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MICROSPECTROPHOTOMETRIC MEASUREMENT OF pH AND pH EFFECT ON COLOR OF PETAL EPIDERMAL CELLS

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Abstract—A microspectrophotometric method has been developed for colorimetric pH determination using indicator dyes. Tissue samples as small as five cells were used. Measurements of standard buffered solutions of known pH were within a standard error of less than $\pm 0{\cdot}04$ pH units. Validity of the technique has previously been established by matching the pH values and absorption spectra of several model systems to that of living cells. A method for spectrophotometric pH determination of single cells is suggested. The pH change seemed to be the major factor in the color change in aging flowers. The epidermal pH, the absorption spectra of living tissue, the anthocyanidins, flavonols, and flavones present in more than 250 plants of many families were determined. These data indicate that pH is only one of numerous parameters determining flavonoid color in the living cell.

INTRODUCTION

In a study of contribution of flavonoids to the color of flowers, we have attempted to measure and characterize the factors affecting color in smaller and smaller parts of the plant, ultimately at the single cell level. Microscopic examination of plant material shows a very compartmentalized and sharply delimited distribution of the colors. For instance, in true petals of most plants, colors due to flavonoids are largely confined to the epidermal cells [1] and adjacent subepidermal cells are colorless. In petals with genetically determined color patterns, adjacent cells may differ in color. The small dark red or purple spots on many flowers usually consist of cells having a whole array of colors [1]. Furthermore, in tissues with uniform color in all cells when young, the color changes associated with aging may proceed at very different rates in contiguous cells [2].

The all-purpose spectrophotometer developed in our laboratories has provided accurate spectrophotometric data on single living cells [3]. The apparent influence of pH on color in tissue [2,4] and in model systems [5] and the pH changes associated with aging of tissue [2] made data on pH on the cellular level important and desirable. We have measured pH of epidermal cells of flowers of more than 250 different plants from many families. The absorption spectra of living tissue of these same samples were recorded in the region from 280 to 700 nm. The anthocyanidins and flavonols present in these tissues were also characterized.

RESULTS

The pH of the pigmented epidermis of petals from young flowers ranged from 2·5 in a begonia cultivar to 7·5 in morning glory cv. Heavenly Blue. Representative pH values and other color-determining characteristics are shown in Table 1.

The epidermal cells of flowers whose only anthocyanins were cyanidin glycosides ranged in pH from 3·1 to 5·5 and the wavelengths of maximum absorption of the whole petal in the visible (λ_{max}) were from 508 to 575 nm (Table 2). Similar ranges of pH, λ_{max}, and absorbance (A) were found

Table 1. Color determining characteristics of some flowers through the pH range

Sample source	pH	Color	Anthocyanidins*	Flavanols†	Flavones‖	Wavelength of absorbers (nm)
Begonia cv. Orange Schwabenland	2·5	Strong red–orange	Cy, Pg	Km, Qu‡	X	436, 508
Azalea cv. Gloria	3·1	Light yellowish–pink	Cy	Az, Qu§		512¶
Impatiens cv. Tangerine	3·5	Moderate orange	Au		X	486
Hydrangea cv. Merrivale	4·0	Moderate purplish–pink	Dp	Km		536
Storks bill	4·4	Light reddish–purple	Mv	Km		562
Delphinium cv. Summer Skies	5·1	Brilliant purplish–blue	Dp	Km	X	540, 582, 629
Cornflower cv. Blue Boy	5·3	Moderate blue	Cy		X	527, 576, 606, 686
Periwinkle	5·5	Light purplish–blue	Dp	Km, My, Qu§		539, 573, 621
Pansy cv. Delft Blue	5·9	Brilliant violet	Dp, Cy§	Qu, Km, My‡	X	512, 552, 581, 634
Bellflower cv. Rose	6·4	Light purplish–pink	Pg		X	496, 531, 571
Morning Glory cv. Heavenly Blue	7·5	Vivid light blue	Pn, Cy§		X	605

* Au aurantinidin; Cy cyanidin; Dp delphinidin; Mv malvidin; Pg pelargonidin; Pn peonidin. † Az azaleatin; Km kaempferol; Qu quercetin; My myricetin. ‡ Present in minor amounts. ‖ Indicated by fluorescence quenchers. § Present in trace amounts. ¶ Absorber with greatest A.

in flowers containing glycosides of other anthocyanidins. Absorbance at λ_{max} of single cells was as high as 2·6 (in the rose cv. Oklahoma). In this paper, A includes light loss by scatter as well as by absorption and is log $(1/T)$, where T is the transmittance.

The pH of epidermal samples from various parts of the same flower were often quite different. The pH of the strong pink calyx epidermis of fuchsia cv. Fanfare was 4·1, and that of the strong red corolla was 3·7. The pH of the strong pink calyx of fuchsia cv. Black Knight was 3·8, and that of the deep purple corolla was 5·4. The epidermis of various parts of the flowers of azalea cv. Orange Red Wing were found to differ in pH and λ_{max}. The pH of petals ranged from 3·0 to 3·2, the filaments from 3·2 to 3·4, the style from 3·6 to 3·8, and the anther sac from 4·4 to 4·5. The corresponding range in λ_{max} was 507–510, 510–514, 522 to 524 and 532–548 respectively. The spectra of the style and anther sac showed absorbers in the UV that were not apparent in the spectra of the petal and filament epidermis. Observations of the range in color within many other flowers strongly suggested similar variation in pH and λ_{max}. The pH of internal tissue was different from that of the epidermis in some flowers. The pH of the epidermis of petals of salvia was 4·7, and that of the whole petal was 5·0. In bracts of poinsettia cv. Stoplight, the epidermal pH was 3·3, and that of the whole bract was 3·5. In poinsettia cv. Pink Stoplight, the epidermal pH was 3·3, and the whole bract 3·8. The upper and lower epidermis of all flowers tested had comparable pH values, and the epidermis of the proximal portion of petals was often slightly higher than that of the distal areas.

In a number of flowers, the pH changed as the flower aged. Many red flowers became more blue and the pH increased as they aged. The greatest change in pH was found in geranium cv. Crimson

Table 2. Characteristics of representative flowers whose only anthocyanins are cyanidin glycosides

Source	Color	Epidermal pH	Whole petal λ_{max}	Whole petal A at λ_{max}
Azalea cv. Skylark	Deep pink	3·1	535	0·66
Azalea cv. Dorothy Gish	Moderate yellow–pink	3·1	508	0·99
Azalea unnamed seedling	Light reddish–purple	3·2	545	0·96
Rose cv. Forever Yours	Deep red	4·1	528	4·25
Rose cv. Pink Van Fleet	Strong pink	4·5	533	0·93
Swamp rose	Moderate purplish–pink	5·3	537	0·62
Cornflower cv. Blue Boy	Moderate blue	5·3	576	0·85
Snapdragon cv. Orchid Rocket	Light reddish–purple	5·4	560	0·42
Petunia cv. Maytime	Strong yellow–pink	5·5	522	0·78

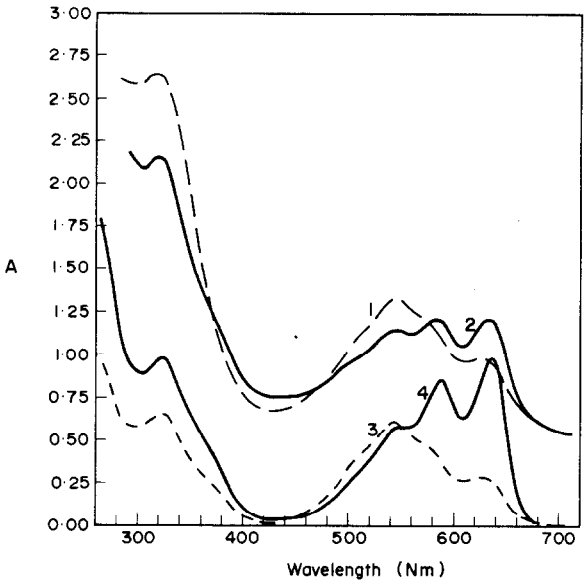

Fig. 1. Absorption spectra of larkspur cv. Dark Blue Supreme. (1) Moderate reddish purple area through whole petal; (2) Light purplish–blue area through same petal; (3) moderate reddish–purple cell from epidermal peel in light purplish area; (4) light blue cell adjacent to (3). Curves (1) and (2) were offset by 0·25 units to reduce overlap. The additional apparent absorbance at 700 nm is caused by light scatter by the internal tissue and air space of the petals.

Carefree flowers. The vivid purplish–red epidermal cells of young flower petals had a λ_{max} as low as 520 nm and a pH as low as 2·9. Light purplish–blue areas in old petals were found with λ_{max} as high as 542 nm and pH as high as 5·4. Areas of flowers of intermediate age were found to have intermediate values for λ_{max} and pH.

Similar changes occurred in the flowers of larkspur cv. Dark Blue Supreme as they aged. Young flowers were uniform color to the eye, with fairly large areas of cells of a uniform moderate reddish–purple color. But even in these flowers there were some areas with redder cells and other areas with bluer cells. In older flowers, the light purplish–blue areas increased and the moderate reddish-purple areas decreased. The pH of epidermal areas (*ca* 5000 cells) of uniform reddish-purple color was around 5·5 to 5·6. The areas with increasing blueness gave higher pH values; the highest pH found in a light purplish–blue area was 6·6. Figure 1 shows the absorption spectrum of a moderate reddish–purple area (pH 5·5), of a light purplish–blue area (pH 6·6), of a moderate reddish–purple cell, and of an adjacent light blue cell. Both red and blue cells are found in bluing areas.

The bluish or purplish petals of cornflower, morning glory, and fuchsia were observed to become more red as they aged. In two fuchsia cultivars, the deep purple corollas of young flowers became more red, and their epidermal pH decreased. Simultaneously, their calyces became more blue, and their epidermal pH increased (Table 3). A similar change of corolla to more red color and change to lower pH of the expressed juice of whole corollas was reported in other *Fuchsia* cultivars [6].

DISCUSSION

Reports of the pH of specific cells or tissue areas of plants [7–9] gave only rough estimates; or where precise values were given, there was no way of checking their validity. For some years it has been possible to determine, with glass pH electrodes, the pH of small amounts of juice expressed from plant tissue, but to obtain even one drop of

Table 3. Characteristics of young and old flowers of two cultivars of *Fuchsia hybrida*

		Calyx			Corolla				
Cultivar	Age	Color	Epidermal pH	Whole petal A at λ_{max} (nm)	Color	Epidermal pH	Whole petal A at λ† (nm)		
Little Beauty	Young	Red	3·6	1·5 at 527	Dark purple	5·0	1·2 at 547	1·1 at 580	0·5 at 638
	Old	Purplish–red	3·8	1·2 at 529	Reddish–purple	3·6	1·3 at 556	* *	* *
Black Prince	Young	Red	3·6	2·8 at 523	Dark purple	4·7	3·4 at 542	2·8 at 582	1·1 at 640
	Old	Purplish–red	4·2	1·7 at 530	Purplish–red	4·0	3·7 at 538	* *	* *

* No apparent absorber. † λ_{max} Of apparent absorbers.

Table 4. Characteristics of indicator dye solutions used in pH determinations

Indicator dye	Useful pH range	Measured wave lengths (nm)	Solution
Bromophenol blue	2·5–4·5	590/435	4 mg/10 ml H_2O*
Bromocresol green	4·4–5·2	615/450	8 mg/10 ml H_2O*
Chlorophenol red	5·0–6·0	573/432	10 mg in 1·2 ml 0·02 N NaOH + 23·8 ml H_2O
Bromophenol red	5·6–6·8	560/432	10 mg in 1 ml 0·02 N NaOH + 24 ml H_2O

* As the Na salt.

juice from a flower petal requires several thousand cells, which would include both epidermal and internal tissue.

The validity of the technique described here was substantiated by four considerations. First, determinations of the pH of buffer solutions were accurate to s.e. 0·038 pH units. Second, the results with many samples of similar tissue gave similar values. Third, values obtained from azalea cv. Orange Red Wing were the same as those determined with a pH microelectrode for expressed sap of "Orange Red Wing". Fourth, and most significantly, use of the pH values determined by this technique has enabled us to reproduce, in model systems, the absorption spectra of the living cells [2,3,10].

The necessity of obtaining pH measurements from as small and specific a tissue sample as possible was indicated by the observed difference in pH between pigmented epidermal and colorless internal tissue in poinsettia and salvia. Similar differences have been described in a *Camellia* graft chimera [11] and in *Rosa*. [2,9] The pH differs between epidermal and subepidermal cells, between the epidermis of different parts of the same flower in fuchsia and azalea, and between adjacent areas of a single petal of larkspur. These pH differences, along with the associated differences in color, strongly suggest that adjacent cells may have quite different pH.

Evaporation of the vacuolar sap within the micropipet determined the minimum sample size. With high relative humidity, a sample of *ca* 1 nl sealed off in the tip of a micropipet evaporated into the vacant part of the pipet slowly enough (*ca* 2 min) to obtain several A readings at the two wavelengths. Smaller samples, such as those from a single cell, were greatly reduced in volume or completely dried up by the time the sample was positioned in the microspectrophotometer. With the largest epidermal cells observed, those of azalea petal, the minimum number of cells required to provide adequate sample size was five.

The principal advantages of the technique described here are the ease and accuracy with which measurements can be made on very small samples from specific areas of the tissue. Appropriate instrumentation can be assembled in most laboratories from generally available or inexpensive components. The technique applies to a wide range of plant material. Using the dual sample procedure where the A of the cell sap plus water is subtracted from that of the cell sap plus dye, one has in effect a dual-beam instrument, and colorimetric pH measurements can be made on colored tissues. One problem was that some indicator dyes were not stable when mixed with cell sap, and the sap of some species caused precipitation when mixed with the dyes. The dyes listed in Table 4 were most generally useful.

Although it was important and informative to determine pH of the exact cells displaying color, it was increasingly apparent that pH was only one of several factors determining color. The only anthocyanin in all the species listed in Table 2 were cyanidin glycosides. The data show that the swamp rose and snapdragon cv. Orchid Rocket, with essentially the same pH, differed in λ_{max} by 23 nm. The two azaleas, whose pH values were not significantly different, differed in λ_{max} by 27 nm. These differences in λ_{max} at the same pH can be explained by differences in the concentration of anthocyanins, the type of copigment, and the ratio of copigment to pigment [12].

The bluing of flowers as they age is a well-known phenomenon and a concurrent increase in pH has often been demonstrated [13]. Bluing with

age is a particularly important problem with cut roses [2,14]. We have not observed color change upon aging without associated change in pH. These observations suggest that change in pH is a major factor in the color changes associated with aging.

The pH values determined with the micropipet or microcapillary techniques represent average values for a number of cells. A comparison of absorption spectra of individual epidermal cells of some tissues indicate their pH may vary considerably from that of the average. The visible spectra of a moderate reddish purple area [pH 5·5, Fig. 1 (1)], and an adjacent light purplish–blue area [pH 6·6, Fig. 1 (2)] of the same petal of larkspur show a loss in absorption in the short-wavelength region and an increase in the long-wavelength region for the higher pH. The spectra of two adjacent cells from the light purplish–blue region show this same shift, with the spectra from the moderate reddish–purple cell [Fig. 1 (3)] matching the tissue spectra for a pH of 5·5, and that of the light blue cell [Fig. 1 (4)] being more blue than the tissue spectra for a pH of 6·6. The pH of this light blue cell is estimated to be 7·1 by comparison of the ratio of absorption at 540 nm to that at 635 nm for the different samples.

These results suggest that the pH of individual cells can be determined by measuring the spectra of the cells and comparing these spectra with the spectra of the tissue samples from which the average pH can be measured. This assumes, of course, that the type and amount of anthocyanin and copigment do not change from cell to cell and that the change in spectra are the result of pH differences.

EXPERIMENTAL

Spectrophotometers. The spectra of the living tissue were determined on a multipurpose spectrophotometer [3]. Most of the *A* measurements for determining pH were made on the microspectrophotometer described earlier [15]. However, the adaption of a microscope for this specific purpose described below was less expensive, less difficult to construct, and more efficient to use.

A microscope with a camera attachment was modified to make the required absorption measurements (abridged microspectrophotometer). Interference filters (10 nm half bandwidth) appropriate for the indicator dye in use, Table 4, were moved in and out of the light path ahead of the specimen. The camera back was replaced with a housing for the phototube, which incorporated a broad-pass filter to remove the radiation outside the range of useful wavelengths. A thin piece of Teflon (0·2 mm) was placed between the photo tube and the filter to minimize errors from cathode sensitivity variations. The housing also included an adjustable aperture (iris diaphragm), which was used to define the area of the sample measured by the photo tube. Any sensitive photometer could be used to measure the transmission, but a unit with digital readout in absorbance is more convenient. A stable light source is required and was achieved with an electronically regulated power supply (0·01% regulation) to power the tungsten light source. The *A* of the tissue + water was subtracted from that of tissue + dye to correct for instrument response and absorption due to the pigment in the vacuolar sap. The ratio of the corrected absorbances at the two wavelengths was compared to the calibration curve to determine pH. Adaptations of microscopes for other photometric purposes have been described [16,17].

Tissue samples—micropipets. To obtain the vacuolar pH, epidermal cells of flower petals were punctured with micropipets controlled with micromanipulators. The micropipets were made from uniform-sized, thin-walled, soft-glass capillaries, 1 mm or less in dia, on an automatic micropipet puller. This puller formed them in reproducible size and shape, with the tip *ca* 10 μm dia. Also, the micropipets were bent in a microforge to provide a more sharply angled approach to the surface of the cells, making penetration of the cell wall easier. The vacuolar sap of punctured cells was forced into about 1 mm of the micropipet by a combination of turgor pressure and capillary action. The contents of *ca* 10 rose petal epidermal cells, or only 5 of the larger cells of azalea petals, were required for adequate sample size, *ca* 0·5 nl. This vol. of vacuolar sap was dil with an eq amount of indicator dye soln by briefly dipping the micropipet into a drop of dye on a glass slide. If the micropipet was then shortened by breaking it off near the sample, and the ends were sealed with a silicone grease, evaporation of the sample was usually slow enough (*ca* 2 min) to obtain the spectrophotometric measurements. A similar sample of vacuolar sap was dil with dist deionized H_2O. Measurements of *A* at the two appropriate wavelengths were made through the samples in the micropipets on our abridged microspectrophotometer at 100× so that the vol of the sample measured was *ca* 40 pl.

Tissue samples—microcapillary. A second procedure used a somewhat larger sample. The epidermis of a flower petal was peeled off, and pieces *ca* 1 × 4 mm were placed in adjacent cavities of porcelain spot-test plates. Depending on the cell size, there were from 2000 to 5000 cells in each sample. One sample was crushed in 3 μl of dye soln and the other in 3 μl of dist, deionized H_2O. The liquid was then taken up in 3 μl disposable microcapillary tubes, and absorption was measured on our abridged microspectrophotometer at 40×. Nearly all petals have a spongy network of internal cells from which the epidermis can cleanly and easily be peeled. The epidermis peeled from other flower or plant parts, usually included considerable hypodermal tissue. Some pH determinations were made on samples of whole petals of *ca* 4 mm^2, so that the epidermis and spongy mesophyll were crushed together. The accuracy of determinations of the pH of buffer solns is shown on the calibration curve. Determinations of pH of cells or tissues from the same region of flowers of the same kind, age and condition gave values as uniform as those with a buffer soln.

Indicator dyes. The absorption spectra of indicator dyes were measured on a spectrophotometer in a series of buffer solns at pH from 2·5 to 7·5. Two wavelengths showing a max opposite change in *A* with change in pH were chosen for each dye. The ratio of the absorbance at these wavelengths was used because the ratio does not change with dye concn, but does change with

pH. The log of this ratio has a linear correlation to pH. Filters with peak transmission at the appropriate wavelengths were used with the abridged microspectrophotometer to measure the same series of dye–buffer solns to establish calibration curves. Typical calibration for bromophenol blue gave a s.e. of ±0·038 pH units with a linear correlation of $r = 0{\cdot}997$ for five determinations of each of nine buffers. The dyes used, the solvent, the wavelengths measured, and the useful pH ranges are given in Table 4.

Identification of flavonoids. Fresh flowers were ext with 1% HCl–MeOH, and the extracts were reduced at 40° under red pres. Aliquots were hydrolyzed by refluxing at 100° for 1 hr in 1 N HCl. Flavonols and anthocyanidins were ext with EtOAc- and *i*-AmOH, respectively. They were characterized by co-chromatography with authentic compounds. The solvents used were: *n*-BuOH–HOAc–H_2O, (6:1:2), HCO_2H–HCl–H_2O (5:2:3), HOAc–HCl–H_2O (30:3:10) and phenol-H_2O, (73:27). Flavones were not characterized, but their presence was suggested by the appearance of quenchers when chromatograms were viewed with UV radiation.

Color notation. Color notations used in the tables and text but not in the plant names are consistent with those of Kelly [18].

Plant materials. The plant materials are: Snapdragon *Antirrhinum majus* L. cv. Rocket Orchid.† *Begonia elatior* Hort. ex Steud. cv. Orange Schwabenland, bellflower *Campanula* sp. cv. Rose,‡ cornflower *Centaurea cyanus* L. cv. Blue Boy,† larkspur *Delphinium ajacis* L. cv. Dark Blue supreme,† delphinium *Delphinium elatum* L. cv. Summer Skies,† storksbill *Erodium cicutarium* (L.) L-Her.,§ poinsettia *Euphorbia pulcherrima* Willd. ex. Klotzsch cv. Pink Stoplight and cv. Stoplight, *Fuchsia hybrida* Voss cv. Black Knight, cv. Black Prince, cv. Fanfare, cv. Little Beauty, *Hydrangea macrophylla* (Thunb.) Ser. cv. Merrivale, *Impatiens aurantiaca* Teysm. ex Koord. cv. Tangerine‡ morning glory *Ipomoea purpurea* (L.) Roth cv. Heavenly Blue,† geranium *Pelargonium hortorum* Bailey cv. Crimson Carefree† *Petunia hybrida* Vilm cv. Maytime,† azalea *Rhododendron* cv. Dorothy Gish, cv. Gloria, cv. Skylark, (unnamed seedling) swamp rose *Rosa caroliniana* Michx.,‖ *Rosa* cv. Forever Yours, cv. Oklahoma, cv. Pink Van Fleet, *Salvia patens* Cav.,‡ periwinkle *Vinca minor* L., pansy *Viola tricolor* L. cv. Delft Blue.†

† George J. Ball, Inc., West Chicago, Il 60185, U.S.A.

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