LXXXV. NATURAL ANTHOCYANIN PIGMENTS.

I. THE MAGENTA FLOWER PIGMENT OF *ANTIRRHINUM MAJUS*.

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FROM her study of the Mendelian factors for flower colour in *Antirrhinum majus*, Wheldale [1907, 1914] showed that, on crossing white with yellow or ivory varieties, plants bearing red or magenta flowers are produced. This led her to suggest that the red and magenta anthocyanin pigments might be derived from the yellow and ivory flavones, and that the white varieties, from which these pigments are absent, might carry the factors which act on the flavones to form anthocyanin.

With a view to testing this hypothesis, Wheldale [1913] commenced an investigation of the chemistry of these four pigments. With Bassett [1913, 1914, 1] she isolated the yellow and ivory flavone pigments and identified them as glucosides of luteolin and apigenin. The yellow flowers contained glucosides of both these pigments, while apigenin alone was present in the ivory variety. From the red and magenta flowers they isolated two amorphous pigments of high molecular weight and uncertain constitution, which were apparently uncombined with any acid [Wheldale and Bassett, 1914, 2].

At this time Willstätter and Everest [1913] published an account of the isolation of the blue anthocyanin from *Centaurea cyanus* (cornflower). In this classical paper, they revealed the constitution and true nature of the anthocyanins, showing that they could be isolated by means of their stable crystalline oxonium salts. Unfortunately, this communication, which revolutionised the methods employed in the extraction and isolation of these pigments, was published too late to be of any use to Wheldale and Bassett, and the question of the relationship between these co-existing flavones and anthocyanins remained unanswered.

More recent research has still left the question open [Onslow, 1925] as to whether anthocyanins are derived directly from flavones or not: it is possible for instance that they are formed independently from the same parent substances.

With a view to throwing more light upon the interrelationships between flavones and anthocyanins in the same varieties, a further attempt has been made to isolate and identify the red and magenta pigments of *Antirrhinum majus*. The pure crystalline chloride of the magenta pigment *antirrhinin* has been isolated by a process based on the methods of Willstätter and his co-workers. The preparation of the red pigment is to be attempted next.

If there were any evidence for the direct origin of anthocyanins from

flavones in the plant, the genetical relationships would point to apigenin as the parent substance of this magenta pigment. On crossing an ivory variety with a white of certain origin, a magenta offspring is produced. In this case the flavone apigenin is the only pigment present in either parent, and only this flavone and magenta anthocyanin are to be found in the offspring. If Wheldale's original hypothesis had been correct, some simple relationship should be apparent between the structures of these two pigments. Wheldale had originally suggested that the reaction occurring on the formation of anthocyanins would be one of oxidation. After the elucidation of the constitution of the anthocyanins, and the production by reduction, *in vitro*, of the anthocyanidin cyanidin from the flavone quercetin [Willstätter and Mallison, 1914], Everest [1918] supported the view that the process *in vivo* was one of reduction. If this second theory is correct, the magenta anthocyanin should prove to be apigeninidin, a pigment which has not yet been found to occur naturally.

The analysis of antirrhinin chloride, however, has shown it to be a 3-rhamnoglucoside of cyanidin chloride, which is closely related, structurally, to the flavonol quercetin and not to apigenin.

Since cyanidin contains one more molecule of oxygen than apigenin, the connection between these two pigments cannot be one of reduction, nor can one explain their relationship by any simple oxidative process. Wheldale and Bassett did not identify the sugar residues attached to the flavones of *Antirrhinum*, so that it is not yet known whether there is any correlation between the carbohydrate portions of these co-existing pigments.

The determination of the identity of antirrhinin therefore throws no further light upon the nature of a relationship, either of oxidation or reduction, between flavones and anthocyanins, which has been indicated by the study of the Mendelian factors.

In only four other cases have the constitutions of the co-existing flavones and anthocyanins been determined.

The red rose contains cyanin [Willstätter and Nolan, 1914], together with the corresponding flavone quercetin [Karrer and Schwarz, 1928], while the author (unpublished work) has recently identified a similar anthocyanin in

brown *Cheiranthus cheiri* (wallflower), from which quercetin and its mono-methyl ether, isorhamnetin, have already been isolated [Perkin and Hummel, 1896]. Thus in these two flowers the constitution of the pigments favours the theory of their interconversion *in vivo*.

The significance of the results in the case of *Viola tricolor* is at present not very clear, since Everest [1919] claims that myricetin is present in this flower together with the quercetin and delphinidin identified by Mandelin, and later by Perkin [1902] and by Willstätter and Weil [1916]. His claim, however, is not based on analytical results.

The flavone kaempferol [Perkin and Wilkinson, 1902] and the anthocyanin delphinin [Willstätter and Mieg, 1914] have been isolated from *Delphinium consolida*: thus here, as in *Antirrhinum*, no close relationship is apparent.

The information derived from the study of the constitution of these flavones and anthocyanins is therefore conflicting. There is no conclusive evidence that the plant produces the latter from the former, or that they are both formed by separate but parallel syntheses from the same parent substance.

The flowers used for this investigation were those of the deep crimson varieties, *Purple King* (Carter), *Purple Queen* (Ryder), and *Giant Crimson* and *Dwarf Crimson* (Sutton). They all presumably contained the magenta anthocyanin together with luteolin and apigenin. The plants, which were grown on a plot of land kindly lent to me by Miss E. R. Sanders of Newnham College, were planted and tended by the staff of the Cambridge Botanic Gardens.

The isolation of the pigment was carried out by a modification of Willstätter and Zollinger's [1916] method for the preparation of keracyanin chloride from the cherry. Dilute methyl alcoholic hydrochloric acid was used for the first extraction, since glacial acetic acid extracts contained large amounts of flavone which formed a gel, and made filtration very difficult. Owing to the flavone present even in the alcoholic extracts, great difficulty was experienced at first in obtaining the chloride of the pigment in a pure enough state for crystallisation, but this was at length achieved.

Purification of the pigment by means of the crystalline picrate was not possible in this case, owing to the extreme solubility of this salt. Use was therefore made of the insoluble lead salt. This was precipitated from the crude alcoholic extracts, and then dissolved in glacial acetic acid, in which the lead salts of flavones and other contaminating substances are not soluble.

The yield of pure pigment was small in proportion to the large amount of material used, but, as flowers were not lacking, it was found to be more economical in labour and reagents to start with large quantities, and subsequently to discard mother-liquors which still retained appreciable amounts of pigment.

The pure chloride of antirrhinin was finally isolated in four hydrated forms. These differed in colour and water of crystallisation, and each of them possessed a characteristic crystalline form.

The chloride of the sugar-free pigment was identified as cyanidin

chloride by means of its appearance, general properties and reactions, combustion analyses and products of alkali fission. In all these respects, perfect agreement was found with the account of this pigment given by Willstätter and Everest [1913].

It has also been possible to compare the pigment from *Antirrhinum* with a sample of pure synthetic cyanidin chloride which was very kindly sent to me by Prof. R. Robinson. They showed a close resemblance both in their absorption spectra and behaviour in a range of buffered solutions. This latter method, which has been introduced by Robertson and Robinson [1929], is an excellent and reliable means of comparing and characterising anthocyanins and anthocyanidins.

The identification of the sugar residue as a combination of a molecule each of rhamnose and glucose was determined by means of the distribution number, and qualitative and quantitative analyses of the sugars produced on hydrolysis.

The position of the sugar residue at position 3 in the antirrhinin molecule is indicated by the violet colour reaction given with sodium carbonate. If the substitution were at 5 or 7, the colour reaction would be either pure blue or violet blue, according to whether the hydroxyls at 7, 3, 3' and 4', or at 5, 3, 3' and 4', were unsubstituted. This decision is strengthened by the fact that, after hydrolysis of the glucoside, one obtains a pure blue reaction with the resulting cyanidin chloride from which the rhamno-glucosidal residue at position 3 has been removed. The validity of these colour reactions as a test for the position of the sugar residue has been studied by Robinson and his co-workers during their extensive syntheses, first of pyrylium compounds [Pratt and Robinson, 1925], and later of substituted anthocyanidins, and even of anthocyanins themselves [Robertson and Robinson, 1927, 1928].

Keracyanin, the pigment of the fruit of the cherry, not only has a similar constitution to that of antirrhinin, but appears from its reactions to be identical. Keracyanin chloride was isolated by Willstätter and Zollinger [1916] who describe two hydrates which resemble the red and brown hydrates of antirrhinin chloride.

The pigment isolated by Karrer and Widmer [1927] from the fruit of *Sambucus niger* (elderberry), and named by them sambucin, is also a rhamnoglucoside of cyanidin, probably together with traces of some other pigment. Many of the reactions of sambucin chloride resemble those of antirrhinin chloride, but no mention is made of the existence of different hydrated forms, and the crystalline picrate, which they isolated without any difficulty, must have been much less soluble than that of antirrhinin.

Antirrhinin chloride may be represented by the following formula:

EXPERIMENTAL.

The extraction and purification of the pigment.

The pigment was extracted with 2 % methyl alcoholic hydrochloric acid (8 litres) from about 10 kg. of fresh crimson flowers, from each of which both calyx and pistil had been removed. After standing for 2 days, the first extract was separated off, and the residue squeezed and re-extracted with a further 7 litres of acid-alcohol. Even after these two extractions some pigment was still retained.

A saturated aqueous solution of lead acetate was slowly added to the filtered extract until there was an excess. Lead chloride precipitated first; the solution then turned from red to purple, green, and then dark blue; finally the pigment was precipitated, together with the flavones, as a dark blue lead salt.

The mixture was allowed to stand for 2 days, by which time most of the pigment had been precipitated. After decantation of the red-brown mother-liquor, the precipitate was filtered, washed with a small amount of methyl alcohol, and sucked as dry as possible. A certain amount of pigment remained in the mother-liquor. Addition of ethyl alcohol precipitated some of this, and more came down on standing, but it was not possible to recover it all.

The moist, dark blue precipitate from two litres of alcoholic extract was extracted with successive amounts of glacial acetic acid, about 300 cc. being used in all. The extraction was incomplete, even on frequent rubbing and stirring. This was probably due to the retention of the pigment by the lead chloride. After standing for some hours, the dark-red acetic acid extract was again filtered from further impurities which had settled out.

On the addition of two volumes of purified ether, the lead salt was precipitated in dark blue granules, which were filtered off, washed with ether, dried and ground. The filtrate, which was green in colour, retained much of the impurity.

The rest of the original lead precipitate was purified in the same manner, about 100 g. of nearly pure lead salt being obtained in all. In some cases the ether precipitated the salt as a gummy mass, which absorbed water from the air. It was partially dried by rubbing with absolute alcohol and filtering. This deliquescence was probably due to the impurities present. It was noted, later on, that precipitation of the impure chloride of the pigment with ether generally gave a dry powdery residue, which quickly became sticky, but as purification proceeded, the pigment obtained was much less deliquescent.

11.0 g. of the finely ground lead salt were dissolved in a minimum of 5-10 % hydrochloric acid in methyl alcohol, and the lead chloride was filtered off. (If left to stand, a gel was formed due to flavone impurity.) One-third the volume of propyl alcohol was next added and the solution left overnight; the impurities which came down were filtered off, and, on addition of purified ether, the pigment was recovered as a flocculent, scarlet precipitate. The purity of the bright red, gummy mass, obtained on filtering, was tested by hydrolysing

a small amount. The sugar-free pigment which separated was mostly amorphous, but a few crystals were found on examination under the microscope.

The glucoside was next dissolved in a few cc. of warm methyl alcohol, made 20 % acid with hydrochloric acid and left to stand overnight. The chloride of the pigment was precipitated as a dark brown amorphous, or microscopically crystalline, substance, and a sample now gave, on hydrolysis, homogeneous needle crystals of the non-glucosidal pigment.

The glucoside was filtered, washed quickly with 20 % methyl alcoholic acid and dried by suction. The yield was 1.9 g.

The mother-liquor still retained much of the pigment. Part of this was later isolated, but great difficulty was experienced, as the impurities still present kept the pigment from precipitating.

The glucoside was recrystallised twice by solution in warm methyl alcohol, and addition of one-fifth the volume of concentrated acid. On standing overnight, the precipitation of the pigment as golden brown crystals with a bronze reflex was almost complete.

1.4 g. of pure antirrhinin chloride were thus obtained, and this method of purification was repeated on the rest of the crude pigment.

The glucosidal pigment, antirrhinin chloride $C_{27}H_{31}O_{15}Cl$.

Antirrhinin chloride can be prepared in at least four different hydrated forms. The appearance, composition, method of preparation and characteristics are given in the accompanying table:

Hydrate	Appearance	Properties	Method of preparation
<i>Yellow hydrate</i> $C_{27}H_{31}O_{15}Cl, 1\frac{1}{2}H_2O$	Orange-yellow ppt. of fine, pale yellow, ill-defined needles. (See Fig. 1). Yellow powder when dry, turning brick-red after several weeks	V. insol. in cold water In hot water dissolves and isomerises instantaneously Barely soluble in cold or hot HCl solution (except in 3 %) Fairly soluble in cold alcohol, more soluble hot Insol. in neutral amyl alcohol, but more soluble when acidified	Precipitated from warm methyl alcohol solutions on standing. Precipitation not complete
<i>Fan hydrate</i> $C_{27}H_{31}O_{15}Cl, 2\frac{1}{2}H_2O$	Dark purple-brown ppt. of feathery clusters of crystals resembling Japanese fans. (See Fig. 2)	Soluble in cold and hot water, alcohol and aqueous hydrochloric acid	Precipitated from strong (20 %) aqueous HCl; never from acid-alcohol. Often contaminated by the brown hydrate
<i>Red hydrate</i> $C_{27}H_{31}O_{15}Cl, 3\frac{1}{2}H_2O$	Scarlet flocculent ppt. of fine needles. Brick coloured powder when dry. (See Fig. 3)	S. soluble in cold water. More soluble on heating Soluble in alcohol and in aqueous hydrochloric acid	Precipitated from aqueous 5 % HCl. A mixture of the red and brown hydrates separates from 10 % acid
<i>Brown hydrate</i> $C_{27}H_{31}O_{15}Cl, 4\frac{1}{2}H_2O$	Brown crystalline ppt. with bronze reflex, composed of pale grey-brown leaflets in clusters. (See Fig. 4) Marks paper with a purple streak	V. soluble in cold water and alcohol V. soluble in dil. HCl, but decreasingly sol. in higher concs. More soluble on heating	Precipitated from 10 % acid methyl alcoholic solutions on cooling and standing

Fig. 1. The yellow hydrate.

Fig. 2. The fan hydrate.

Fig. 3. The red hydrate.

Fig. 4. The brown hydrate.

The following are the chief reactions of antirrhinin chloride:

<i>Ferric chloride.</i>	Fairly stable, good blue in alcohol. Violet, and less stable, in water.
<i>Sodium acetate.</i>	Violet colour.
<i>Potassium acetate.</i>	Plum-coloured solution.
<i>Sodium carbonate.</i>	Blue-violet to red-violet.
<i>Sodium hydroxide.</i>	Sapphire blue to green-blue.
<i>Lead acetate.</i>	Blue precipitate.
<i>Perchloric acid.</i>	Dissolves on warming. On standing, pale red-brown crystals of perchlorate separate in sheaves.
<i>Picric acid.</i>	Very soluble, no crystalline picrate separates.
<i>7% sulphuric acid.</i>	Soluble on heating. On standing, clusters of fine orange needles separate.
<i>Fehling's solution.</i>	Reduced.

The violet reaction with sodium carbonate indicates that the sugar-residue is attached to the molecule at position 3.

A solution of the colourless *pseudo*-base is easily produced on adding the pigment to a large volume of hot water, and heating. If acid is added to this solution, it reddens again, even in the cold. The purple colour base is partially precipitated when a concentrated water solution of the glucoside is heated.

A Zeisel estimation showed the complete absence of methoxyl groups.

The colour reactions and stability of antirrhinin chloride in a series of buffered solutions were determined by Robertson and Robinson's method.

The 17 buffer solutions, starting from p_H 3.2 and each increasing by 0.6 p_H , were prepared by the addition of suitable amounts of 0.2 *N* sodium hydroxide (CO_2 -free) to 50 cc. of "Universal Buffer Solution" supplied by the British Drug Houses. The p_H values up to 11.0 were calculated by means of the formula supplied. Equal amounts (10 cc.) of each buffer were placed in a series of boiling-tubes, 1 cc. of an alcoholic solution of the pigment (25.0 mg. in 100 cc. 98 % alcohol) was added to each tube from a burette, and the tubes well shaken and examined, both at the moment of mixing, and at intervals during the next 48 hours. The results are given in the following table:

	p_H	Colour reaction and stability
10 % HCl		Salmon red
1 % HCl		Pink
I	3.2	Pink, fades quickly, but still pale pink after 3 days
II	3.8	Pink, fades less quickly
III	4.4	Pink, fades slowly
IV and V	5.0 and 5.6	Mauve pink, fades more slowly
VI	6.2	Mauve pink, more stable
VII	6.8	Brownish pink, fades slightly
VIII and IX	7.4 and 8.0	Brownish cherry, fades slightly
X to XII	8.6 to 9.8	Brownish cherry, very stable even after 16 hours
XIII and XIV	10.0 and 11.0	Bordeaux red, very stable even after 16 hours
XV		Cherry with blue tinge
XVI		Permanganate colour, slowly destroyed, pale brownish pink in 16½ hours
XVII		Deep violet, fades slowly
10 % NaOH solution		Pure blue, very unstable

The following figures were obtained for the loss in weight of the four hydrates, on removal of their water of crystallisation, by drying *in vacuo* over phosphorus pentoxide at 100° and 105°.

The 'yellow' hydrate

I. 77.8 mg. 'yellow' hydrate lost 1.8 mg. on drying at 100°	1.93 %
72.5 mg. 'yellow' hydrate (dried at 100°) lost 1.7 mg. on drying at 105°	2.35 %
Total loss in weight on drying at 105°	4.28 %
II. 179.1 mg. yellow hydrate lost 8.1 mg. on drying at 105°	4.52 %
Calculated for $C_{27}H_{31}O_{15}Cl, 1\frac{1}{2}H_2O$	4.12 %

The 'fan' hydrate

271.4 mg. 'fan' hydrate lost 17.7 mg. on drying at 105°	6.52 %
4.837 mg. 'fan' hydrate lost 0.320 mg. on drying at 105°	6.5 %
Calculated for $C_{27}H_{31}O_{15}Cl, 2\frac{1}{2}H_2O$	6.52 %

The 'red' hydrate

53.4 mg. 'red' hydrate lost 4.8 mg. on drying at 105°	9.0 %
34.0 mg. 'red' hydrate lost 3.0 mg. on drying at 105°	8.8 %
Calculated for $C_{27}H_{31}O_{15}Cl, 3\frac{1}{2}H_2O$	9.0 %

The 'brown' hydrate

0.0790 g. 'brown' hydrate lost 0.0074 g. on drying	9.36 %
Calculated for $C_{27}H_{31}O_{15}Cl, 4H_2O$	10.2 %

Micro-analyses of the substances after drying for carbon, hydrogen and chlorine gave the following figures:

4.821 mg. 'yellow' hydrate gave 8.960 mg. CO_2 and 2.18 mg. H_2O	
5.20 mg. 'yellow' hydrate gave 1.09 mg. AgCl	
4.517 mg. 'fan' hydrate gave 8.250 mg. CO_2 and 2.03 mg. H_2O	
6.963 mg. 'fan' hydrate gave 1.623 mg. AgCl	

The percentage compositions are therefore:

	C	H	Cl
'Yellow' hydrate	50.6	5.04	5.13
'Fan' hydrate	49.8	4.99	5.76
Calculated for $C_{27}H_{31}O_{15}Cl$ (cyanidin rhamnoglucoside)	51.39	4.92	5.62

The combustion analyses for the C and H content of antirrhinin chloride and the sugar-free pigment were carried out by Dr Ing. A. Schoeller of Berlin-Schmargendorf, and those for chlorine by A. Colwell of this laboratory.

The sugar-free pigment, cyanidin chloride, $C_{15}H_{11}O_6Cl$, H_2O .

The sugar-free pigment was obtained by hydrolysis of antirrhinin chloride with boiling 20 % hydrochloric acid for 4 minutes. After 2 minutes pale red-brown crystals began to separate in long needles (see Fig. 5). On cooling, filtering, washing with a little 20 % acid, and drying, a reddish chocolate-brown powder was obtained.

If dissolved in methyl alcohol, an equal volume of 10 % aqueous hydrochloric acid added, and left to evaporate, the pigment precipitates almost

Fig. 5. Cyanidin chloride from *Antirrhinum*.

quantitatively in well-formed crystals. These are not long and tapering as before, but are more sturdy and collect in stars and clusters.

When the pigment is dissolved in methyl alcohol, acid added and the alcohol boiled off, the salt reprecipitates in a curious symmetrical S-shaped form with curled offshoots.

The sugar-free pigment is slightly soluble in cold water, giving a mauve-red solution, which on heating turns purple, and then isomerises. It is difficultly soluble in aqueous hydrochloric acid of all strengths: on heating, it has a maximum solubility in 0.3 % acid, and in the cold in 0.03 % acid, a colour base being precipitated if this solution is heated. A hot saturated solution in 5.0 % acid does not contain much pigment, but on cooling even this is precipitated in fine needles.

The pigment is very soluble in methyl and ethyl alcohol, giving beautiful red-violet solutions which turn an intense cerise colour on acidification.

If water and not acid be added, a precipitate of the purple colour-base is immediately formed, and a green sheen appears on the sides of the tube. This behaviour is typical of cyanidin chloride.

Solutions in amyl alcohol are violet, and isomerise on standing. On acidification with an equal quantity of dilute acid, the alcohol layer turns crimson. On addition of alkali, the pigment passes into the water layer and turns blue. An acetone solution is violet.

Ethyl acetate dissolves the pigment to a small extent, giving a crimson solution on acidification, and a clear green-blue on addition of sodium hydroxide.

The chief reactions are:

<i>Ferric chloride.</i>	Blue colour in alcohol. Purple on addition of water. Unstable on addition of excess of reagent.
<i>Sodium carbonate.</i>	Violet and then pure blue.
<i>Sodium hydroxide.</i>	Clear green-blue, unstable.
<i>Sodium acetate.</i>	Red-purple solution in alcohol, purple in water.
<i>Potassium acetate.</i>	Dichroic red-blue in alcoholic solution. Colour base precipitated on standing.
<i>Lead acetate.</i>	Blue precipitate of the lead salt.
<i>Perchloric acid.</i>	Very soluble. No crystals of perchlorate are formed.
<i>Picric acid.</i>	Soluble on warming. Burr-like clusters of orange-red picrate crystals separate on standing.
<i>7 % sulphuric acid.</i>	Slightly soluble on heating. Brown hair-fine needles of sulphate separate on standing.
<i>Fehling's solution.</i>	Reduced slowly on heating.

It did not melt when heated up to 300°.

Acid amyl alcohol solutions gradually lose their colour on standing in the light. In a few days most of the pigment is destroyed.

A purple colour-base can be prepared by heating strong aqueous solutions of the pigment, from which it is quantitatively precipitated as an amorphous flocculent precipitate.

A solution of the colourless *pseudo*-base is obtained on adding an alcoholic solution of the pigment to a large volume of hot water. Addition of acid will not reproduce the red oxonium salt in the cold, but will do so on heating—a phenomenon peculiar to cyanidin chloride.

On fusion of the pigment with 50 % potassium hydroxide, small amounts of the breakdown products were isolated and found to be phloroglucinol and protocathechuic acid. The phloroglucinol was identified by means of its characteristic orange-red diazobenzene compound, the red colour given with a pine shaving and concentrated hydrochloric acid, the brick-red phloroglucide of methylfurfuraldehyde precipitated after heating with rhamnose and 20 % hydrochloric acid, and the darkening due to oxidation on addition of potassium hydroxide. The protocathechuic acid gave a green colour with ferric chloride followed by the very characteristic violet changing to violet-red on addition of weak sodium carbonate solution.

The crystalline chloride of the sugar-free pigment was dried at 105° *in vacuo* over phosphorus pentoxide, and eventually lost all its water of crystallisation. The last traces were very difficult to remove, and the whole amount was quickly absorbed again on standing in air. Combustion analyses were

therefore carried out on the crystalline and the dried pigment. The materials used in the third and fourth analyses were dried and undried samples of the same specimen. In the fourth analysis the agreement with the calculated values was better than that of the first three: a fact which points to the retention of a small proportion of water of crystallisation by the dried pigment.

On analysis, the following values were obtained:

- 0.1462 g. chloride dried at 105° lost 0.0082 g. = 5.58 %
 0.1784 g. chloride dried at 105° lost 0.0092 g. = 5.16 %
 $C_{15}H_{11}O_6Cl, H_2O$ dried at 105° would lose 5.28 %
 I (a). 4.854 mg. chloride dried at 105° gave 9.51 mg. CO_2 and 1.73 mg. H_2O
 I (b). 5.904 mg. chloride dried at 105° gave 2.596 mg. $AgCl$
 II. 4.009 mg. chloride dried at 105° gave 1.756 mg. $AgCl$
 III. 4.682 mg. chloride dried at 105° gave 9.36 mg. CO_2 and 1.64 mg. H_2O
 IV. 4.666 mg. chloride (air-dried) gave 8.995 mg. CO_2 and 1.70 mg. H_2O

The percentage compositions are therefore:

		C	H	Cl
By experiment.	Sample I	54.38	3.72	10.87
	Sample II	—	—	10.83
	Sample III	54.55	3.90	—
	Sample IV (air-dried)	52.57	4.05	—
Calculated for $C_{15}H_{11}O_6Cl$		55.81	3.42	11.00
Calculated for $C_{15}H_{11}O_6Cl, H_2O$		52.94	3.82	—

Fig. 6.

The sugar-free pigment was further identified as cyanidin chloride by means of its colour reactions, power of isomerisation and stability in the series of buffers. On comparing the pigment side by side with a sample of pure cyanidin chloride, the behaviour of these two pigments over the whole range was almost identical. Above p_H 8.6 the colour given by cyanidin chloride was slightly more violet, but this was only noticeable on very careful comparison. The reactions were similar to those described for cyanidin chloride by Robertson and Robinson.

The absorption spectrum of the chloride of the sugar-free *Antirrhinum* pigment was also compared with that of a synthetic sample of cyanidin chloride. From the readings taken with a spectrophotometer, the log. absorption curves were plotted over the visible spectrum (see Fig. 6), and were found to be very similar. The maximum absorption of the dark band in the green region is, in each case, at a wave-length of 550μ . Another band in the violet is also indicated. Acid (HCl) alcoholic solutions were used of such a strength that the maximum value found for the log. absorption lay between 1.75 and 2.0. Since the concentration of the synthetic cyanidin chloride solution was less than that of the *Antirrhinum* pigment, the curves, though parallel, were not identical.

The sugar residue.

The identification of the glucosidal residue of antirrhinin was carried out by means of the "distribution number," and by qualitative tests and quantitative estimations of the free reducing sugars and cyanidin chloride liberated on hydrolysis.

The "distribution number" for antirrhinin chloride was found by dissolving 0.005 g. of pigment in 25 cc. 5 % hydrochloric acid and shaking with two successive amounts of 25 cc. pyridine-free amyl alcohol. The amount of pigment extracted in each case was measured colorimetrically, by comparison with a standard made from 0.005 g. of pigment, dissolved in 1 cc. methyl alcohol, and made up to 25 cc. with amyl alcohol, a trace of acid being added to prevent isomerisation.

The mean value for the two extractions was 8.07, which compared well with the values for other rhamnoglucosides, which all lie between 6.5 and 10.

Qualitative sugar tests were carried out on the hydrolysis mixture, after filtering off the crystalline sugar-free pigment and removing any remaining traces of colour by extraction with amyl alcohol. A distinct smell of methylfurfuraldehyde could be detected during the hydrolysis. This is typical of rhamnoglucosides.

A portion of the filtrate was tested for pentose by the orcinol test. The solution turned pink (as for rhamnose) on boiling with orcinol and hydrochloric acid, and, on adding a drop of ferric chloride gave a green which was extracted by amyl alcohol. No blue precipitate (as for ordinary pentose) was obtained on boiling. The green colour was similar to that found with rhamnose, and was not of the vivid blue-green obtained with arabinose.

On hydrolysing for some time with strong hydrochloric acid, the distillate gave no pink with aniline acetate paper (therefore not normal pentose), but, on adding phloroglucinol, gave the characteristic yellow colour, and, on standing, a brick-red precipitate of the phloroglucide of methylfurfuraldehyde.

After neutralising the original filtrate with lead carbonate, filtering, and removing excess lead with hydrogen sulphide, the mixed osazone was prepared and compared with that from a mixture of pure rhamnose and glucose.

Quantitative estimations of the percentage of cyanidin chloride and reducing sugars obtained on hydrolysis of antirrhinin chloride were next carried out.

Weighed quantities of antirrhinin chloride (dried at 105°) were dissolved in 3 to 5 cc. of 1 % hydrochloric acid, and hydrolysed by boiling with 10 to 20 cc. 20 % acid for 2½ to 3 minutes. After standing in the ice-chest until the mother-liquor was barely pigmented, the crystalline cyanidin chloride was collected on a weighed Pregl glass filter, washed with a small amount of 20 % acid, dried *in vacuo* at 105° over P₂O₅, and weighed. One yield was air-dried, an allowance being made, in the calculation of the percentage, for the presence of one molecule of water.

After extraction of the remaining pigment with amyl alcohol, and subsequent removal of traces of the latter with pure ether, the filtrate was nearly neutralised with a small quantity of sodium hydroxide, and made up to a definite volume. The sugars were then estimated both polarimetrically and by Bertrand's method, it being assumed that rhamnose and glucose were present in equimolecular proportions. The polarimetric values obtained confirmed the identification of the hexose as glucose. Accurate polarimetric readings were taken by Mr B. Woolf of this laboratory, to whom I am much indebted.

The rhamnose content was estimated by the phloroglucide method [Ellett and Tollens, 1905], an aqueous solution of a weighed quantity of the glucoside being treated directly with 12 % aqueous hydrochloric acid.

I.	31.1 mg. antirrhinin chloride gave 16.80 mg. cyanidin chloride	
	31.1 mg. " "	13.59 mg. rhamnose + glucose (26.52 mg. Cu)
II.	187.7 mg. " "	104.20 mg. cyanidin chloride (air dried)
	187.7 mg. " "	82.30 mg. rhamnose + glucose (161.7 mg. Cu)
III.	490.2 mg. " "	262.50 mg. rhamnose + glucose (50 cc., $l=4$ dm., $\alpha_D = +0.65^\circ$)
IV.	135.8 mg. " "	65.02 mg. rhamnose + glucose (25 cc., $l=2$ dm., $\alpha_{H_2O} = +0.19^\circ$)
V.	231.4 mg. " "	62.40 mg. rhamnose (33.00 mg. phloroglucide)

The percentage yields are therefore:

	% cyanidin chloride	% combined sugars	% rhamnose
By experiment I	54.00	43.75	—
II	53.00	43.80	—
III	—	53.30	—
IV	—	47.80	—
V	—	—	27.00
Calculated for C ₂₇ H ₃₁ O ₁₅ Cl	51.11	54.57	28.86

An appreciable amount of sugar was broken down during the hydrolysis of the glucoside, the rhamnose being more easily destroyed than the glucose. The extent of this breakdown, which is shown in the low values obtained by both methods of analysis, is not so apparent in the polarimetric as in the copper reduction estimations, since, as the rotation of rhamnose (+ 9.0°) is small compared with that of glucose (+ 52.7°), a considerable loss of the pentose does not lower the combined rotation to a proportional extent. Willstätter found a similar discrepancy in the values obtained on estimating these sugars from keracyanin chloride and the flavone rutin by the same two methods.

SUMMARY.

1. The magenta anthocyanin, *antirrhinin*, has been isolated from the crimson flowers of *Antirrhinum majus* as a crystalline chloride.

2. Antirrhinin is shown to be a 3-rhamnoglucoside of cyanidin. It is probably identical with keracyanin, the pigment of the fruit of the cherry, but differs from sambucin from the elderberry.

3. The identification of antirrhinin as a cyanidin compound contradicts the theory of a simple relationship between this pigment and the ivory flavone apigenin, which is indicated by a study of the Mendelian factors for flower colour in *Antirrhinum*.

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