38. NATURAL ANTHOCYANIN PIGMENTS

3.¹ FLOWER PIGMENTS OF *VERBENA HYBRIDA*

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IN connexion with genetical studies of flower colour in *Verbena hybrida* [Beale, 1940] we have isolated the anthocyanins from flowers of two colour types, purple and maroon. The pigment of the purple is a delphinidin 3:5-diglycoside (I) and of the maroon a delphinidin 3-monoglycoside (II); the sugars are probably glucose:

Both pigments on acid hydrolysis gave a hexahydroxyflavylium chloride which proved to be delphinidin chloride. That obtained from purple *Verbena* was fused with caustic potash; the products were phloroglucinol and gallic acid.

Delphinidin 3:5-diglucoside (delphin) has been isolated from the bright blue flowers of *Salvia patens* by Reynolds *et al.* [1934]; it has also been synthesized by the same workers. The colours of the pure natural and synthetic products, in a range of buffer solutions from pH 3.2 to 14.0, were identical. The pigments of *S. patens* and purple *Verbena* similarly showed no significant divergence. This does not however establish the identity of their sugars. The anthocyanins most frequent in nature are glucosides, and since we obtained glucosazone from the sugars produced by acid hydrolysis of the purple *Verbena* pigment, this is probably delphinidin 3:5-diglucoside.

Delphinidin-3-glucoside has also been synthesized [Reynolds & Robinson, 1934], but apparently it has not yet been isolated in the pure condition from natural sources. However, the occurrence of a delphinidin 3-monoside mixed with other pigments has been noted in several plants, including *Viola tricolor* and *Vaccinium Myrtillus*. A delphinidin 3-monoside has also been isolated from Awobana paper by Kuroda [1933]. The pigment of the maroon *Verbena* gave the same colours over a range of pH as those described for synthetic delphinidin 3- β -glucoside by Reynolds & Robinson [1934]. The distribution number of the natural pigment to isoamyl alcohol was a little higher than that of the synthetic product, but this is probably not significant, as Reynolds & Robinson found a similar variation between the natural and synthetic 3:5-diglucosides with *n*-butyl alcohol.

¹ Part 1 (1930) *Biochem. J.* **24**, 753; Part 2 (1930) *Biochem. J.* **24**, 767.

EXPERIMENTAL

A. Isolation of pigment from purple *Verbena* flowers

The fresh flowers were extracted with 1 % aqueous HCl, and the solution shaken repeatedly with ethyl acetate to remove flavones and tannins. After several extractions with ethyl acetate, solid anthocyanin began to separate from the acid solution. This increased in amount as the flavone was removed. When the extractions were completed the solution was allowed to settle overnight. The liquid was then decanted and the remaining sludge centrifuged. The solid was collected and boiled with 0.5 % aqueous HCl, which dissolved the pigment and left behind some white insoluble material which was discarded. On cooling the filtered solution, golden-brown crystals of semi-pure anthocyanin separated. This was further purified by dissolving in hot 0.5 % aqueous HCl, cooling and making the acid concentration up to 5 %. The pigment obtained by this method was clean but amorphous. Two or three crystallizations from 0.5 % aqueous HCl gave the pure anthocyanin in lens-shaped leaflets, blue by transmitted light and with a gold reflex.

The anthocyanin was not extractable from an aqueous acid solution by *isoamyl* alcohol, and it showed all the reactions of a delphinidin 3:5-dimonoside; for example it gave a pure blue colour with Na_2CO_3 , violet with FeCl_3 in neutral solution, and with NaOH blue which faded rapidly. The addition of a large volume of hot water to an alcoholic solution of the pigment transformed it to the colourless pseudo-base, from which the coloured form was restored by acidification. (Found, after drying at 105° over P_2O_5 *in vacuo*: C, 48.8; H, 4.8; Cl, 3.9 %. $\text{C}_{27}\text{H}_{30}\text{O}_{17}$, 0.75 HCl, 0.25 H_2O requires C, 49.2; H, 4.75; Cl, 4.04 %.)

There is a loss of HCl on drying at 105° in a vacuum. This was also noticed [Reynolds *et al.* 1934] when the pigment of *Salvia patens* was dried under similar conditions. In this case there is partial replacement of HCl by water.

The colours in buffered solutions of graded pH were compared with those of a pure specimen of delphin from *S. patens*. There were no marked divergences, but the purple *Verbena* pigment faded rather more rapidly between pH 3.4 and 5.0, and the solutions were slightly redder than those of the *Salvia* pigment between pH 3.8 and 8.6. The differences were too slight to be of any structural significance.

Preparation of anthocyanidin. The diglycoside was hydrolysed by boiling with 16 % aqueous HCl for 3 min. The anthocyanidin separated as a dark brown amorphous precipitate, which was dissolved in the minimum quantity of cold 0.5 % aqueous HCl, heated to boiling and the acid concentration increased to 10 %. On cooling, the anthocyanidin separated in clusters of rod-shaped crystals. These were filtered, dried *in vacuo* at 105° over P_2O_5 and analysed. (Found: C, 52.3; H, 4.1; Cl, 6.9 %. $\text{C}_{15}\text{H}_{10}\text{O}_7$, $2/3$ HCl, 1.0 H_2O requires C, 52.3; H, 3.7; Cl, 6.8 %.)

The anthocyanidin gave all the qualitative reactions of delphinidin chloride. It was extracted by *amyl* alcohol from 1 % aqueous HCl. The *amyl* alcohol on shaking with sodium acetate became violet blue, and on addition of a drop of FeCl_3 solution this changed to pure blue. The pigment was not extracted by either the "cyanidin reagent" or the "delphinidin reagent" [Robinson & Robinson, 1931]. The comparison of this anthocyanidin with that from the maroon *Verbena* and with synthetic delphinidin chloride are described below (p. 271).

The amounts of sugar and anthocyanidin produced on acid hydrolysis were estimated as follows. A weighed amount of anthocyanin was hydrolysed, cooled

and left overnight to ensure as complete a precipitation of the anthocyanidin as possible. It was then filtered, the residue washed with a small quantity of 16% aqueous HCl and dried to constant weight over P_2O_5 at 105° . The filtrate was shaken with amyl alcohol to remove the last traces of anthocyanidin, then with ether to remove amyl alcohol, and finally neutralized with caustic soda. The sugars were then estimated by Bertrand's method. In the first hydrolysis 30.2 mg. of the air-dried delphinidin 3:5-dimonoside (28.0 mg. calculated as anhydrous) gave 13.8 mg. anthocyanidin (49.3%) and 11.11 mg. hexose, calculated as glucose (40.0%). In a second estimation 42.6 mg. of the diglycoside (dried *in vacuo* over P_2O_5 at 105°) gave 20.6 mg. anthocyanidin (48.3%) and 18.53 mg. glucose (43.5%). $C_{27}H_{31}O_{17}Cl$ requires $C_{15}H_{11}O_7Cl$, 51.1%; $C_6H_{12}O_6$, 54.2%. These results are similar to those obtained by Reynolds *et al.* who found values for sugar 9–12% below the theoretical expectation.

In a larger scale experiment 0.395 g. of anthocyanin was hydrolysed and the sugar converted into its osazone in the usual way. This was crystallized from pyridine—ethyl alcohol—water mixture and identified as glucosazone by its M.P., $205\text{--}206^\circ$, which was not depressed by admixture with an authentic specimen.

Fusion with potash. 0.4 g. of air-dried anthocyanidin chloride was heated for 2 min. at 200° with 5 g. solid KOH and 5 ml. water. The mixture was cooled, diluted with water, acidified and extracted several times with ether. The acidic and phenolic degradation products were separated with bicarbonate in the usual manner and found to be phloroglucinol and gallic acid. The former was identified by its transient violet colour with $FeCl_3$, red colour with a pine shaving and conc. HCl, the formation of an orange-red diazobenzene compound, and by the brick-red phloroglucide of methylfurfuraldehyde obtained by heating rhamnose with 20% HCl and distilling the vapour into a solution of the phenol in water. The gallic acid gave a blackish-blue precipitate with $FeCl_3$, a deep blue colour with $FeSO_4$, and its alkaline solution darkened rapidly on exposure to air.

B. Isolation of pigment from maroon *Verbena* flowers

The fresh flowers were extracted with 1% aqueous HCl, and the solution shaken with successive amounts of *n*-butyl alcohol; about 5 l. of the alcohol were used in all. The combined butyl alcoholic extracts were shaken with an equal volume of light petroleum (B.P. $60\text{--}80^\circ$) and 500 ml. of 1% aqueous HCl. The concentrated aqueous solution so obtained was extracted several times with ethyl acetate in order to remove flavones. 250 ml. absolute alcohol and 2 l. of ether were then added, when the pigment was precipitated as a dark red syrup. The ether-alcohol was decanted, the syrup warmed gently on the water bath to remove any remaining ether and left for 16 days. It then contained dark red crystals of the pigment together with some colourless crystalline material. The crystals were filtered and boiled with 1% aqueous HCl, which dissolved the pigment but not the white substance, the latter being removed by filtration of the hot solution. The filtrate was shaken with benzene to remove traces of alcohol, an equal quantity of conc. HCl added and the solution left for several days, when clean amorphous pigment separated. This was dissolved in the minimum quantity of hot 0.5% aqueous HCl, the solution filtered, cooled and 0.1 vol. 25% ethyl alcoholic HCl added. Only slight separation had taken place after 2 days, and the bulk of the pigment was recovered by the addition of $\frac{1}{3}$ vol. of conc. HCl. Crystallization was effected by solution in warm methyl alcohol and addition of an equal volume of 10% methyl alcoholic HCl. Irregular masses of crystals separated, bluish-purple by transmitted light with a copper

reflex. These were dried at 105° *in vacuo* over P_2O_5 , and analysed. (Found: C, 48.86; H, 4.5; Cl, 7.2%. $C_{21}H_{21}O_{12}Cl \cdot H_2O$ requires C, 48.6; H, 4.44; Cl, 6.85%.)

The distribution number (isoamyl alcohol and equilibrated 0.5% aqueous HCl) was determined by the standard method [Levy & Robinson, 1931] with the following results:

		Distribution no.
I. 5.0 mg./100 ml. mixed solvents:	1st shaking	12.6
	2nd shaking	13.8
II. 4.0 mg./50 ml. mixed solvents:	1st shaking	11.4
	2nd shaking	12.1

The anthocyanin is therefore monoglycosidic. Its colour reactions in solutions of graded pH [Robertson & Robinson, 1929] were examined, and found to be the same as those of synthetic 3- β -glucosidyl delphinidin chloride [Reynolds & Robinson, 1934]. The pigment was only slightly soluble in hot ethyl alcoholic HCl. It gave a blue colour with Na_2CO_3 , violet with $FeCl_3$ in neutral solution, blue violet with sodium acetate, and a pure blue, which faded rapidly, with 10% NaOH.

Preparation of anthocyanidin. 23.4 mg. of anthocyanin (dried *in vacuo* at 105° over P_2O_5) were dissolved in 0.5% aqueous HCl, and hydrolysed by boiling for 3 min. with an equal volume of conc. HCl. The anthocyanidin chloride separated in clusters of crystals on cooling. These crystals were air-dried, weighed and analysed. (Found: C, 49.4; H, 4.2; Cl, 9.5%. $C_{15}H_{11}O_7Cl \cdot 1.5 H_2O$ requires C, 49.2; H, 3.8; Cl, 9.7%.)

Willstätter & Weil [1916] described four hydrates of delphinidin chloride, dependent upon the condition of crystallization. Among them was one with $1.5 H_2O$ crystallizing from 20% HCl.

The sugars produced in the above hydrolysis were determined by Bertrand's method. The results were as follows: hexose calculated as glucose, 5.887 mg., 25.2%; anthocyanidin, 17.13 mg., air-dried, 73.2% (calculated as anhydrous). $C_{21}H_{21}O_{12}Cl$ requires $C_6H_{12}O_6$, 36%; $C_{15}H_{11}O_7Cl$, 67.6%.

A 1% aqueous HCl solution of the anthocyanidin gave the same qualitative reactions as those of delphinidin chloride from the purple *Verbena* (see above, p. 269).

The colours of the anthocyanidin chlorides from the purple and maroon *Verbena* were compared with those of a specimen of synthetic delphinidin chloride. The colours were identical except from pH 8.6 to 9.2, at which points the anthocyanidin from the purple type was slightly more violet than the other two.

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