97. *Nitrogenous Anthocyanins. Part IV. The Colouring Matter of* Bougainvillæa glabra.

By J. R. Price and Robert Robinson.

In a survey of anthocyanins (Robinson and Robinson, *Biochem. J.*, 1932, **26**, 1653; 1934, **28**, 1713) it was noted that the purple bracts of *Bougainvillæa glabra* contained a pigment resembling betanin, and a variety with scarlet bracts was found to contain a different anthocyanin of the same group. Through the kindness of Prof. A. K. Macbeth of Adelaide University, to whom we express our warmest gratitude, a considerable quantity of the dried bracts was available and it has been possible to carry out a preliminary investigation. Dr. W. S. Rapson has kindly collected further large quantities at Cape Town and the investigation will be continued; in the meantime we have established certain facts in regard to the pigment and these are now placed on record.

The greater part of the material was used up in the trial of alternative methods of isolation of the pigment, but eventually the bracts were extracted with 1% methyl-alcoholic hydrogen chloride, and the solution shaken with alumina, ammonia being added cautiously until the correct p_H was reached. The colouring matter was removed from the alumina by solution in sodium carbonate or disodium hydrogen phosphate, regenerated by acidification, and separated into anthocyanidin and anthocyanin fractions by extraction with butyl alcohol. The anthocyanidin, which may be termed *bougainvillæidin chloride*, could sometimes be crystallised from 2% hydrochloric acid; on other occasions it separated in an indefinitely crystalline or amorphous form. The analyses of different specimens were moderately consistent and showed a far higher carbon nitrogen ratio than in the case of betanidin (preceding paper). Assuming a molecule comparable with that of the anthocyanidins, it can contain only one nitrogen atom. The carbon nitrogen ratio varied from C_{20} : N to C_{24} : N, the most reliable value being C_{22} : N. This was obtained from the better analyses after allowance had been made for the methoxyl content, which was about 29% of 1MeO on the basis of the suggested formula; this methoxyl is considered to be due to partial esterification of a carboxyl group by the methyl-alcoholic hydrogen chloride used for the initial extraction. The chlorine content was again low and represented a loss of about

68% of 1HCl. Some of this loss may occur on drying in a vacuum and at 80° in a vacuum, and it must be remembered that the nitrogenous flavylium salts are now represented as containing carboxyl groups and thus may exist as betaines, the hydrochlorides of which are sometimes very unstable. A correction being made for this, the analyses are in good agreement with the formula $C_{22}H_{26-30}O_{10}NCl$, losing 68% HCl and gaining 29% CH_2. This applies to material dried at 80° in a high vacuum and it is not certain that this is anhydrous, a consideration that affects the number of oxygen atoms to be associated with the flavylium nucleus. The *N*-methyl derivative of a condensation product of delphinidin chloride or an isomeride and leucine (loss of $1H_2O$) would have the composition $C_{22}H_{24}O_8NCl$ and the occurrence of $2H_2O$ in an anthocyanidin dried at 80° is quite possible. Unfortunately decomposition occurred at higher temperatures and for this reason we were unable to establish the composition of the anhydrous substance. The distribution number of bougainvillæidin chloride (as isolated) is 50 towards equilibrated *n*-amyl alcohol–0·5% hydrochloric acid. The absorption spectrum resembles that of betanin.

Quercetin also has been isolated from the bracts.

EXPERIMENTAL.

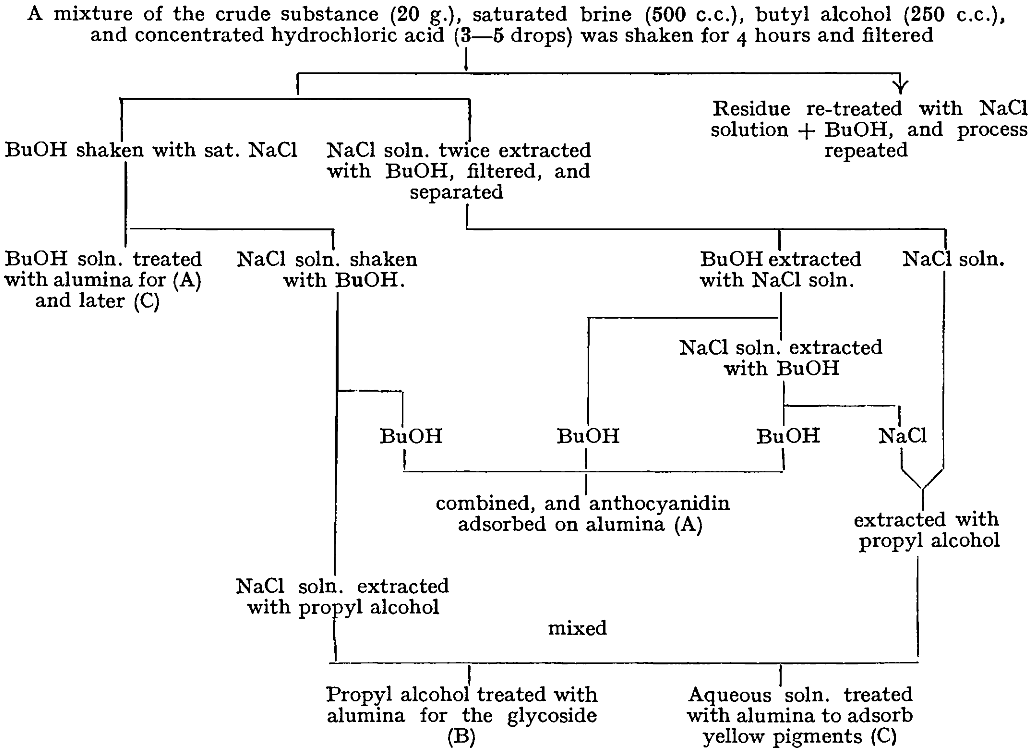
Extraction of the Bracts.—The bracts of the variety most commonly grown in S. Australia (exact variety unknown) were dried on wire-gauze trays in a rapid stream of air at 45° for 24 hours or more. The material was powdered and stored in sealed containers; it suffered little change in transit. The powder (1000 g.) was placed in a 10 l. aspirator bottle and covered with 1% methyl-alcoholic hydrogen chloride. The solution was drawn off periodically from the bottom and replaced with fresh solvent; after 1—4 days the whole of the solution was drained away and a fresh extract was made which was used to start the extraction of a second kilogram of the bract powder. The first extract (*ca.* 6 l.) was mixed with ether–light petroleum (2 : 1), the colouring matter being almost completely precipitated. After a few hours, the liquid was decanted, the residue dissolved in 1% methyl-alcoholic hydrogen chloride, and the pigments reprecipitated from the filtered solution. This operation was carried out from five to eight times in different experiments. The viscous, intensely bluish-red residue was dried over calcium chloride in a vacuum desiccator and obtained as a dark purple solid. The yields were variable [26 g., 66 g., 70 g., and (from 1400 g.) 77 g.], probably depending on the time expended in the first extraction.

The crude substance was insoluble in benzene, chloroform, ether, ethyl acetate or acetic acid, partly soluble in propyl alcohol, and readily soluble in methyl and ethyl alcohols. It was not completely soluble in water or dilute acids and the addition of salt increased the proportion of insoluble material. The solution in brine contained a mixture of monoglycoside and diglycoside; the distribution to *iso*amyl alcohol was negligible, that to butyl alcohol was higher but still quite low. With propyl alcohol the greater part of the pigment could be extracted from saturated brine, but there remained a small part, considered to be diglycosidic, that could not be extracted. The precipitated pigment had non-glycosidic character and a useful step in the purification of this fraction was to shake the crude extract with butyl alcohol and brine. A considerable amount of impurity, insoluble in butyl alcohol, was eliminated at the same time. The crude product contained much material of high molecular weight (protein, polysaccharide). No crystalline salts, such as picrate, perchlorate, or ferrichloride, could be precipitated and in the crude form the colouring matter was very unstable both in acid and in alkaline solution.

The first attempted purification was by means of extraction with *cyclo*hexanone and picric acid; the colouring matter could be concentrated in this way, but the method was wasteful and was abandoned.

Adsorption of the Pigments on Alumina.—Chromatographic adsorption in a column of alumina (Merck's, *nach* Brockmann) gave two well-defined zones, an upper deep purple and a lower bright yellow. It was not practicable to separate the anthocyanidin from the anthocyanin in this way, although the latter was more completely adsorbed from alcholic than from aqueous solutions. The yellow pigment was a mixture of a glucoside with its aglucone.

On the basis of the preliminary experiments several schemes of purification were tried : that finally adopted is shown in the diagram. The solutions used for adsorption were all neutralised and adsorption was carried out by shaking with successive small quantities of the alumina. The treated alumina was well washed with water and methyl alcohol until the washings were colourless. The extraction with propyl alcohol was used, as it gave a good separation of monofrom di-glycoside, and the latter was discarded; also the adsorption was very effective from the propyl-alcoholic solution.



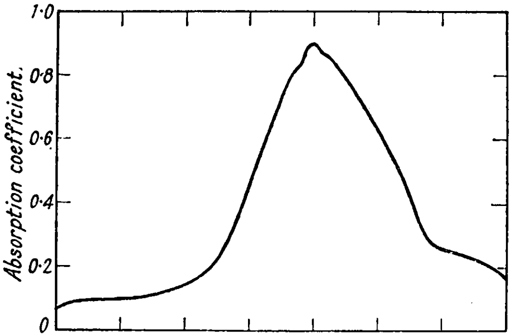
An analysable product has not yet been isolated from (B), but since adsorption of the more readily accessible beet colouring matter on alumina from methyl-alcoholic solution has afforded the glucoside, not the anthocyanidin, and as the material thus purified possesses considerable stability, we are concentrating on this aspect of the problem. The experiments described below refer to (A) and (C).

Separation of Bougainvillæidin Chloride.—The colouring matter could be eluted from the alumina by methyl-alcoholic or aqueous hydrogen chloride, but the methods were wasteful owing to decomposition. A specimen thus prepared and further purified by evaporation of the solution and repeated extraction with alcohol, followed by evaporation, was analysed with results similar to those cited below, but the purity was certainly not equal to that achieved later. Aqueous sodium carbonate or ammonia was much better than an acid reagent, but there was a tendency to decomposition when the liquid was heated to effect solution of the colouring matter. This disadvantage was overcome by the use of disodium hydrogen phosphate (7·5%, $Na_2HPO_4,12H_2O$), a hot solution of which removed all the colouring matter without decomposition.

The sodium phosphate extract was saturated with sodium chloride, filtered, acidified with hydrochloric acid, and extracted with butyl alcohol, and the butyl-alcoholic layer was shaken with a little water and just sufficient sodium carbonate to effect transference of the colour to the aqueous solution. These processes were repeated and on acidification of the concentrated aqueous solution a semi-crystalline precipitate was thrown down; this crystallised from 2% hydrochloric acid in black needles possessing a green reflex. Specimens (i and ii) from two different preparations were dried in a vacuum desiccator [Found in (i) and (ii) : loss at 80° in a high vacuum, 10·8, 10·7% respectively. Found in (i), dried at 80° : C, 55·28; H, 6·13; N, 2·93%. Found in (ii), dried at 80° : C, 55·57; H, 5·64; N, 2·66; MeO, 1·86%].

A third specimen (iii) was prepared by a simplified process. The bracts were extracted during 3 days and the solution was shaken with alumina, ammonia being cautiously added until adsorption of pigment occurred. The elution was by means of cold aqueous sodium carbonate (the pigment not removed in this way was dissolved in dilute hydrochloric acid and, after neutralisation, re-adsorbed on fresh alumina) and the pigment was passed to butyl alcohol as described above (the glycosides remained in the acid aqueous solution). The further treatment was as above, and the yield from the bracts was about 0·1%. The substance obtained in this way did not crystallise, or at least the crystals were of very indefinite form, but it was purified by three solutions in, and separation from, 2% hydrochloric acid and dried in a vacuum over phosphoric oxide (Found : loss at 80° in a high vacuum, 5·5%. Found in material dried at 80° : C, 55·36; H, 4·96; N, 3·03; Cl, 2·32; MeO, 1·83%).

Bougainvillæidin chloride dissolves in hydrochloric acid to a deep purple solution, which becomes bright red on neutralisation; the addition of more alkali causes a change of colour through blue and violet to yellow. The distribution number with equilibrated *n*-amyl alcohol and 0·5% hydrochloric acid was 50·0. The absorption in the visible region was determined with a solution of 1·636 mg. in 25 c.c. of 0·05% methyl-alcoholic hydrogen chloride in a 2 cm. cell. The curve (see figure) shows points of similarity with those of crude betanin and the pigment of *Atriplex hortensis* (cf. Robinson and Robinson, J., 1932, 1439).



Discussion of the Analytical Results.—The average estimations for the three specimens are : C, 55·40; H, 5·58; N, 2·87; Cl, 2·32; MeO, 1·85%. On the basis of a formula containing one nitrogen atom, 1MeO corresponds to 6·35%. In harmony with our conception of the underlying chemistry and also in view of the method of preparation, we may assume that this material is a mixture of a certain substance with about 29% of its methyl derivative. Then, if all the substance had been methoxyl-free, the figures would be : C, 55·15; N, 2·90%. This gives the ratio C : N as 22·2 : 1. The value for chlorine is low, indicating loss of hydrogen chloride, and correcting for the chlorine content, and calculating for the methoxyl-free, chlorine-free base, we find C, 56·51; N, 3·0%. The carbon value is regarded as the more accurate and from this the molecular weight found is 467. From this we find $C_{22}H_{27}O_{10}N$ (*M*, 465) to be the best formula for the free base. The next stage is to note that the ratio MeO : Cl is almost 1 : 1, and this suggests that our specimens were mixtures of about 2 parts of bougainvillæidin (betaine) with about 1 part of bougainvillæidin methyl ester chloride [2($C_{22}H_{23}O_8N,2H_2O$), $C_{23}H_{26}O_8NCl,2H_2O$, or $C_{67}H_{84}O_{30}N_3Cl$ requires C, 55·6; H, 5·8; N, 2·9; Cl, 2·5; MeO, 2·15%, in good agreement with the analytical results]. This hypothesis is inherently probable from the chemical point of view, because methylation of the carboxyl group greatly reduces the ease of betaine formation. Phenol-betaines would still be possible, but these are not stable in acid solution in the flavylium series. Similar formulæ based on $C_{22}H_{23}O_7N$ (for example, anhydro-cyanidin-leucine) are also feasible (H, 6·2%), but require $3H_2O$ to be retained at 80°. Formulæ based on $C_{22}H_{23}O_6N$ are improbable.

Isolation of Quercetin.—The alumina (C, p. 451) on which the yellow colouring matters were adsorbed was extracted with 5% methyl-alcoholic hydrogen chloride, the filtered solution mixed with a little water, and the methyl alcohol and some of the water removed under diminished pressure. The brown solid that separated was collected and extracted with ether (Soxhlet), and the crude product precipitated from the concentrated ethereal solution by the addition of light petroleum. It was crystallised from aqueous alcohol and then from alcohol–benzene, forming yellow needles, m. p. 308—310°, and m. p. 309—311° when mixed with synthetic quercetin, m. p. 310—313°. The identity was confirmed by a comparison of dyeing properties on cotton mordanted with iron and aluminium (weak and strong), and the results were identical. Further, an acetyl derivative was prepared from the two specimens as follows. A mixture of the colouring matter or quercetin (0·3 g.) with acetic anhydride (4 c.c.) and pyridine (2 drops) was kept for 1 hour at room temperature. The product crystallised from aqueous acetone in colourless needles, m. p. 169—170° (from the colouring matter, m. p. 166—168°; from synthetic quercetin, m. p. 167—168° when mixed). There is some doubt as to the composition of this derivative (cf. Perkin, J., 1899, **75**, 449; Kubota and Perkin, J., 1925, **127**, 1889), and the question of the acetyl derivatives of quercetin is being re-investigated. The penta-acetate was also prepared from the *bougainvillæa* pigment, m. p. 191—193° after crystallisation from acetone–alcohol, m. p. 192—194° when mixed with penta-acetylquercetin, m. p. 194—195°.

The brown solid not extracted by ether in the Soxhlet apparatus could not be crystallised; it probably consisted of decomposition products of bougainvillæidin, because the yellow pigments increased in amount when the solutions constituting the first extracts were kept.

The authors thank the Royal Commissioners for the Exhibition of 1851 for a Scholarship (Overseas) awarded to one of them.

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[*Received, January* 14*th*, 1937.]