XXVI. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. III. THE RED AND MAGENTA PIGMENTS.

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An account of the Mendelian factors for flower colour in *Antirrhinum*, of the preparation of the pigment and of the analyses of the yellow pigments has been given in previous communications [Wheldale, 1913; Wheldale and Bassett, 1913, 1914].

Of the yellow pigments in *Antirrhinum*, only two occur in quantity and are concerned in the inheritance of colour. One is present in the so-called ivory variety and has been identified with the flavone, apigenin, $C_{15}H_{10}O_5$; the other is present (in addition to apigenin) in the yellow variety and has been identified with the flavone, luteolin, $C_{15}H_{10}O_6$. The ivory variety is dominant to the yellow and contains a factor "I" which is absent from yellow and may be expressed as the power to inhibit the formation of luteolin. The true white or albino variety does not contain either apigenin or luteolin.

The remaining varieties contain anthocyanin in addition to flavones and may be enumerated as follows:

1. Yellow tinged with bronze.	5. Yellow tinged with crimson.
2. Ivory tinged with rose doré.	6. Ivory tinged with magenta.
3. Bronze.	7. Crimson.
4. Rose doré.	8. Magenta.

It has hitherto seemed probable that, in addition to flavones, one anthocyanin pigment only—red—is responsible for varieties 1–4, rose doré being coloured by red anthocyanin and apigenin, and bronze by red anthocyanin and luteolin. This assumption has now been confirmed and also the similar view that varieties 5–8 contain only magenta anthocyanin; crimson being coloured by magenta anthocyanin and luteolin, and magenta by the same anthocyanin and apigenin.

The purification of red and magenta anthocyanins was carried out as follows:—The crude pigment (obtained by hydrolysis of the glucosides from flowers containing, of course, flavone in addition to anthocyanin) is finely powdered and extracted with absolute alcohol and in this way is purified from a considerable amount of brownish insoluble substance which is probably a decomposition product produced by boiling with sulphuric acid during the hydrolysis of the glucoside. The alcohol extract is taken to dryness again, finely ground, and extracted in a Soxhlet thimble suspended immediately above boiling ether. After some months most of the flavone pigments are removed by the ether, the process being continued with fresh quantities of ether till the latter remains practically colourless after boiling for a few days. The residue consisting almost entirely of anthocyanin, is then taken up in the minimum amount of absolute alcohol and a large volume of ether added which precipitates the anthocyanin, any flavone present remaining in solution. The precipitated anthocyanin is filtered off, dried, and again extracted with ether to remove traces of flavone.

Red anthocyanin thus prepared is an Indian red powder. We have not yet been able to obtain anthocyanin as a satisfactory crystalline product. It does not melt even when heated to 350° . It is readily soluble in absolute alcohol and pyridine, slightly soluble in dilute acids, ethyl acetate and acetone, almost insoluble in water and insoluble in ether, chloroform and benzene. In concentrated sulphuric acid it forms a reddish solution with a slight green fluorescence. It is soluble in alkalis to a green solution. With ferric chloride solution it gives a green colouration changing to brown. Lead acetate forms a brownish yellow precipitate, but barium, silver, cadmium and mercury do not form precipitates.

Pure magenta anthocyanin is a magenta red powder with similar properties and solubilities to the red. In concentrated sulphuric acid it gives a red solution with a slight greenish fluorescence. It is soluble to a green solution in alkalis. With ferric chloride solution it gives a green colouration changing to blue. With lead acetate it forms a dull green precipitate of a lead salt. In alkaline solutions it is strongly fluorescent, green by transmitted, red by reflected light.

The combustion results for pure red anthocyanin derived from different varieties were:

			C	H	O (by difference)
From rose doré	...	...	51.93 %	5.02 %	43.05 %
From bronze	...	...	51.37	5.05	43.58
“ “	...	...	52.12	4.97	42.91
		Mean	51.81	5.01	43.18

Of magenta anthocyanin from different varieties :

From magenta	...	...	50.26 %	4.89 %	44.85 %
From ivory tinged with magenta			50.68	5.54	43.78
From crimson	...	...	50.56	4.90	44.54
		Mean	50.50	5.11	44.39

The corresponding numbers for the yellow pigments are as follows :

Apigenin	...	...	66.66 %	3.70 %	29.64 %
Luteolin...	...	...	62.90	3.49	33.61

The anthocyanins were always obtained in amorphous form, but although we were unable to get a crystalline product, the combustion results, combined with the failure to effect any separation even though a large number of solvents were used, made it appear certain that the pigments were single substances and not constant mixtures, a view confirmed by the fact that the same substance could be obtained from different varieties.

This note is suggested by the following observations. In working with the yellow pigments we, for a long time, obtained a mixture of apigenin and luteolin of constant melting point and great difficulty was experienced in separating the constituents, but in this case the mixture gave very variable combustion results, quite in contrast with those of the anthocyanins quoted above.

Attempts were made to determine the molecular weight by depression of freezing point, using phenol as a solvent, but the results, though consistent in a series of experiments with anthocyanin from different varieties, were obviously far too low. In the case of magenta, the average value was 150, of red 380. Since the value for magenta is clearly too small, it is probable that the red is also below the correct value. The remarkable agreement between the results in the series of determinations carried out suggests however that the numbers obtained bear a simple relation to the molecular weights and that the low values are to be explained by some type of dissociation in the phenol.

Acetic acid and various other solvents did not dissolve sufficient substance to give readable depressions.

Attempts to determine the molecular weight by elevation of boiling point in absolute alcohol gave mean values of 572 for red and 717 for magenta. The elevation of the boiling point, for the concentrations it was possible to use, was so slight that the possible experimental error might be very considerable. Added to this, there appeared to be some change in the anthocyanin during the experiment and towards the end varying amounts were precipitated.

The combustion results give as simplest formulae, $C_8H_5O_5$ for the red and $C_{15}H_{18}O_{10}$ for the magenta. The boiling point determination of the molecular weight would appear to indicate that the molecule is $3(C_8H_5O_5)$, i.e. $C_{24}H_{27}O_{15}$ which has a molecular weight of 555 for the red, and $2(C_{15}H_{18}O_{10})$, i.e. $C_{30}H_{36}O_{20}$ which has a molecular weight of 716 for the magenta.

Lead salts of both the anthocyanins were prepared by mixing alcoholic solutions of lead acetate and anthocyanin. The precipitates were washed with hot alcohol, dried at $130^\circ C.$ and the lead determined as $PbSO_4$.

In the case of red, 1 gram-atomic weight of lead was found to be equivalent to 224 g. of anthocyanin. Taking the molecular weight of the anthocyanin to be 555, this would indicate that 5 atoms of lead combine with 2 molecules of the pigment and the molecular weight of red anthocyanin calculated in this way from the lead equivalent would be 560.

In the case of magenta, 1 gram-atomic weight of lead was found to be equivalent to 204 g. of anthocyanin. Taking the molecular weight of magenta anthocyanin to be 716, this would indicate that 7 atoms of lead combine with 2 molecules of the pigment and the molecular weight of magenta anthocyanin calculated from the lead equivalent would be 714.

An attempt was made to estimate the number of hydroxyl groups present in the anthocyanin molecule by means of Zerewitinoff's [1907] modification of Hibbert and Sudborough's method. This consists in dissolving the substance in thoroughly dried pyridine, treating it in a suitable apparatus with a considerable excess of methyl magnesium iodide dissolved in dry amyl ether, and collecting the gas evolved. Each hydroxyl group causes the evolution of a molecule of methane. It should be noted that "hydroxyl groups" as determined by this method include those forming part of carboxyl groups, and also such ketone groups as can give rise to hydroxyl by a tautomeric change.

The values obtained indicate that the red anthocyanin, assuming the formula to be $C_{24}H_{27}O_{15}$, contains twelve hydroxyl groups as defined above, while the magenta, taking the formula as $C_{30}H_{36}O_{20}$ contains fifteen hydroxyl groups.

It will be seen that in the formation of the lead salts, a number of the hydroxyl groups do not react.

As far as our researches are concerned, it still remains to be shown that anthocyanin is a flavone derivative. The order of magnitude indicated for the molecular weights suggests that the anthocyanins of *Antirrhinum*, if flavone derivatives, are considerably more complex than flavones and may be formed by condensation of two molecules accompanied by oxidation or by condensation of a flavone with phenols or hydroxybenzoic acids.

In a recent paper, Willstätter [1913] gives some account of the extraction and analyses of pigments (and their derivatives) from the Corn-flower. Three pigments are identified; a purple pigment which he considers to be a flavone derivative and which is an acid; a blue pigment, the potassium salt of the purple, and a red pigment which is a salt, of the oxonium type, of the anthocyanin with an acid present in the plant.

The relation between the magenta and red pigments of *Antirrhinum* cannot be of the nature of that suggested by Willstätter for the pigments of the Corn-flower. It seems that the anthocyanins of the two genera differ considerably from each other. For instance Willstätter's anthocyanins form oxonium salts with mineral acids with great ease, whereas we have entirely failed to obtain such salts by any method. The very dissimilar range of colour varieties thrown off in the two cases also lends support to the view that the anthocyanins concerned are different in character.

From the standpoint of genetics, the chief items of interest contained in the present paper are the following:

1. There are only two kinds of anthocyanin in *Antirrhinum*, red and magenta. Mixed with ivory pigment (apigenin) the colours of red and magenta are unaffected, but a mixture with yellow pigment (luteolin) gives two other colours, bronze and crimson respectively. Both red and magenta anthocyanin may also occur in varying amounts giving rise to tinged, pale and deep varieties.

2. Both red and magenta anthocyanin contain a considerably higher percentage of oxygen than the flavones, and of the two, the percentage in magenta is the higher. Hence if the anthocyanins are derived from flavones, the process must be in part one of oxidation.

3. There is evidence also that the anthocyanin molecules are larger than the flavone molecule. Hence, again, if a flavone constitutes the chromogen, condensation must take place, either of two flavone molecules, or of a flavone with one or more molecules of an aromatic acid or phenol, etc.

We were unfortunately obliged to discontinue work at this point owing to our supply of pure material being exhausted, but we hope to carry out further investigations and to try to elucidate the constitution of anthocyanins.

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