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INVESTIGATION OF THE *IN VIVO* ORGANIZATION OF ANTHOCYANINS USING RESONANCE RAMAN MICROSPECTROMETRY

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Key Word Index—*Vitis vinifera* cv. Pinot noir; Vitaceae; *Malva sylvestris*; Malvaceae; common mallow; plant colour; Raman microspectrometry; anthocyanoplast; anthocyanin; carotenoid.

Abstract—Resonance Raman (RR) microspectrometry constitutes a new means for studying the organization of anthocyanins in living tissues. RR spectra of the pigments present in a single cell have been recorded from the skins of the mature berries of the 'Pinot noir' grape, as well as the petals of the common mallow. Comparison of these spectra with those obtained from model solutions of anthocyanins permits us to conclude that in the 'Pinot noir' berries, malvidin 3-glucoside is the main pigment. Furthermore, inside the skin it is essentially in the quinonoidal base form, whereas in the outer face of the skin it is mainly in the flavylium form. In the upper epidermis of petals of the common mallow, only malvidin 3,5-diglucoside could be detected, entirely in the cationic flavylium form. Since self-association or co-pigmentation processes do not seem to contribute much to the RR spectra, we conclude that they perturb the electronic excited state of the monomeric anthocyanin chromophores much more than they modify the corresponding electronic ground state. In the case of the skins of the mature berries of the 'Pinot noir' grape, dark grains, which we believe to be anthocyanoplasts, have been observed. *In vivo* RR spectra of the dark grains have been recorded.

INTRODUCTION

Flavonoids are important constituents of plant cell vacuoles [1]. Whereas most flavonoids are colourless, anthocyanins strongly absorb visible light and, thus, confer colour to many parts of higher plants [2, 3]. For a better understanding of the plant pigmentation processes, it is important to obtain a deeper insight into the natural organization of these pigments. This can now be achieved essentially by two main approaches.

(a) In the first approach, one has to extract, purify and identify anthocyanins from convenient plant sources and then place them under conditions as similar as possible to the natural conditions, in order to reproduce the natural colour patterns. The natural physical conditions of the vacuoles are easily reproduced in the laboratory using slightly acidic or neutral aqueous solutions held at room temperature. However, anthocyanins exist in the vacuolar sap, among many other biochemicals, whose interaction with anthocyanins can be demonstrated or, at least, postulated. What are these compounds and what are their effects on the colours due to anthocyanins chromophores? It is too early to give a full answer to this question, but rapid progress in this field can now be made, since species present in vacuoles originating from many plant tissues can now be analysed with safety and accuracy [4]. Effective isolation of intact mature flower or fruit epidermal cell vacuoles has now been achieved [5–8]. Interference with constituents arising from other organelles is, thus, limited if not completely avoided. Therefore, compounds present in anthocyanin-containing vacuoles can be identified, isolated and mixed with anthocyanins in model solutions.

(b) When feasible, the use of a microprobe at the subcellular level constitutes a second approach to the study of anthocyanin pigmentation [9]. This second approach, which is not too damaging to the natural medium, has the advantage of indicating how the pigment exists *in vivo*. In particular, its application does not necessitate the destruction of the cell and gives, therefore, a real view of the *in vivo* situation. Nevertheless, the microprobe method has to be based on a characteristic property of the compounds investigated to the exclusion of all the other compounds present in order to be efficient.

Until recently, the only microprobe used for studying anthocyanins in their natural sites was visible microspectrometry, which is based on absorption of visible light by anthocyaninchromophores [10–12]. A short time ago, we demonstrated that resonance Raman (RR) microspectrometry can also be used for the same purpose [13–15]. It is clear now that Raman spectrometry has been widely applied in biology [16]. In this paper, we now extend the RR microspectrometry technique to the studies of skins of mature *Vitis vinifera* 'Pinot noir' berries and epidermal tissues of the flowers of wild mallow (*Malva sylvestris*). Since 'Pinot noir' grapes and *Malva sylvestris* are good sources of malvidin 3-glucoside and malvidin 3,5-diglucoside (malvin), respectively, comparisons of the RR spectra obtained on the berries and flowers tissues are made with RR spectra of these two pure pigments in model experiments under various conditions.

RESULTS AND DISCUSSION

RR spectra of pure aqueous anthocyanin solutions

Structural modifications of anthocyanins in water are essentially due to the high reactivity of their aglycone

moieties [17]. In particular, it has been demonstrated that under natural conditions, many anthocyaninchromophores exist in fast acid–base equilibrium. These chromophores are the flavylium cation AH^+, the neutral quinonoidal bases A and the ionized quinonoidal base A^- (Fig. 1). Usually, hydration of the flavylium cation AH^+ also takes place in pure water, a process which leads to colourless structures in equilibrium with the visible light absorbing chromophores. By varying the pH of the solution and by using visible light excitation, one can obtain the RR spectra associated with AH^+, A and A^-, even if their concentrations are lower than the concentrations of the colourless structures. As long as the overall anthocyanin concentrations are kept sufficiently low, aggregation phenomena do not occur and the spectra recorded are characteristic of the monomeric species.

At low pH the flavylium cation dominates and the RR spectrum of an aqueous solution of malvin chloride, at pH close to 1, can be considered as characteristic of this chromophore (Figs 2a and 2c). Since the electronic delocalization, which is responsible for the strong visible absorption band of the flavylium cation, only affects the aglycone moiety, no Raman line directly assignable to the glucosyl residues can be observed in the RR spectra of malvin chloride. The same kind of spectrum has been recorded from methanolic solutions containing 0.1% hydrochloric acid, but lines characteristic of methanol then perturb the malvin aglycone spectrum (Fig. 2b). However, by comparing the spectra in Figs 2(b) and 2(c), for instance, one can easily ascertain that the malvin aglycone (malvidin), retains the same flavylium structure under both aqueous and methanolic conditions. The peak intensity changes observed mainly in the 1500–1650 cm^{-1} spectral range, can be related to the shift of the visible maximum of absorption from 519 nm (aqueous solution) to 533 nm (methanolic solution) leading to different resonance conditions.

In order to determine the influence of the glycosylation pattern of the benzopyrylium part of the molecule (rings A and C), we have also recorded the RR spectrum of malvidin 3-glucoside chloride in water at a pH close to 1 (Fig. 2d). We observe similar spectral features and very close wavenumber coincidence for the lines in the 1200–1650 cm^{-1} spectral range in both cases, except for the 1338 cm^{-1} line which shifts towards 1353 cm^{-1}, when the molecule is 3,5-diglucosylated. In the low wavenumber range (500–900 cm^{-1}), more changes appear which can be considered characteristic of the anthocyanin glycosylation pattern. In particular, for malvidin 3-glucoside chloride the most intense band is recorded at 540 cm^{-1}, whereas for malvin chloride the most intense band lies at 628 cm^{-1}. This observation seems to be general and constitutes a new means for distinguishing between the two main classes of anthocyanins, i.e. the 3-glycosides and the 3,5-diglycosides. Monoglycosides will always exhibit a strong RR diffusion line close to 540 cm^{-1} while 3,5-diglycosides will have their strongest RR feature in the lower frequency range close to 630 cm^{-1} [18].

A solution of malvin chloride at pH 6 is blue when freshly prepared but fades in a few minutes at room temperature. At this pH, the neutral quinonoidal bases $A_{4'}$ and A_7, are the only existing chromophores, but they slowly evolve towards an equilibrium state which is completely dominated by the colourless structures [17]. Drastic changes appear when we compare the RR spectra of a malvin solution at pH 6 (Fig. 2e)—the spectrum being taken immediately after preparation of the solution—and a malvin solution at pH 1 (Fig. 2c). The most important changes are observed for the relative intensities of the lines in the 1500–1650 cm^{-1} spectral range where vibrational modes of rings are expected. The strong band at 1651 cm^{-1}, which appears at pH 6, can be assigned to the carbon–carbon double bond stretching vibration of the quinone methide rings of $A_{4'}$ and A_7. It is also clear that numerous new bands appear in the 1200–1500 cm^{-1} spectral range in the solution at pH 6. On the other hand, between 500 and 900 cm^{-1}, very minor changes are observed; both spectra are characterized by lines having

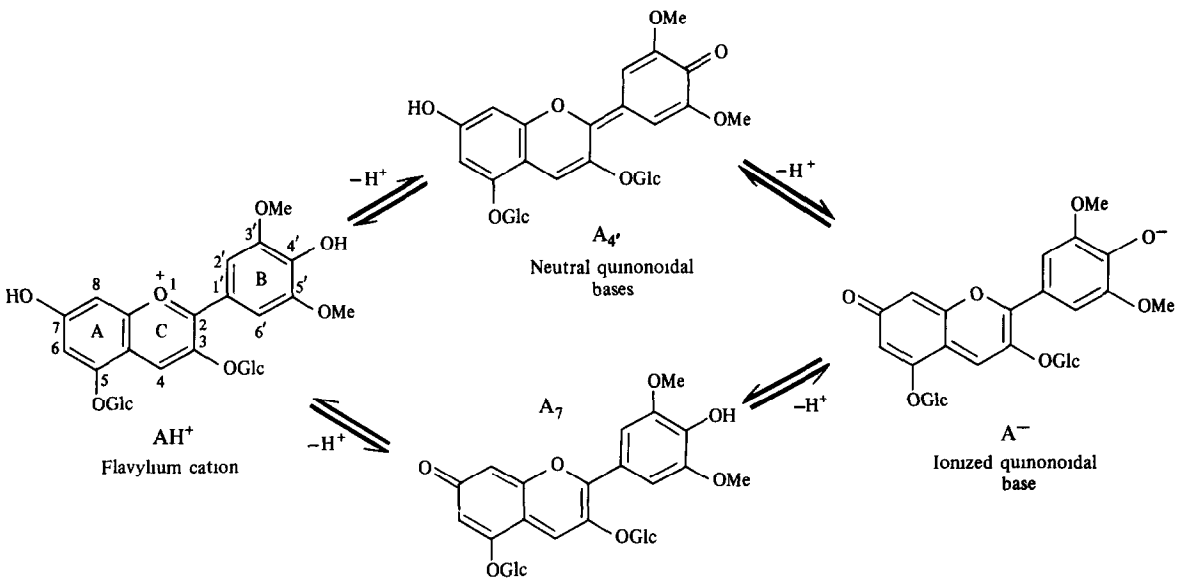

Fig. 1 Structural modifications of malvin chromophores as a function of pH [17].

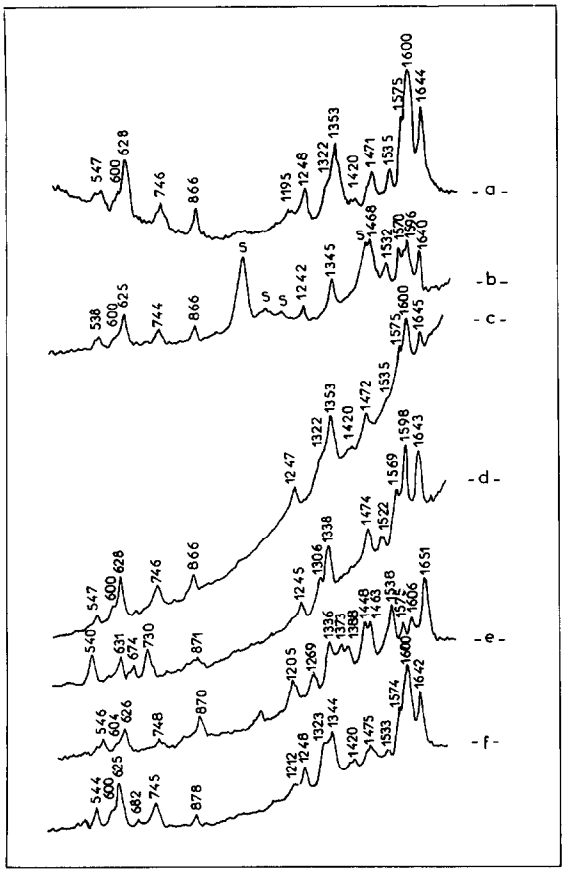

Fig. 2. RR spectra of anthocyanin solutions. (a) Malvin in water at pH 1; 457.9 nm excitation; (b) malvin in methanol with hydrochloric acid (0.1 %); 488 nm excitation; S indicates the solvent lines; (c) malvin in water at pH 1; 488 nm excitation; (d) malvidin 3-glucoside in water at pH 1; 488 nm excitation; (e) malvin in water at pH 6; 488 nm excitation; (f) cyanin in water at pH 1; 488 nm excitation. Numbers indicate cm^{-1}.

nearly the same wavenumbers and the same relative intensities. As these lines seem to be closely related to the structure and substitution pattern of the benzopyrylium group, one can conclude that rings A and C are not too much perturbed when malvin chloride transforms from the flavylium cation into the neutral quinonoidal bases. Consequently, A_4· would be largely dominant over A_7 and ionization would take place only at the flavylium cation OH-4' group. However, another explanation for the great similarities in the lines found in the 500–900 cm^{-1} range is possible. The 488 nm laser excitation radiation falls on the short wavelength side of the large visible absorption band due to the neutral quinonoidal bases. This band has a flat summit from 525 to 565 nm [17]. The shape and the extent of this very large absorption band (450–700 nm), indicate the probable existence of more than one electronic absorption process and it is likely that the A_4· neutral base is mostly responsible for the 488 nm absorption. If this is the case, the RR spectrum shown in Fig. 2(e) is mainly characteristic of the neutral base A_4·. In order to complete our RR investigation, it would be necessary to use an excitation radiation lying not too far from 600 nm, which corresponds to the long wavelength side of the visible absorption band at pH 6.

Cyanin is of interest in this study because it has the same structural pattern for the benzopyrylium part as malvin, but a different substitution pattern in ring B. The RR spectrum of cyanin chloride at pH 1 in water is shown on Fig 2(f). Compared to the spectrum of malvin chloride, no drastic changes appear for either peak positions or relative intensities. This observation points to the fact that the lines measured in the RR spectra are probably more related to the vibrational modes of the benzopyrylium moiety than to any other vibrational modes of the remainder of the molecule. The Raman active vibrational modes associated with the B ring are, perhaps, too weak to be observed.

In the 500–900 cm^{-1} region, mainly out-of-plane deformations of ring and carbon–hydrogen bonds are expected. In the 1200–1650 cm^{-1} spectral range, where most of the in plane ring vibrations and substituent vibrations are expected, very small changes in wavenumber and relative intensity occur when the substitution patterns of both the benzopyrylium group and the B ring are modified. All flavylium cation spectra are dominated by strong lines near 1575, 1600 and 1645 cm^{-1}. The other lines are due to aromatic ring vibrations. The methoxy stretching modes (1160–1210 cm^{-1}) and the methyl deformations are not observed. Hydroxyl deformations, if Raman active, appear in the 1200–1300 cm^{-1} region. A more complete assignment of the observed lines is not possible at this stage; we need experimental results on other flavylium cations.

In vivo *studies*

By using the Raman microspectrometer it is possible to obtain vibrational spectra of anthocyanin pigments directly by illuminating a vacuole of a single cell without special preparation of the sample [14].

'Pinot noir' grape. The RR spectra obtained from vacuoles of the skin of the 'Pinot noir' mature berries are shown on Fig. 3. The spectral features are very similar to those observed for dilute aqueous solutions of malvidin and cyanidin glucosides (Fig. 2). These spectra can be considered characteristic of the state of the anthocyanin mixture in the grape skin vacuoles. Other cell constituents, which do not show noticeable absorption at the laser emission wavelength, are characterized by lines which are not resonance enhanced and, generally, are too weak to appear in the spectrum. On the other hand, constituents which absorb 488 nm excitation light, will contribute to the overall *in vivo* RR spectrum. Therefore, lines characteristic of carotenoids are clearly seen in Fig. 3(a) (lines at 1010, 1161, 1200 and 1531 cm^{-1}).

The anthocyanins present in the 'Pinot noir' grape (*Vitis vinifera*) are well documented. All the anthocyanins found in *Vitis vinifera* sp. are 3-monoglucosides of malvidin, delphinidin, petunidin, cyanidin and peonidin [19]. Their relative abundance depends largely upon the vine plant and also upon grape maturity. In the case of the 'Pinot noir' grape, malvidin 3-glucoside is the main pigment with significant amounts of peonidin 3-glucoside, whereas delphinidin, petunidin and cyanidin derivatives are very minor components [20]. Figures 3(a) and 3(b) exhibit the strong feature at 545 cm^{-1} expected of 3-glucosides [18]. The ratio of the intensities of the 545 and 651 cm^{-1} lines

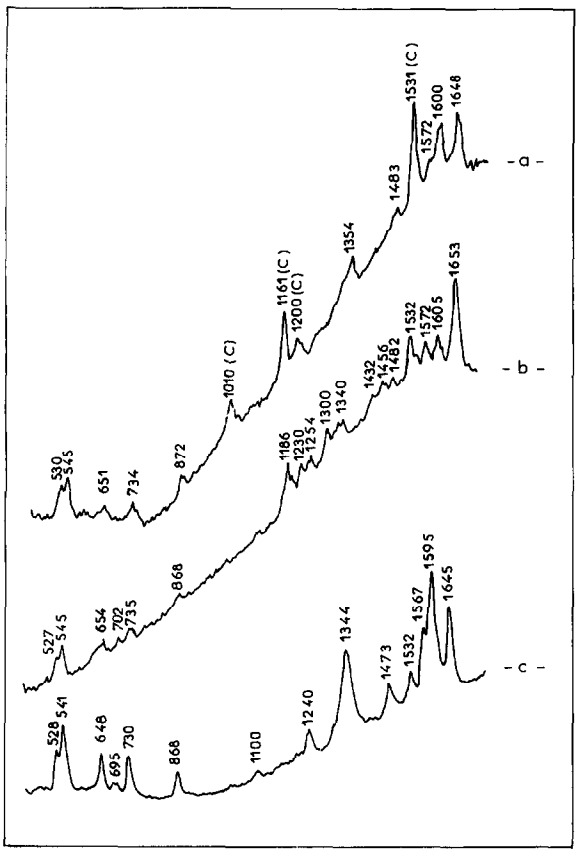

Fig. 3. *In vivo* RR spectra of the epidermal tissue of a berry of 'Pinot noir' grape; 488 nm excitation. (a) Outer cells vacuoles; C indicates the carotenoid lines; (b) dark grains of the skin inner cells; (c) crude extract aqueous solution slightly acidified with hydrochloric acid (0.1%).

indicates that no or very few diglucosides are present in our 1983 Alsace 'Pinot noir' sample. However, these spectra are very different. For instance, in the lower spectrum (Fig. 3b), the lines characteristic of carotenoids have disappeared. The RR spectra of carotenoids are very intense and these compounds can be detected in this way at levels as low as 10^{-7} M, and even lower. Thus, the amount of these light harvesting pigments in the mature epidermal cells of our grape berries probably remains very low. Nevertheless, it is noteworthy that our method permits carotenoids and anthocyanins to be distinguished when these pigments are found in the same tissue. A similar co-occurrence of carotenoids and anthocyanins has been reported previously in cultured tissues of red cabbage (*Brassica oleracea*) [15]. It was further demonstrated that, during ageing of the red cabbage cells, carotenoids are lost and, at the same time, significant structural changes in the anthocyanin chromophores are observed [21]. It is not clear here whether or not carotenoids are to be found in the vacuole since they are lipid soluble and usually not constituents of this organelle.

It is seen, from the light microscope, that on the inner side of the berry skin, dark grains are visible in the vacuoles. These dark grains are no longer observed on the outer side of the skin. It is demonstrated that anthocyanins are the main pigments of the dark grains. According to previous reports on dark grains in pigmented vacuoles [22], biosynthesis of the 'Pinot noir' anthocyanins takes place at this subcellular level and the dark grains are anthocyanoplasts. RR spectra of anthocyanins in the dark grains have been recorded and a typical spectrum is shown in Fig. 3(b). The laser beam impact on the sample delineates the portion of the tissue which is investigated. It has an approximately cylindrical form with a diameter of 2–3 μm and a depth of a few micrometres. The dark grains are spherical, their diameters ranging from 10 to 20 μm. Structural modifications of the anthocyanins in the dark grains and the outer cell vacuoles are reflected in the spectral changes observed in Figs 3(a) and 3(b). In the dark grains of the inner cells of the skin, carotenoids are lacking, as mentioned above, but anthocyanins are mainly in the neutral quinonoidal base form (strong feature at 1653 cm^{-1}) whereas, in the outer cell vacuoles anthocyanins are essentially in the flavylium cationic form (lines at 1572, 1600 and 1648 cm^{-1}). The Raman spectrum obtained from outer cell vacuoles can be compared to that recorded from the slightly acidified crude extract aqueous solution (Fig. 3c).

One can conclude that the pH of the dark grains is close to neutrality whereas, in the vacuoles of the outer cells, where no anthocyanin synthesis occurs, the pH is probably below 3–4. Another interesting conclusion is that the ultimate step in anthocyanin biosynthesis in 'Pinot noir' grapes probably produces neutral quinonoidal bases and not flavylium cations as usually depicted. This result explains why all natural anthocyanins known so far carry a hydroxyl group at either the 5-, 7- or 4'-positions. However, an argument against this hypothesis is that neutral bases and flavylium cations are in fast acid–base equilibrium (Fig. 1) and the ultimate step in anthocyanin biosynthesis could well be the formation of a flavylium cation which, at neutral pH, cannot accumulate and is instantaneously transformed into the neutral bases, the only chromophores existing in this pH range. Moreover, neutral conditions are, perhaps, a prerequisite for the stability of the enzymes participating in anthocyanin biosynthesis and too acidic a medium perhaps inhibits their activities.

In our model experiments dilute anthocyanin solutions were used. In living tissues anthocyanin concentrations are frequently much higher and molecular association processes usually occur. Is this reflected in our RR spectra? In RR as in normal Raman spectrometry, the position of bands is a property solely of the electronic ground state. Consequently, aggregates in which the electronically excited state of the monomeric molecule is strongly perturbed, with the ground state much less affected, will exhibit RR spectra with features having approximately the same frequencies as the corresponding features of the free molecule. In this case, concentration effects will only slightly modify the RR spectra. This is probably the reason for the great similarities between our *in vitro* and *in vivo* RR spectra. In fact, in the vacuoles of the mature 'Pinot noir' skins, molecular aggregation of malvidin 3-glucoside and peonidin 3-glucoside chromophores with themselves and with co-pigments does take place when critical concentrations of the pigments are met. Further experiments in this field should use UV–visible absorption spectrometry and fluorescence spectrometry combined with Raman spectrometry in

order to obtain more information about the anthocyanin association processes.

Malva sylvestris. *Malva sylvestris* is one of the best known sources of malvidin 3,5-diglucoside (malvin). The RR spectrum, which is not presented here, of the crude pigment extract obtained by maceration of a petal in aqueous 0.1 % hydrochloric acid exhibits exactly the same spectral features as those observed for pure dilute acidic solutions of malvin chloride. The *in vivo* RR spectra, obtained by exciting one petal epidermal cell at different wavelengths, are shown in Fig. 4. Laser wavelengths are 457.9, 488 and 514.5 nm. At 457.9 nm, poor pre-resonance conditions occur and, therefore, there is a low signal-to-noise ratio (Fig. 4c). At 488 nm good resonance between the electronic and vibrational levels is achieved and, at the same time, fluorescence scattering does not interfere too much with Raman scattering (Fig. 4b). Fluorescence scattering is so low in *Malva sylvestris* petals that it was even possible to obtain the RR spectrum using the 514.5 nm argon laser excitation line. Of course, the fluorescence scattering was then more important in the case of the 488 nm excitation radiation, but the RR spectrum was still observable. In acidic aqueous solutions of malvin, use of the 514.5 nm laser line is precluded due to the existence of a very intense fluorescence scattering phenomenon (see Experimental). Consequently, it can be concluded that in the mallow petals fluorescence quenching occurs. It was recently demonstrated that the fluorescence intensities of the model compounds 7,4'-dihydroxy- and 4'-hydroxy-flavylium chlorides are strongly affected by the presence of compounds acting as co-pigments [23]. Attenuation of the fluorescence intensity when going from the pure malvin solution to the malvin *in vivo*, may well be explained by formation in the petals of a loose malvin–co-pigment complex.

Comparison of Figs 2(c) and 4(b) shows that they are very similar both in frequencies and relative intensities. They only differ by a stronger fluorescence emission in the case of the pure dilute malvin chloride solution. Moreover, no other pigments absorbing light in the 400–600 nm range are found in these flower petals. For instance, carotenoids are completely lacking. We can conclude that in the vacuoles of the upper epidermal cells of the common mallow petals, the only existing malvin chromophore is the cationic flavylium form. Again, the pH of the vacuoles must be lower than 4. Of course, colourless malvin forms can exist in equilibrium with the cationic form, but they are not detected with the RR technique using visible excitation. Due to the flavylium cation concentration of malvin in the upper epidermis cells of the mature wild mallow petals, two cases should be taken into consideration in order to account for the extraordinary similarity between Figs 2(c) and 4(b). (a) If this concentration is of the same order of magnitude as our model solutions ($\simeq 5 \times 10^{-5}$ M), no aggregation occurs and the flavylium chromophore exists solely in the monomeric state. (b) If this concentration is high enough ($> 10^{-4}$ M), weak association phenomena probably exist which are reflected only in the fluorescence spectra, not in the Raman spectra. Consequently, the electronic ground state of the flavylium cation within the aggregates is only slightly disturbed compared to the modifications of their first electronic excited state. Fluorescence attenuation in the *in vivo* state clearly favours the second hypothesis.

EXPERIMENTAL

Plant material. Berries of the 'Pinot noir' grape were collected from the Alsatian vineyard during the autumn of 1983. Flowers of the common mallow were gathered on the campus of the University of Lille.

Origin of anthocyanins. Malvin and cyanin chlorides were purchased from Roth and were used without further purification. Malvidin 3-glucoside chloride was extracted from the 'Pinot noir'

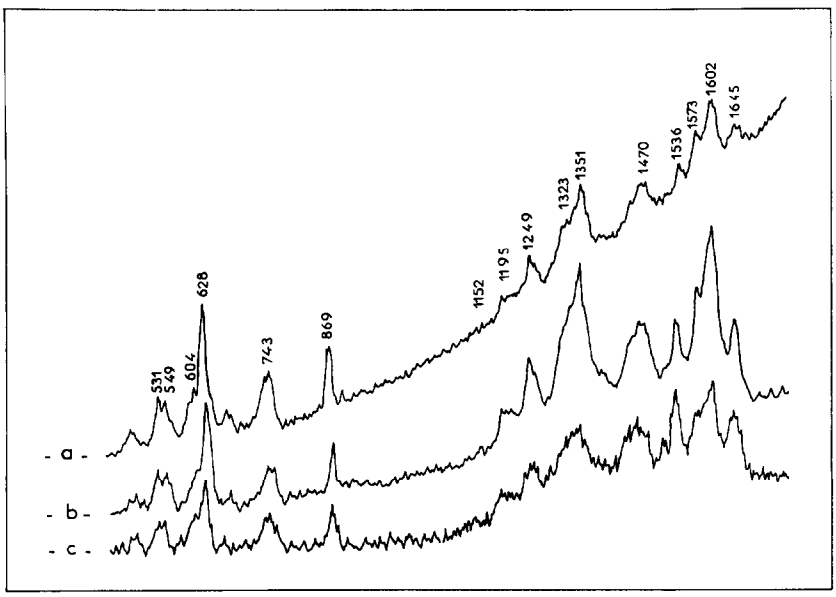

Fig 4. *In vivo* RR spectra of a petal epidermal cell of the common mallow with different excitation wavelengths (a) 514.5 nm; (b) 488 nm; (c) 457 9 nm.

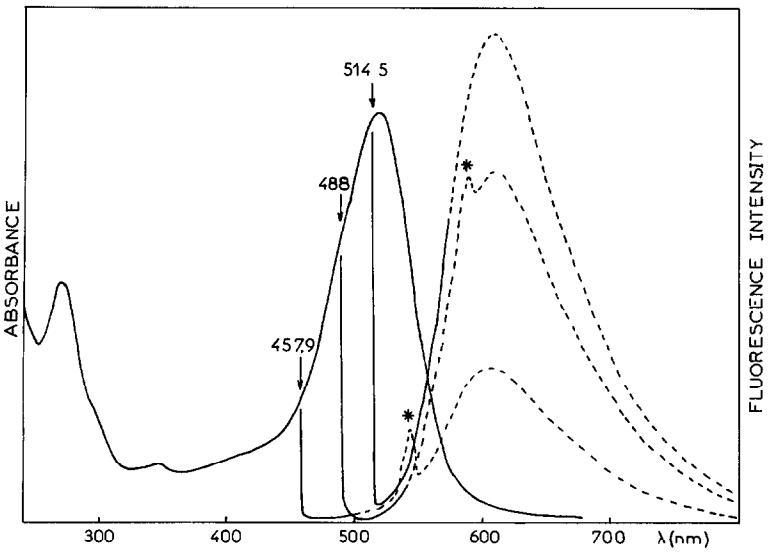

Fig. 5. Absorption and fluorescence spectra of malvin in water at pH 1. The solid parts of the fluorescence spectra (broken curves) represent, for each excitation wavelength used in this study (indicated by arrows), the 0–2000 cm^{-1} Raman investigated spectral range *Indicates the Raman lines of water. The *A* and fluorescence scales are in arbitrary units.

grape using well-known procedures. Its purity was checked both by PC and by UV–visible absorption spectrometry following Harborne [2].

Raman spectrometry. In a Raman expt a monochromatic laser beam is focused into or onto the sample The resulting scattered light is collected and focused on the entrance slit of a monochromator. The dispersed lines are detected and recorded sequentially by a cooled photomultiplier followed by a d.c. amplifier or a photon counter. The spectrum is traced on a chart recorder.

RR spectra of stable solns were obtained using a conventional DILOR RT 30 spectrometer When time evolution of the sample occurred, the RR spectra were obtained with a Ramanor HG2 spectrometer equipped with a rapid scanning device and a data averaging system (interzoom SEIN computer) [24]. A scan time of 30 sec for a region of 400–1700 cm^{-1} was used for our expts. Averaging of many sets of recording, each obtained from a freshly prepared soln, allows the signal-to-noise ratio to be increased.

The Raman microanalysis technique was first described by Delhaye and Dhamelincourt [25]. The arrangement of the Raman microprobe, developed at the 'Laboratoire de Spectrochimie Infrarouge et Raman', consists of a conventional optical microscope associated with an optical filter and a monochannel detection when the instrument functions as a microspectrometer. The same microscope objective is used both to focus the laser beam into a small spot (1–2 μm^2) on the component of the sample to be studied and to collect the scattered light The sample of plant tissue is placed directly at the focus of the laser beam between a slide and a cover glass A drop of H_2O allows minimization of the thermal effect produced by the absorption of the laser beam.

Ionized Ar lasers (Spectra Physics 164 AC or Lexel model 95) emitting 514 5, 488 and 457 9 nm radiations were used. The choice of the excitation wavelength was made by considering both the absorption spectrum and the fluorescence emission of the sample. For malvin acidic solns (Fig. 5), the expected maximum intensity enhancement should occur with the 514.5 nm line, but the strong fluorescence background obtained with this excitation hinders the RR observation With the 488 nm line, the resonance process is less important, but the consequent reduction of fluorescence allows the Raman signal to be observed with a rather good signal-to-noise ratio. This reduction is even more important with the 457.9 nm excitation.

In all cases, reproducibility of all spectral features was observed as a function of time and as the power of the laser beam, thus showing that no significant denaturation of the solns or the cells occurred under the exptal conditions employed.

REFERENCES

1. McClure, J. W. (1975) in *The Flavonoids* (Harborne, J. B., Mabry T. J. and Mabry, H., eds) p. 970. Chapman and Hall, London.
2. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids.* Academic Press, New York.
3. Britton, G. (1983) *The Biochemistry of Natural Pigments* Cambridge University Press, Cambridge.
4 Matile, P. (1976) in *Plant Biochemistry* (Bonner, J. and Varner, J. E., eds) 3rd edn, p. 189. Academic Press, New York
5. Wagner, G. J., Butcher, H. C. and Siegelman H. W. (1978) *Bioscience* **28**, 95.
6. Wagner, G. J. (1979) *Plant Physiol.* **64**, 88.
7. Wagner, G. J., Mulready, P. and Cutt, J. (1981) *Plant Physiol* **68**, 1081.
8. Moskowitz, A. H. and Hrazdina, G. (1981) *Plant Physiol* **68**, 686.
9. Brouillard, R. (1983) *Phytochemistry* **22**, 1311.
10 Saito, N. (1967) *Phytochemistry* **6**, 1013.
11. Asen, S., Stewart, R. N. and Norris, K. H. (1975) *Phytochemistry* **14**, 2677
12. Ishikura, N. (1978) *Plant Cell Physiol.* **19**, 887.
13. Statoua, A., Merlin, J. C, Delhaye, M. and Brouillard, R. (1982) in *Raman Spectroscopy Linear and Nonlinear* (Lascombe, J and Huong, P V., eds) p 629. John Wiley, Chichester.
14. Statoua, A., Merlin, J. C., Brouillard, R. and Delhaye, M

(1983) *C.R. Acad. Sci. Paris* **296**, 1397.
15. Merlin, J. C. (1983) *Spectros. Int. J.* **2**, 52.
16. Carey, P. (1982) *Biochemical Applications of Raman and Resonance Raman Spectroscopies.* Academic Press, New York.
17. Brouillard, R. (1982) in *Anthocyanins as Food Colors* (Markakis, P., ed.) p. 1. Academic Press, New York.
18. Statoua, A. (1982) Thése de 3è Cycle. Université des Sciences et Techniques de Lille.
19. Ribéreau-Gayon, P. (1982) in *Anthocyanins as Food Colors* (Markakis, P., ed.) p. 209. Academic Press, New York.
20. Fong, R. A., Kepner, R. E. and Webb, A. D. (1971) *Am. J. Enol. Viticul.* **22**, 150.
21. Morinville, P. (1983) Thèse de 3è Cycle. Université des Sciences et Techniques de Lille.
22. Pecket, R. C. and Small, C. J. (1980) *Phytochemistry* **19**, 2571.
23. Santhanam, M., Hautala, R. R., Sweeny, J. G. and Iacobucci, G. A. (1983) *Photochem. Photobiol.* **38**, 477.
24. Berry, J. M , Sombret, B. and Wallart, F. (1978) *J. Mol Struct.* **45**, 349.
25. Delhaye, M. and Dhamelincourt, P. (1975) *J. Raman Spectrosc.* **3**, 33.