ON THE BEHAVIOUR OF "ANTHOCYANINS" AT VARYING HYDROGEN-ION CONCENTRATIONS.

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(*From the laboratory of the Royal Horticultural Society, Wisley Gardens.*)

(With One Text-figure, Three Tables and One Coloured Plate.)

One is given to understand in botanical literature that "anthocyanins" are red in acid, and blue in neutral or alkaline media, but on testing, in a very rough and ready way, the petals of several blue or red flowers at different hydrogen-ion concentrations, we were surprised to find that although for the most part the anthocyanins would show red in acid, and blue in neutral or slightly alkaline media, yet in many instances they did not appear to turn blue at all on passing from acid to alkali.

After some preliminary tests the following method of dealing with the matter was adopted and carried out systematically.

Buffered water at varying hydrogen-ion concentrations was obtained from the British Drug Houses, and found to work very satisfactorily, there being no appreciable change in the *p*H values after addition of the petals, undiluted extracts from which usually have an acid reaction *in vitro* of about *p*H 4·5 to 5. The reaction *in vivo* is not necessarily the same as *in vitro*, since osmotic and adsorption factors, about which little is known, may have some influence. Pearsall[1] for instance distinguishes *in vivo* between the xylem *p*H 4–5, and the phloem, *ca.* *p*H 7. *In vitro* the two would be inextricably mingled.

Two grams of the petals to be tested were pounded in a mortar with one gram of pumice; the pulp then divided into nine equal parts, and the portions dropped one by one into nine tubes, each containing 10 c.c. of the buffered water from *p*H 3 to 11. In addition, a small piece of petal, not mashed, was put into each of nine small tubes, and 1 c.c. of the required *p*H pipetted in. The small pieces of entire petals served very well as controls, but are not again referred to in the text.

The 10 c.c. tubes were then well shaken, and observations taken as soon as the larger particles had subsided. It is necessary to make a set

[1] "H-ion concentration and growth," *Science Progress*, 1925, xx, 58 with references.

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of observations as early as possible, as in most cases the colours soon fade and in an hour or two the issues may be obscured on that account.

Full tables of all the flowers tested are given at the end of the article, and on consulting them it will be seen that the blue flowers, as a rule, run the course red to pink and violet to blue at neutral. As the medium becomes alkaline at pH 8 the blue takes on a greenish tinge, showing green to yellowish green at pH 10 and 11. Row I on Plate VIII gives a typical instance. The green colour usually fades very rapidly, and in a few hours has disappeared, leaving the fluid a more or less deep yellow. The yellow tint in the alkaline media appears to be due to the presence of flavones, for if a white flower is treated in the same way, it is found that the fluid at pH 3, 4, 5 remains white, but at about pH 6 or 7 a pale yellow tint appears, deepening to orange yellow at pH 10 and 11. Such whites are those which turn yellow in ammonia vapour, and are known as ivory whites.

It seems certain that the green, commencing usually about pH 8, is due to the effect of the yellow upon the blue, and that, in the absence of flavones, the blue would not change to green. Four examples of white flowers are given at the end of the table, and of them, one—*Iris Kaempferi*—appears to be a nearly pure white, as there is only a slight change to yellow at the alkaline end of the series. The "anthocyanin" of the purple *Iris Kaempferi* shows blue at pH 8 and 9, and only turns to a blue-green at pH 10 and 11, as is shown in Row VI of Pl. VIII. The blue in this case is not affected by the slight yellow tinge at pH 8 and 9, and only partially so at pH 10 and 11.

Turning now to the group of clear red flowers, their behaviour is found to be very different. At pH 3 the colour is vermilion, of a tint very distinct from the lake red of the blues. From the vermilion the changes are through pale vermilion and pink to orange or brownish red at pH 7 and 8, and a deeper brownish purple at pH 9, 10, 11, between which three there is no appreciable difference. The appearance of the brownish tinge at about pH 8 or 9 to 11 is probably due to the yellow of the flavones, but there is no blue or green at any stage. On Pl. VIII, Row II gives a typical instance. It is evident, therefore, that there are two very distinct groups of anthocyanins which can be differentiated by their behaviour in media of varying hydrogen-ion concentration.

Besides these two main groups of flowers, a third, intermediate in character, may be distinguished, with flowers ranging from purple to magenta or pink—a colour series which appears to be due to a mixture of blue and red. The most salient feature of this group is that, after

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reaching blue or violet at about pH 7, the colours pass through a purple or brownish stage at pH 8 and 9 before reaching the green at pH 10 and 11. It appears that there is sufficient red to retard the appearance of the green, but not enough to suppress it altogether as in the clear reds.

Two sub-groups can be recognised:

A. Purple with blue predominant, the members of which are blue at pH 7.

B. Magenta or pink with red predominant, which do not show any clear blue but are violet at pH 7.

A third sub-group C, however, must also be distinguished in which the blue appears at pH 9, and is followed immediately by green. This peculiarity requires some further consideration, necessitating a short digression.

In a previous article (*R.H.S. Journal*, January 1929), it was shown that when dried petals of coloured or white flowers are macerated in neutral water ·5 grm. to 100 c.c. and filtered clear, the fluid in two or three days becomes cloudy; the cloudiness increasing, and followed by complete precipitation (of proteins) in the course of a month or so, leaving the fluid quite clear again. During this time there are very marked changes in the pH of the extract; at first, as the cloudiness appears, a decided increase in the acidity to pH 5–5·5 from the initial pH 6·2–6·7 (averaging about 6·4), usually followed by a decrease to about pH 7 or 8, but in some cases the acidity is persistent, remaining throughout in the neighbourhood of pH 5. A generalised graph, Fig. 1, is given below in order to illustrate the point.

The graph brings out the rather remarkable fact that of the magentas or pinks it is precisely the flowers belonging to the acid group which constitute sub-group C, and turn to blue at pH 9. The corresponding clear reds, *e.g.* of Pelargonium and Rhododendron, do not show any difference from the colours of the neutral group. It may here be remarked that varying colour shades, including whites, of the same species, or even genus, never showed appreciable differences in the reactions of their extracts.

Of the different families *Lathyrus* (? in graph) has not been tested in this way, but conforms to the neutral group in respect of its colour series. *Rosa* (? in graph), according to its pH reactions, was also originally grouped with the acids, but some recent tests indicate that this was an error, and that it should more properly belong to the neutral group. Its colour reactions seem to confirm this later view. Sub-group B will

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have to be tested more fully, but Row III of Plate VIII is probably fairly representative of the intermediate magentas and pinks.

There are also certain differences to be observed among the groups themselves in regard to shade of colour, but more particularly in the degree of fading. Except for those at *p*H 3, which never fade to an appreciable extent, the colours of most petals from *p*H 4 onwards may all fade rapidly, so that in 24 hours there is none left except the yellow of

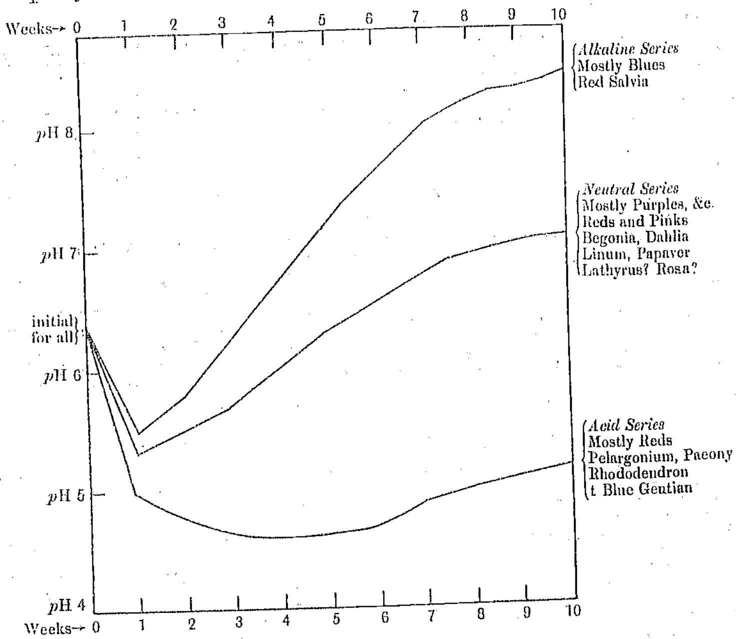

Fig. 1.

the flavones. In some there is little or no appreciable fading except for the greens, which in 24 hours have almost always entirely disappeared.

The greens and *p*H 3 are not taken into account in the accompanying list, but for the pinks, violets, blues and purples it gives a rough idea of the grouping according to their relative stability. It may here be noted that the initial colours at *p*H 4 and 5, for some unaccountable reason, are almost invariably paler than at other *p*H values, and this is true also, but less frequently, at *p*H 6. The purples are listed with the blues, and the magentas with the reds.

Degrees of fading—little in 24 hours.

Blues: *Iris Kaempferi*, *Iris sibirica*, Primrose, *Viola*.

Reds: *Papaver*, Primrose, *Tropaeolum*.

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Degrees of fading—complete or almost complete in 24 hours.

Blues: *Aconitum, Anchusa, Campanula, Delphinium, Digitalis*, Gentian, *Lathyrus, Linum*, Lupin, *Salvia.*

Reds: *Begonia, Dahlia*, Paeony, *Pelargonium, Rhododendron*, Rose, *Salvia.*

Since the fading takes place so irregularly, it would seem to be due to some inherent differences in sub-groups of the anthocyanins. Willstäter considers that the fading is caused by change to a colourless isomere and occurs *in vitro* among the blues but not among the reds, *i.e.* according to him in alkaline but not in acid media; but since there are some blues which do not fade except when they occur as greens towards the alkaline end, and some reds which soon fade, this rule cannot be said to have a universal application unless the acid or alkali is relatively strong[1].

SUPPLEMENTARY REMARKS.

1. On Pl. VIII, besides the three typical examples and *Iris Kaempferi* which have already been referred to, two series of *Salvia* are given to show the remarkable differences between them. It seems clear that in the blue *Salvia patens* the red anthocyanin, and in the red *Salvia splendens* the blue one, has been entirely eliminated. On the other hand, a lavender-coloured *Salvia* (*S. farinacea*) ranks with the intermediates, though it is not altogether typical, and probably contains both blue and red elements. The blue *Salvia* is of itself remarkable in that the blue colour extends over such a wide range, pH 4–8, a quite unique experience, since in no other case has blue been observed over more than three pH integers, and usually one or two only.

2. The red *Linum* and red Primrose belong to sub-group B of the intermediates. They are neither of them clear red, although they could hardly be called magenta or pink, except for the paler shades of the Primrose, and have the peculiarity of turning purple on pounding to a pulp, or on drying out—indicating that the cell contents may be more acid than the sap in the vessels external to the cells. Selective permeability of the cell membrane may possibly account for this phenomenon.

3. There are certain flowers classed among the blues, *e.g. Clematis, Iris Kaempferi, Lathyrus odoratus*, which would be considered purple rather than blue, and be expected to rank with the intermediates. In these instances the purple is perhaps due to some deviation *in vivo* from

[1] Willstäter: numerous articles in German chemical journals, and extensively quoted by Onslow in *The Anthocyanin Pigments of Plants*, 2nd edition, 1925.

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the usual reaction of the sap, and not to admixture of red. If this is the case, one could not expect to get a really blue *Lathyrus* for example, and the so-called blues are probably diluted purple. The slight differences noted in the table between the purple and blue *Lathyrus* are probably due to the more diluted colour of the latter.

Genetically the bicolor purple wild pea has been shown to contain both blue and red pigment, but the garden self-purple tested by us shows no evidence of red, at least as judged by the standard suggested here.

4. Among the intermediates one would not expect to find *Anchusa*, but if the sap is of the right *p*H for blue, the influence of the red in the flower itself might be masked, although, *in vitro*, the purple at *p*H 8 and 9 seems to indicate its presence, and it is significant also that the flowers are a purple-red at an early stage and only appear to be a pure blue when fully open, as with the Borage family in general. The blue Lupin also affords evidence of the presence of red, and one can surmise that the recently introduced red strains are due to elimination of the blue together with intensification of the original red, but a red Lupin has not yet been tested.

5. At the end of the table a special class has been provided for aberrants. *Anagallis* (blue) is the only flower so far tested which cannot be placed among the regular groups. A red *Anagallis* should give interesting results, but has not yet been available. Perhaps the lavender *Salvia* and red *Linum* should have been classed as aberrant, since neither of them is quite typical of the group to which it has been assigned.

Although these experiments are not in any sense genetical of themselves, they seem to have some bearing on the subject, and to explain to some extent the nature of the colour factors, viewed from a different standpoint to that taken by Onslow (*op. cit.*). On our original assumption that all anthocyanins are red in acid and blue in neutral or slightly alkaline media, it has always seemed difficult to understand how colour mutations could arise, since one can hardly suppose that the actual reaction of the sap could be changed all at once, without affecting the general growth of the plant in any way[1]. But on realising that there are two distinct groups of anthocyanins, one of which is never blue at any *p*H value, the question of sap reaction does not arise.

[1] Lathouwers, for example, in *Campanula medium* (*Acad. Roy. des sciences de Belgique*, 1922, T. IV, fasc. 8), recognises two main colours, violet and red, ascribing to the violet a factor which renders the sap more alkaline than that of the red flower; but he offers no proof that this is actually the case.

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NOTE.

In Haas and Hill, *Chemistry of plant products*, Fourth ed. 1928, p. 336, we find: "The occurrence of a red, blue, or purple pigment, either dissolved in cell sap—the exact colour depending on the acid, alkaline or neutral reaction of the cell sap—(or in an amorphous or crystalline state as in Delphinium and many other plants) is generally ascribed to the presence of anthocyanins."

(In another paragraph, p. 345, they except the beet, and Chenopodiaceae generally, which give a purple reaction with acid and yellow with alkali.)

Again, on p. 345, "Solutions are turned red by acid and blue by alkali; owing to the almost universal contamination by flavonols, the crude aqueous extracts of anthocyanins usually give a green coloration with alkali, due to simultaneous production of blue and yellow."

Onslow (*op. cit.*), p. 50, also discusses the nature of the green coloration and gives references. Haas and Hill's views are largely taken from Willstätter and Onslow.

SUMMARY.

According to the reaction of coloured flowers in media of varying hydrogen-ion concentration, their anthocyanins can be differentiated into two main groups, which may conveniently be called the blue and the red.

The anthocyanin of the blue group is a lake red at *p*H 3, passing through pink and violet to blue at about *p*H 7, and to green at the alkaline end of the series; the green being due to influence on the blue of flavones, which are yellow in alkaline media.

That of the red group is a vermilion red at *p*H 3, and passes through varying shades of red and pink to brownish purples in the more alkaline media. There is no blue or green at any hydrogen-ion concentration, and the brownish tints are caused by admixture of the yellow flavones.

Flowers of intermediate shades of purple to magenta or pink contain for the most part both blue and red anthocyanins in varying proportions.

It need not therefore be assumed that the sap of the purple or red flower is necessarily more acid than that of the blue flower, although in some cases the blue pigment alone may be present, but appear violet or purple-red on account of the acidity of the sap.

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EXPLANATION OF PLATE VIII.

The number at the head of each column indicates the *p*H hydrogen-ion concentration.

Row I. Typical example of a clear blue flower; blue at about *p*H 7, changing to green at the alkaline end on account of the yellow flavones.

Row II. Typical example of a clear red flower; no blue or green, but with brownish tints towards the alkaline end, due to yellow flavones.

Row III. Typical example of an intermediate magenta or pink flower; both blue and red pigments are present.

Row IV. *Salvia patens*. Clear blue. Unusually wide range of blue.

Row V. *Salvia splendens*. Clear red. No blue or green.

Row VI. *Iris Kaempferi*. Purple blue. Blue at *p*H 8 and 9. Little flavone and therefore little green.

For the original of the coloured plate we are indebted to Mr A. J. Wise of the R.H.S., Wisley Gardens.

TABLE I

Blue flowers.

*p*H→	3	4	5	6	7	8	9	10	11
Aconitum	Pink	Pink-violet	Violet	Violet-blue	Blue	Green	Green	Green	Green
Aquilegia	Red	Violet-pink	Violet-blue	Blue	Blue	Green-blue	Blue-green	Green	Green
Campanula	Red	Violet-pink	Violet	Violet-blue	Blue	Green-blue	Green	Green	Green
Clematis	Pink	Pink	Violet	Violet	Blue	—	Green	Green	Green
Delphinium	Red	Violet	Blue	Blue	Blue	Green-blue	Green	Green	Green
Gentian (*acaulis*)	Pink	Violet	Violet-blue	Blue	Green-blue	Blue-green	Green	Green	Green
Gentian (*asclepiada*)	Pink	Violet	Violet-blue	Blue	Green-blue	Blue-green	Green	Green	Green
Iris Kaempferi	Red	Pink	Violet-pink	Violet	Violet-blue	Blue	Blue	Green-blue	Green-blue
Lathyrus (Purple)	Red	Pink	Violet	Violet	Blue	Blue	Green-blue	Blue-green	Green
Lathyrus (Blue)	Pink	Pink	Violet	Violet	Blue	Green-blue	Blue-green	Green	Green
Linum	Red	Pink	Pink-violet	Violet	Blue	Green-blue	Blue-green	Green	Green
Primrose	Red	Pink	Violet	Violet	Blue	Blue	Green-blue	Blue-green	Green
Salvia	Pink	Blue	Blue	Blue	Blue	Blue	Green-blue	Green	Green

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TABLE II. *Clear red flowers.*

pH→	3	4	5	6	7	8	9	10	11
Begonia	Verm.	Verm.-pink	Pink	Pink	Purple	—	Brown-purple	Purple	Purple
Dahlia	Verm.	Orange	Orange	Orange-red	Orange-red	Red	Red	Red	Red
Lathyrus	Verm.	Verm.-pink	Pink	Pink	Pink	Pink	Purple	Purple	Purple
Papaver (orientalis)	Verm.	Verm.-red	Pink-red	Red	Purple-red	Purple-red	Red-purple	Red-purple	Red-purple
Papaver (Rheas)	Verm.	Verm.	Pink	Pink	Orange-brown	Orange-brown	Red-purple	Red-purple	Red-purple
Rose	Verm.	Pink	Pink	Pink	Purple	Red	Red	Pink	Brown
Salvia	Verm.	Verm.-pink	Verm.-pink	Red	Purple-red	Purple-red	Purple	Purple	Purple
Tropaeolum	Verm.	Verm.	Red-verm.	Red	Purple-red	Purple-red	Purple-red	Purple-red	Purple
Acid *Pelargonium*	Verm.	Pink	Pink	Pink	Violet	Violet	Purple	Purple	Purple
Rhododendron	Verm.	Red	Pink	Violet	Violet	Purple-red	Purple-red	Purple-red	Brown

Note. The so-called Purples have in reality a brownish tinge, but not sufficient to be designated as Brown purple.

TABLE III. *Intermediate flowers.*

Sub-group A. More blue than red.

	3	4	5	6	7	8	9	10	11
Anchusa	Red	Pink	Violet	Blue	Blue	Purple	Purple	Blue-green	Green
Digitalis	Red	Pink	Pink	Violet-pink	Violet-blue	Pink	Brown-pink	Green	Green
Iris siberica	Red	Violet-pink	Pink-violet	Violet	Blue	Blue	Purple	Green	Green
Lupin	Red	Violet-pink	Violet	Violet	Blue	Blue-brown	Brown	Green-brown	Green
Salvia (farinosa)	Violet-pink	Brown-yellow	Yellow-brown	Violet-brown	Violet-brown	Violet	Blue-green	Green	Green
Viola	Red	Pink	Violet	Violet-blue	Blue	Purple-blue	Purple	Green	Green

Sub-group B. More red than blue.

	3	4	5	6	7	8	9	10	11
Dahlia	Red	Pink	Pink	Violet-red	Violet	Red	Red-brown	Green-brown	Brown-green
Linum	Verm.	Red-pink	Violet	Violet	Blue	Purple-red	Purple-red	Purple	Purple
Primrose	Red	Red	Pink	Violet	Violet	Violet	Purple	Purple	Green
Rose	Verm.	Pink	Pink	Violet-pink	Violet	Red	Purple-red	Green	Green

Sub-group C. Acid.

	3	4	5	6	7	8	9	10	11
Paeony	Red	Pink	Pink	Pink	Violet	Violet	Blue	Green-blue	Green
Pelargonium	Verm.	Pink	Pink	Violet	Violet	Violet	Blue	Green-blue	Green
Rhododendron	Red	Pink	Violet-pink	Violet-pink	Violet	Violet-blue	Blue	Green-blue	Green

White flowers.

	3	4	5	6	7	8	9	10	11
Dahlia	White	White	White	Yellow, pale	Yellow, pale	Yellow, deeper	Yellow, deep	Yellow, deep	Yellow, deep
Iris Kaempferi	White	White	White	White	White	White	Yellow, slight	Yellow, slight	Yellow, slight
Rhododendron	White	White	White	Yellow, slight	Yellow, slight	Yellow, pale	Yellow, deep	Yellow, deep	Yellow, deep
Rose	White	White	White	Yellow, slight	Yellow, slight	Yellow, pale	Yellow, deep	Yellow, deep	Yellow, deep

Aberrant.

	3	4	5	6	7	8	9	10	11
Anagallis (Blue)	Red	Violet	Blue	Purple	Green	Green	Purple	Purple	Purple

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