

The Widespread Occurrence in Nature of Anthocyanins as Zwitterions

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An electrophoretic survey of anthocyanins in flowers of 81 species belonging to 27 plant families indicated that malonated or similarly substituted zwitterionic anthocyanins occur in half the sample. In particular, almost all species surveyed in the Compositae and the Labiatae were positive. The same results were also indicated by HPLC separations. Detailed examination of the anthocyanins of *Dahlia variabilis* and *Plectranthus argentatus* confirmed the presence of malonylation through glucose. It appears that anthocyanins substituted by aliphatic dicarboxylic acids may be widespread in the angiosperms.

Introduction

Until recently, anthocyanins containing acyl substitution were recorded in a small number of plant families and the acyl group present was usually an aromatic organic acid, such as *p*-coumaric, or acetic acid [1, 2]. However, there have been several recent reports of malonated anthocyanins, *e.g.* of pelargonidin 3-malonylsophoroside in flowers of *Papaver nudicaule* [3] and of cyanidin 3-(6-malonylglucoside) in leaves of *Cichorium intybus* [4]. More significantly, Japanese workers investigating anthocyanins responsible for blue flower colour in *Commelina communis* and *Centaurea cyanus*, discovered the pigments to have malonic and succinic acids respectively, where previous investigations had failed to show such acylation to be present [5, 6]. Such acyl groups are labile in solutions containing mineral acid, so that in the standard procedures for extracting anthocyanins from plant tissues which use methanolic HCl, these substituents are likely to be lost. Only by using alcoholic solvents where the HCl is replaced by acetic or formic acids is it possible to isolate the pigments with the dicarboxylic acid substituents intact.

Malonated derivatives have been described among other flavonoids; *e.g.* malonated flavone glycosides occur in *Petroselinum crispum* plant and cell culture extracts [7] and malonated isoflavone glycosides have recently been reported in *Cicer arietinum* roots and stems [8]. In almost all cases, malonic acid is linked through sugar and commonly is attached to the 6-position of glucose. In the case of flavones and isoflavones, the attachment of malonic acid confers a

negative charge on the molecule and this may be important to the way the compound is stored in the vacuole or in protecting the glycoside from enzymic hydrolysis by glycosidases. In the case of anthocyanins, malonylation balances the cationic charge on the flavylium ion, so that the malonated anthocyanin is effectively a zwitterion. Thus, the need to assume that anthocyanins occur in the cell vacuole in loose ionic association with organic acids, as suggested in the earlier literature, is removed for such pigments.

Because of their lability, such zwitterionic anthocyanins might well have been overlooked in earlier studies. We therefore set out to investigate whether malonylation or similar acylation might not occur quite widely among the anthocyanins of the angiosperms.

Results

The observation of Cornuz *et al.* [3] that the malonated pelargonidin 3-sophoroside of *Papaver nudicaule* could be distinguished from the related nonacylated pigment by its mobility on paper electrophoresis at pH 4.4 provided us with a simple general screening procedure for such pigments. We found that at this pH anthocyanin colours tend to fade rapidly, so that after development, we dipped the papers briefly in 1% HCl in order to stabilize the colour. Using an extraction medium based on aqueous methanolic acetic acid, we surveyed the flower tissues of some 81 species and obtained positive results in 40 samples (Table I).

These results considerably extend earlier records of malonated anthocyanins in plants. Of the 27 angiosperm families surveyed, positive records were made in 10. In fact, the number of families contain-

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Table I. Electrophoretic survey of angiosperm flowers for zwitterionic anthocyanins.

Plant species	Mobile Pigment Present*	Plant species	Mobile Pigment Present*
Boraginaceae		Iridaceae	
<i>Borago officinalis</i> L.	—	<i>Iris cristata</i> Soland.	—
<i>Echium vulgare</i> L.	—	<i>Schizostylis coccinea</i> Backh. & Harv.	—
Bromeliaceae		Labiatae	
<i>Billbergia decora</i> Poepp. & Endl.	—	<i>Hyssopus officinalis</i> L.	+
Campanulaceae		<i>Lavandula stoechas</i> L.	+
<i>Campanula cervicaria</i> L.	—	<i>Monarda didyma</i> L.	+
<i>Codonopsis cardiophylla</i> Diels ex Kom.	—	<i>Plectranthus argentatus</i> Blake	+
Cannaceae		<i>Prunella vulgaris</i> L.	+
<i>Canna edulis</i> Ker-Gawl	—	<i>Salvia nemorosa</i> L.	+
Caryophyllaceae		<i>S. verticillata</i> L.	+
<i>Dianthus deltoides</i> L.	+	<i>S. virgata</i> Ait.	+
<i>Dianthus sylvestris</i> Wulf.	+	<i>Stachys macrantha</i>	+
<i>Lychnis flos-jovis</i> Desr.	+	<i>S. sylvatica</i> L.	+
Commelinaceae		<i>Thymus praecox</i> Opiz ssp. <i>arcticus</i>	+
<i>Tradescantia navicularis</i> Ortgies	—	<i>T. pulegioides</i> L.	+
<i>Tradescantia</i> cv. <i>Leonora</i>	—	Leguminosae	
Compositae		<i>Lathyrus latifolius</i> L.	—
<i>Ageratum</i> cv. <i>Ocean</i>	+	<i>L. odoratus</i> L.	—
<i>Arctotis</i> sp.	+	<i>Ononis spinosa</i> L.	—
<i>Bellis perennis</i> L.	+	<i>Vicia sativa</i> L.	—
<i>Bidens</i> sp.	—	Lobeliaceae	
<i>Callistephus hortensis</i> Cass.	+	<i>Lobelia</i> cv. <i>Mrs. Clibran</i>	+
<i>Catananche caerulea</i> L.	+	Malvaceae	
<i>Centaurea montana</i> L.	+	<i>Abutilon hybridum</i> Hort.	—
<i>C. nigra</i> L.	+	<i>Hibiscus rosa-sinensis</i> L.	—
<i>Cicerbita plumieri</i> (L.) Kirschleger	+	Onagraceae	
<i>Cichorium endivia</i> L.	+	<i>Epilobium angustifolium</i> L.	—
<i>C. intybus</i> L.	+	<i>Fuchsia fulgens</i> DC.	—
<i>Cirsium</i> sp.	+	Papaveraceae	
<i>Dahlia variabilis</i> Desf.	+	<i>Eschsholtzia</i> cv.	—
<i>Helenium</i> cv. <i>Bruno</i>	+	<i>Papaver orientale</i> L.	—
<i>Helianthus annuus</i> L. cv.	+	Polemoniaceae	
<i>Liatris spicata</i> Willd.	+	<i>Phlox drummondii</i> L.	+
<i>Senecio coccinaeflorus</i> Rowl.	—	<i>Polemonium caeruleum</i> L.	—
<i>Stokesia laevis</i> Hill	+	Punicaceae	
<i>Tanacetum coccineum</i> Willd.	+	<i>Punica granatum</i> L.	—
<i>Zinnia</i> cv. <i>Persian Carpet</i>	+	Ranunculaceae	
Convolvulaceae		<i>Aconitum napellus</i> L.	—
<i>Convolvulus arvensis</i> L.	—	<i>Clematis integrifolia</i> L.	+
<i>Convolvulus</i> cv.	+	Rosaceae	
Crassulaceae		<i>Potentilla atrosanguinea</i>	—
<i>Crassula schmidtii</i> Regel	—	<i>Loddiges</i> ex D. Don	—
Dipsacaceae		<i>P. nepalensis</i> Hook	—
<i>Knautia arvensis</i> (L.) Coult.	—	<i>Rosa multiflora</i> cv. <i>Crimson Rambler</i>	—
Ericaceae		Scrophulariaceae	
<i>Daboecia cantabrica</i> (Hudson) C. Koch	—	<i>Digitalis purpurea</i> L.	—
Geraniaceae		<i>Hebe</i> cv.	+
<i>Geranium maderense</i> Yeo	—	<i>Penstemon</i> cv. <i>Skyline</i>	—
<i>G. pratense</i> L.	—	Solanaceae	
<i>G. himalayense</i> Klotzsch.	—	<i>Atropa belladonna</i> L.	—
Gesneriaceae		<i>Browallia demissa</i> L.	+
<i>Streptocarpus</i> cv.	+	<i>Nicandra physalodes</i> (L.) Gaertn.	—
<i>S. saxorum</i> Engl.	—	Valerianaceae	
		<i>Centranthus ruber</i> (L.) DC.	—

* + = Pigment moves ca 3 cm on electrophoresis in acetate buffer, pH 4.4, at 40 V/cm for 2 h.
 — = Pigment remains at origin.

ing malonated anthocyanins is higher than this, since such pigments have been recorded in individual species of the Commelinaceae [5], Leguminosae [9] and Papaveraceae [3]. In some families such as the Leguminosae (see Table I) malonated anthocyanins may be relatively rare, but in others, such as the Compositae and Labiatae, malonated anthocyanins appear to be almost universal. The regular occurrence (Table I) of malonated pigments in the Labiatae is a little surprising since the anthocyanins in this family are usually acylated with hydroxycinnamic acids; clearly malonylation can occur irrespective of any other type of acylation present.

In order to confirm these findings, detailed studies were made on the anthocyanins in a representative species of the Compositae, in *Dahlia variabilis*, and of the Labiatae, in *Plectranthus argentatus*. In the case of *Dahlia*, earlier investigations have established the presence of pelargonidin 3,5-diglucoside (pelargonin) and cyanidin 3,5-diglucoside ^{extensively} in garden forms [1, 2]. Extraction under mild conditions of the major pelargonidin derivative from petals of a scarlet form of *D. variabilis* gave an acylated pigment, different in R_f from pelargonin (see Experimental). On mild hydrolysis, it gave pelargonin while on complete hydrolysis, it gave pelargonidin and glucose. Confirmation of the presence of a malonyl group was obtained by H₂O₂ oxidation, which gave a malonylglucose, liberated from the 3-position, which co-chromatographed in 4 solvents with authentic 6-malonylglucose, prepared similarly from the cyanidin 3-malonylglucoside of chicory leaves. The original *Dahlia* pigment can thus be formulated as pelargonidin 3-(6-malonylglucoside)-5-glucoside, which has not been described before. Similar studies on the zwitterionic leaf pigment of *Plectranthus argentatus* showed the presence of peonidin 3,5-diglucoside, substituted with both malonic and *p*-coumaric acids.

HPLC was also used to confirm the acylated nature of these pigments. Using a C₈ column and gradient elution with aqueous methanolic acetic acid mixtures, malonated pigments consistently eluted later than the corresponding unacylated anthocyanins (Table II). Attempts to use HPLC for direct screening of plant extracts for these pigments have not so far been successful, but the use of ion-exchange columns may make this possible. It was of interest that a flower extract containing a malonated pigment, when placed on an ion exchange column (Amberlite

Table II. HPLC separation of Anthocyanins from their malonyl derivatives.

Compound	t _R [min]*
Cyanidin 3-glucoside	10.77
Malonate from <i>Cichorium intybus</i> leaf	15.49
Delphinidin 3,5-diglucoside	5.14
Dimalonate (?) from <i>Cichorium intybus</i> petals	12.23
Pelargonidin 3,5-diglucoside	7.37
Malonate from <i>Dahlia variabilis</i> petals	10.33
Peonidin 3,5-diglucoside	6.76
<i>p</i> -coumaryl malonate from <i>Plectranthus</i> leaf	21.11

* On a Partisil 5 CCS C₈ column (25 cm × 4 mm) eluted with A, 5% aq HOAc; B, MeOH-H₂O-HOAc (18:1:1), initial concentration 20% B in A, increasing by 2% B per min, flow rate 1.7 ml min⁻¹, detection at 546 nm.

IRC 50), was not retained in the usual way [11], but passed straight through.

Discussion

These results together indicate that anthocyanins substituted with aliphatic dicarboxylic acids may be quite widespread in nature, particularly among the more highly evolved angiosperm families. Of the known organic acids, malonic would seem to be the most common, since it has been reported most frequently. However, succinic acid has been reported as the acylating acid in the *Centaurea* anthocyanin [6] and it is possible that other aliphatic dicarboxylic acids will be found as well.

The present studies were conducted mainly on flower pigments but there is little doubt that malonated pigments occur in other parts of the plant as well. Where pigmentation is found in both leaf and flower, it is likely that malonated anthocyanins will be found in both parts. This is true in the chicory, *Cichorium intybus*, where cyanidin 3-(6-malonylglucoside) has already been identified in varieties with reddish leaves [4]. Our preliminary examination of the delphinidin derivative of the blue flowers indicates that it is a mixture of mono- and dimalonated delphinidin 3,5-diglucoside and not delphin, as previously recorded [10].

The present data do now indicate the need to check for the presence of malonyl groups in any modern investigation of plant anthocyanins. This can be done using a weak acid extraction medium and

electrophoresis or HPLC. Clearly, further work is needed to substantiate the findings reported here and more detailed investigations of the anthocyanins in the Compositae and Labiatae are in progress.

Experimental

Floral material was freshly collected either from the University of Reading botanic gardens or from wild plants collected locally. Pigments were extracted into MeOH-HOAc-H₂O (19:2:19), filtered and the solution concentrated in vacuo at 30°. Electrophoresis was conducted on Whatman No. 3 paper in acetate buffer pH 4.4 for 2 h at 40 V/cm. The papers were immediately removed, dipped briefly in 1% MeOH-HCl and then dried. Cationic anthocyanins remained close to the origin, whereas zwitterionic anthocyanins were clearly distinguished by their movement (3–4 cm) towards the anode.

The malonated pigments in individual species were isolated from similar crude extracts by prep. paper chromatography using the solvents BAW (*n*-BuOH-HOAc-H₂O, 4:1:5, top) and 15% HOAc, repeating where necessary to remove impurities. *R_f* measurements were determined in these two solvents, as well as in BuHCl (*n*-BuOH-2 *N* HCl, 1:1, top) and HOAc-HCl (HOAc-HCl-H₂O, 15:3:82). Details of HPLC separations are given in Table II.

Pelargonidin 3-malonylglucoside-5-glucoside isolated from scarlet blooms of *Dahlia variabilis* had the following *R_f* (×100) (pelargonin values in paren-

theses) in BAW 41 (36), in BuHCl 55 (36), in 1% HCl 40 [26] and in HOAc-HCl 66 [53]. The spectral properties were identical to those of pelargonin. On electrophoresis at pH 4.4 for 2 h, it moved 3.4 cm, but some pelargonin (0.8 cm) was formed as a result of hydrolysis. On H₂O₂ oxidation, it gave a malonylglucose, identical in 4 solvents with 6-malonylglucose prepared from cyanidin 3-(6-malonylglucoside) from chicory leaves. *R_G* values were 1.2 in BAW, 0.17 in *n*-BuOH-toluene-pyridine-H₂O (5:1:3:3), 0.34 in *n*-BuOH-EtOH-H₂O (4:1:2:2, top) and 0.36 in PhOH-H₂O (3:1). These agree with lit. values [3].

The peonidin 3,5-diglucoside acylated with both *p*-coumaric and malonic acids from red leaves of *Plectranthus argentatus* had the following *R_f*s (×100) (*p*-coumaroylpeonidin 3,5-diglucoside values in parentheses) in BAW 55 (63), in BuHCl 47 (43), in 1% HCl 12 (12) and in HOAc-HCl 58 (59). The spectral properties, $\lambda_{\text{max}}^{\text{MeOH-HCl}}$ 282, 315 (*E*₃₁₅/*E*_{max} 84%), 440 (*E*₄₄₀/*E*_{max} 15%), 528 nm indicated disubstitution with *p*-coumaric acid and on acid hydrolysis, *p*-coumaric acid, peonidin and glucose were readily detected. Deacylation with alkali gave peonidin 3,5-diglucoside. H₂O₂ oxidation gave 6-malonylglucose, identified as above. These results suggest that the *p*-coumaroyl groups are attached to the 5-glucose residue, while the malonyl group is present on the 3-glucose

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