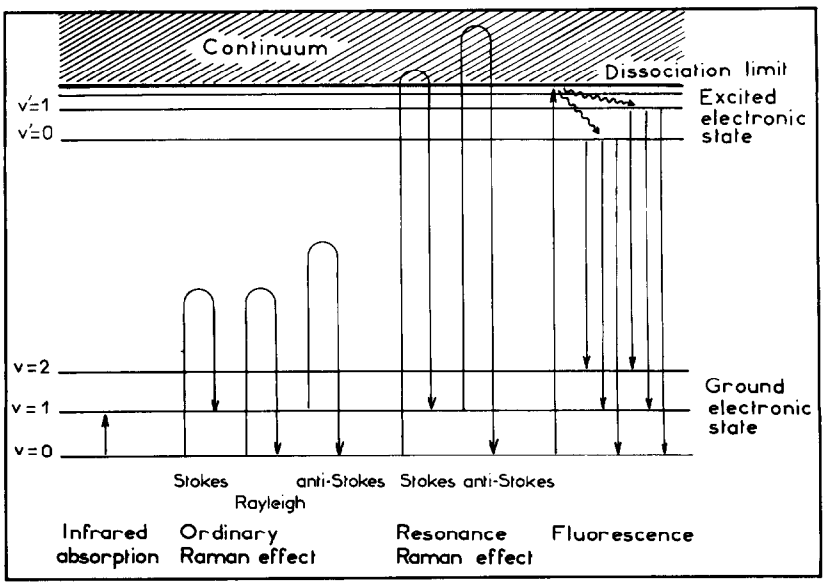

Fig. 8. Energy level diagram illustrating the Raman and resonance Raman scattering phenomena.

vibrational state at room temperature. A Raman spectrum is characteristic of a compound in a given chemical state and in a given environment. Water is a poor Raman scatterer and, therefore, constitutes an ideal medium for Raman spectroscopy. Ordinary Raman spectra are performed at wavelengths where the sample does not absorb the excitation light and, owing to the weakness of the effect, a high light density, as well as sufficiently concentrated solution (0.1 M or greater), are necessary in order to obtain a measurable Raman effect. Another drawback of ordinary Raman spectroscopy, when studying natural media, is the large number of lines associated with the numerous chemical and biochemical species present in such media. Resonance Raman spectroscopy does not suffer from these limitations. Resonance takes place when the incident light falls within an absorption band. Owing to the coupling of the electronic and vibronic transitions, the Raman lines relating to the chromophore are strongly enhanced, whereas the remainder of the spectrum is generally sufficiently attenuated to disappear completely. Dilute solutions (10^{-5} M or even less) can be studied and strongly light-absorbing pigments are especially well-suited for such spectroscopic investigations. Consequently, the resonance Raman effect is a sensitive probe for structural and environmental elucidations in complex media. Fluorescence emission may compete with the Raman effect and, sometimes, seriously hinders its observation.

The resonance Raman spectra—not corrected for the fluorescence background—of pure malvin and pure malvidin 3-glucoside in aqueous solutions at pH 1 are shown in Fig. 9 [A. Statoua, J. C. Merlin, R. Brouillard and M. Delhaye, unpublished]. The Raman lines measured in this way relate only to the portion of the molecule responsible for the colour, i.e. the aglycone. Malvin shows an intense line at 628 cm^{-1}, and the monoglucoside an intense band at 540 cm^{-1}. This fact seems to be general since delphinin and cyanin, and their corresponding 3-glucosides, also exhibit strong Raman features close to 630 and 540 cm^{-1}, respectively. In Fig. 10, the resonance Raman spectra of the malvin flavylium cation, on one hand (a), and its quinonoidal bases, on the other hand (b), are shown [A. Statoua, J. C. Merlin, R. Brouillard and M. Delhaye, unpublished]. Changing the chromophore from the flavylium state to the quinonoidal states greatly affects the Raman spectrum of malvin. Such *in vitro* investigations can only give further insight into the organization of anthocyanins in their natural environment if similar Raman spectra can be recorded in the case of plant tissues, plant cells or even parts of a plant cell. This goal can now be achieved using the Raman microprobe/microscope named Molecular Optical Laser Examiner—in short MOLE—developed at the University of Lille by Delhaye and Dhamelincourt [105]. A schematic diagram of the Raman microprobe/microscope MOLE appears in Fig. 11. A microscope is coupled to the Raman spectrophotometer and spectra of areas as small as a few μm^2 can conveniently be obtained. Microanalyses of plant tissues and plant cells have been successfully achieved using this technique [106, 107]. The resonance spectrum of the skin of a berry from the grape 'Muscat de Provence' (Fig. 12) matches the spectrum of malvidin 3-glucoside in water at pH 1 [33]. However, it should be pointed out that preliminary experiments demonstrate that similarly glycosylated derivatives of malvidin, cyanidin and delphinidin, show little variation in their Raman spectra. Therefore, the antho-

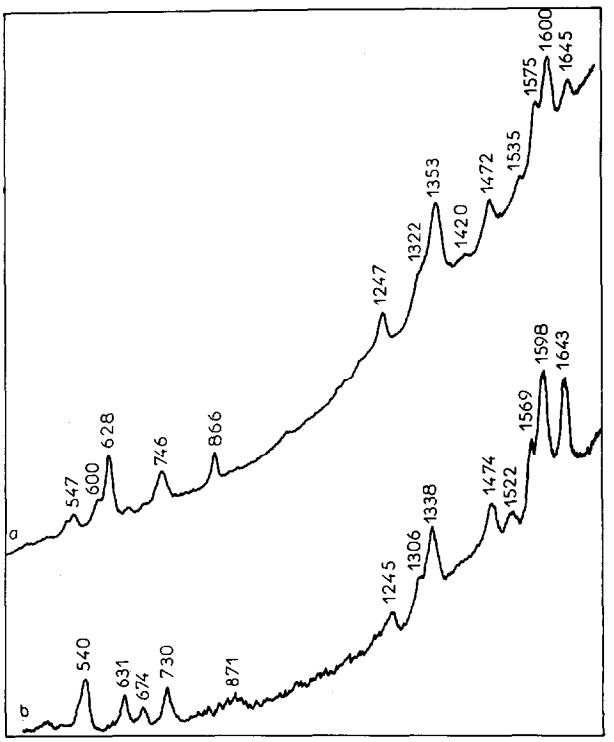

Fig. 9. Resonance Raman spectra in water at pH 1 of: (a) malvin extracted from *Malva sylvestris*; (b) malvidin 3-glucoside. 488 nm excitation. Numbers indicate cm^{-1}.

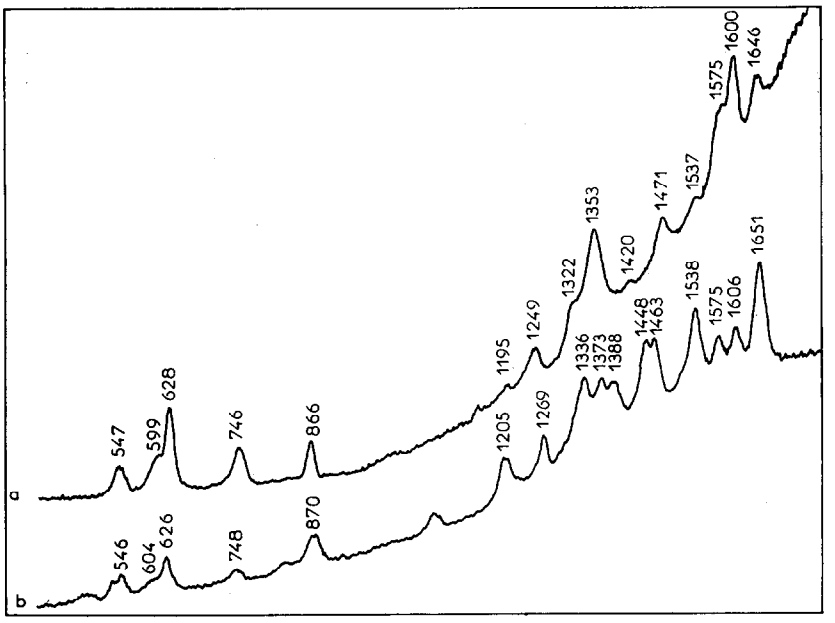

Fig. 10. Resonance Raman spectra of malvin in water at pH 1 (a) and pH 6 (b). 488 nm excitation. Numbers indicate cm^{-1}.

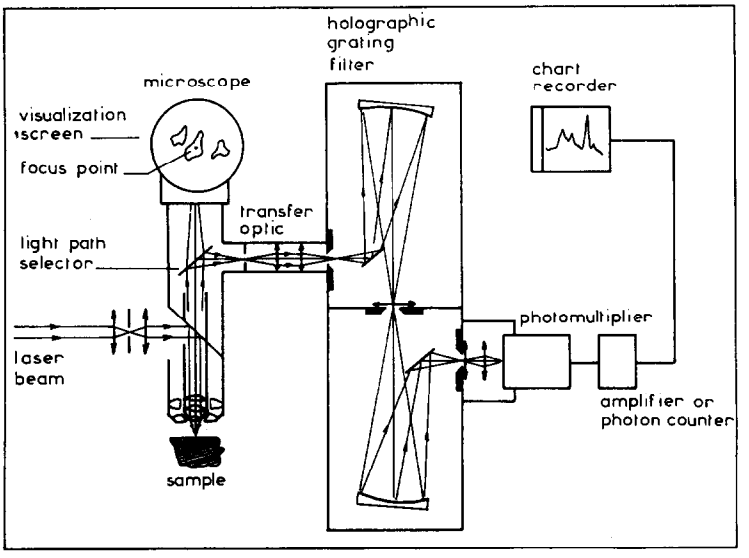

Fig. 11. Optical scheme of laser Raman microprobe (MOLE) used as a Raman microspectrometer.

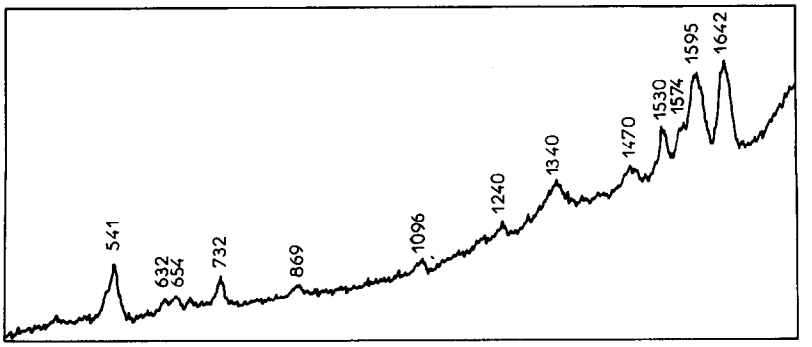

Fig. 12. Resonance Raman spectrum of the epidermal tissue of a berry of 'Muscat de Provence'. 488 nm excitation. Numbers indicate cm^{-1}.

cyanins of the commercial grape 'Muscat de Provence' are probably 3-glucosides, but at the moment it is difficult to distinguish between them. Nevertheless, it can be concluded that they are largely in the flavylium form. A similar conclusion was reached by Hrazdina [108, 109]. It is also suggested that the resonance Raman effect originates essentially from the benzopyrylium moiety of the molecule when the pigment is in the flavylium state. Assignment of the Raman lines to the different structural elements of the anthocyanin chromophores is currently under investigation. Application of the Raman microprobe to many flower and fruit tissues, in combination with the usual *in vitro* methods, will be of great help in solving some of the major problems remaining in flower and fruit pigmentation.

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