242. *Leucoanthocyanins. Part III. Formation of Cyanidin Chloride from a Constituent of the Gum of* Butea Frondosa.

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Leucoanthocyanins occur widespread in plant material, but the anthocyanidins into which they may be converted have hitherto been identified by means of a system of qualitative tests and comparisons in solution. It is now shown that the gum of *Butea frondosa* contains a substance that can be converted into cyanidin chloride, and the anthocyanidin has been isolated, analysed and compared with an authentic specimen. Cyanidin is not formed from the gum by the action of hydrochloric acid alone; preliminary oxidation is necessary and under special conditions the use of picric acid has been found to be advantageous.

Although it is thought that the qualitative examination of anthocyanins and anthocyanidins by the methods of Robinson and Robinson (*Biochem. J.*, 1931, **25**, 1687; 1932, **26**, 1647) affords reliable results, this type of investigation has obvious limitations and is no substitute for the more precise and formal study of the pigments isolated in a pure condition. The two methods are complementary and the value of the qualitative tests as an auxiliary resides in the power that they confer of covering a wide range, a particularly useful application being the analysis of anthocyanins in varieties of the same species in connection with genetical investigations.

The anthocyanidins derived from the widely distributed leucoanthocyanins (Robinson and Robinson, *Biochem. J.*, 1933, **27**, 206) were examined by the qualitative methods alone and, in general, the behaviour of the colouring matters tallied with that of the known anthocyanidins of flower pigments. In this case, however, the background of

systematic investigation was lacking and it was therefore deemed desirable to attempt the isolation of the presumed anthocyanidins from typical leucoanthocyanins. Experimentally the problem proved to be a difficult one and several rich sources of leucoanthocyanins were found in preliminary work to be unsuitable. Eventually the gum of *Butea frondosa* (Bengal kino) was selected and, although all attempts to isolate the leucoanthocyanin itself have been fruitless, conditions have been found which promote the formation of cyanidin chloride from it.

If the gum is boiled in an open vessel with aqueous or alcoholic hydrogen chloride, some colouring matter of anthocyanidin type is produced, but if air is carefully excluded a red coloration is observed which is not due to anthocyanidin. The colouring matter formed in the latter way gives a characteristic violet solution in aqueous sodium carbonate. Aerial oxidation is nevertheless unsatisfactory and, although more colouring matter is produced by boiling with concentrated hydrochloric acid along with acetone, ethylene glycol or glycerol, the cyanidin appears to be modified in these cases. Bromine as an oxidant gives good results, but this method was not adopted in view of the risk of nuclear bromination.

Treatment of the gum with boiling saturated aqueous picric acid (not effective in the presence of hydrochloric acid) brings about a change that facilitates the subsequent formation of anthocyanin and ultimately a preliminary treatment with aqueous sodium acetate and zinc chloride was found to be advantageous.

The cyanidin chloride, isolated as explained in the experimental section, was persistently contaminated with a colourless by-product. The composition of this must be closely similar to that of cyanidin chloride, because a specimen containing only about 67% of the anthocyanidin gave approximately correct results on analysis.

The fully purified cyanidin chloride showed all the characteristic properties of authentic specimens of the natural and the synthetic anthocyanidin and no divergence was observed in any respect. Direct comparisons of distribution ratios and colorations in solutions of definite p_H were carried out.

Experimental.

Preliminary Experiments.—The gum of *Butea frondosa*, as supplied by Messrs. British Drug Houses, Ltd., is moderately easily soluble in boiling water, probably in part in the colloidal state; on drying at 100° it becomes insoluble in boiling water. Also when the dried kino is extracted in a Soxhlet apparatus with benzene or acetone, only a small quantity of waxy matter passes into solution. The prolonged action of cold 10% alcoholic hydrogen chloride on the gum results in the formation of a deep red solution, from which ether extracts a yellow substance (bright red coloration in sulphuric acid) and then ethyl acetate extracts a red substance which dissolves in aqueous sodium carbonate to a violet solution and in aqueous sodium hydroxide to a blue solution. This is not a known anthocyanidin, although it may well be a related substance.

If the gum is heated with 5·5% alcoholic hydrogen chloride, the same colouring matter is produced along with a substance exhibiting a bright blue fluorescence. The latter is removed from an amyl-alcoholic solution by aqueous sodium carbonate but not by aqueous sodium hydrogen carbonate. Prolonged boiling with hydrochloric acid results in the formation of a little anthocyanidin, but, as already explained, some pre-treatment is essential and picric acid was found to give the best results.

The gum was boiled with saturated aqueous picric acid, the mixture concentrated on the steam-bath, washed with benzene, and heated with alcoholic hydrogen chloride. The anthocyanidin was isolated as described below except that the washing with hot ethyl acetate was omitted because the necessity for it was not realised at this stage. The flat needles from 7% methyl-alcoholic hydrogen chloride had the appearance of cyanidin chloride (Found: C, 53·5; H, 4·6; Cl, 9·5%). Colorimetric comparisons showed that this specimen contained 66·6% of cyanidin chloride; the distribution ratios tallied with those of cyanidin chloride after adjustment of the concentrations of the solutions so as to obtain colorimetric equivalence.

Isolation of Cyanidin Chloride.—A mixture of the finely powdered gum (2·0 g.; larger quantities give poorer results) with water (30 c.c.) and crystallised sodium acetate (2·5 g.) was boiled for 10 minutes; powdered zinc chloride (3 g.) was then added, and boiling continued for 1 minute. The solid was collected and refluxed with cold-saturated aqueous picric acid

(14 c.c.) for 8 minutes; almost the whole had then passed into solution. Alcoholic hydrogen chloride (250 c.c. of 5·5%) was added, and refluxing continued for 30 minutes, the liquid becoming dark red. Finally, after the addition of 5% hydrochloric acid (50 c.c.), the liquid was boiled for 15 minutes. The product was diluted with twice its volume of water, washed repeatedly with benzene, and extracted with amyl alcohol. The separated organic layer was shaken with water containing a trace of hydrochloric acid, and light petroleum added. The aqueous solution was successively washed with ether, ethyl acetate, and benzene, aqueous picric acid was added, and on keeping in the ice-box cyanidin picrate (0·19 g.) separated in slender needles. By working up the aqueous solution, a further quantity of picrate (0·09 g.) was obtained as well as cyanidin chloride (0·03 g.). The latter was isolated, after no further picrate separated, by several transferences of the chloride from hydrochloric acid to amyl alcohol and vice versa and finally by precipitation from an aqueous solution by the addition of concentrated hydrochloric acid. The product gave the usual reactions for cyanidin chloride.

The fact that the gum contains about 5% of moisture and about 15% of inorganic matter (mainly sodium silicate with magnesium, potassium, calcium, and iron) being taken into consideration, the yield of cyanidin chloride was about 14% of the weight of the dry, ash-free gum. This is only a rough estimate, but is probably an understatement in view of the inevitable losses sustained in the isolation processes. The picrate was converted into chloride and regenerated in aqueous solution. It was then dissolved in 7% methyl-alcoholic hydrogen chloride, and the solution concentrated under diminished pressure. The crystals of cyanidin chloride were collected and washed with ether and with cold and *hot* ethyl acetate; the substance was again crystallised from 7% methyl-alcoholic hydrogen chloride and washed as before. The washing with hot ethyl acetate removes a colourless substance. This gave no characteristic colour reaction in acid or alkaline solution, but on heating a red coloration was developed in both cases. The cyanidin chloride was dried in a vacuum desiccator at room temperature (Found: C, 52·9; H, 4·1; Cl, 10·1; MeO, 0·0. Calc. for $C_{15}H_{11}O_6Cl,H_2O$: C, 52·9; H, 3·8; Cl, 10·4%). The flat, microscopic needles were chocolate-brown in mass and orange-red by transmitted light; they exhibited all the characteristics and reactions of cyanidin chloride.

Comparison with a Specimen of Cyanidin Chloride from Cyanin Chloride.—The specimen (*B*) from the gum was dried over potassium hydroxide in a desiccator, but at the ordinary pressure; it was probably hydrated to a slight extent. Colorimetric comparison with a very pure specimen of cyanidin chloride (*C*) from cyanin which had been completely dried showed that (*B*) had 95% of the pigment content of (*C*). After the small corresponding adjustment of the concentration of the solutions in 0·5% hydrochloric acid (6·099 mg. in 50 c.c.) they were colorimetrically identical.

In making up the solutions it was noted that both were more intensely coloured when cold than when hot and to the same extent. Addition of alcohol produced the same blueing effect in each case. The following distribution ratios were compared, equal volumes of organic solvent and aqueous solution being used; the organic layers were directly compared and no divergence exceeding 0·4% was observed in the colorimeter: *cyclo*hexanol–toluene (1 : 5), *cyclo*hexanol–toluene (1 : 4), *cyclo*hexanol–benzene (2 : 3), ethyl acetate. In the case of the specimen, mentioned above, containing 66·6% of cyanidin chloride a few more solvents were employed with the same result.

The behaviour with ether–picric acid, sodium acetate, sodium carbonate, sodium hydroxide, ferric chloride, amyl alcohol–sodium acetate–ferric chloride, and concentrated sulphuric acid was identical with (*B*) and (*C*). In the latter case the slow development of a weak green fluorescence was observed and the rate of appearance, colour, and intensity of the fluorescence were indistinguishable.

Dilute aqueous ferric chloride (the bench reagent) was diluted five-fold, and 10 c.c. added to 20 c.c. of each of the solutions diluted with 80 c.c. of 0·5% hydrochloric acid at 17·5°. The solutions showed an identical behaviour; after mixing they became slightly deeper in colour and tinged violet. After 15 minutes the colour was almost discharged and after 30 minutes the solutions were colourless. Addition of concentrated hydrochloric acid did not restore the colour, but gave a yellow solution due to the ferric salt alone, as was proved by a parallel experiment without cyanidin. The colours developed in two ranges of buffered solutions were also observed and no divergences were noted (cf. Robertson and Robinson, *Biochem. J.*, 1929, **23**, 35). The ranges used were of p_H 3·1, 4·3, 5·5, 6·65, 7·8, 9·0, 10·2, 11·4 and 3·1, 3·34, 3·58, 3·82, 4·05, 4·29, 4·53 and 4·77; the latter range was used to observe the rate of formation of

the ψ-base. This was very slow at p_H 3·1 but rapid at p_H 3·3 and most rapid at about p_H 3·6; with increasing p_H complete decolourisation occurred up to p_H 4·8 in 30 minutes, but at p_H 5·0 a pale colour remained after this period. Addition of hydrochloric acid restored the colour in all cases to the same extent, but after the solutions had been kept for 15 minutes or more they had to be boiled in order to produce regeneration of the oxonium salt.

These results prove that the pigment isolated after treating the gum of *Butea frondosa* in the prescribed manner is cyanidin chloride. The difficulties in the purification do not arise from the necessity for separating two anthocyanidins but from the presence of impurities of an entirely different character.

Notes on Occurrence of Leucoanthocyanins.—In addition to the sources already disclosed (*loc. cit.*), the following observations have been made by means of the method of qualitative tests. The conclusion previously reached that the majority of leucoanthocyanins yield cyanidin has been confirmed. Instead of treating the materials with hot hydrochloric acid, it is now preferred to use boiling 5% alcoholic hydrogen chloride. Cyanidin chloride is derived from the woods of *Endriandrh Palmerston* (Queensland walnut) and *Eucalyptus tereticornis* (also *Eucalyptus kino*). All parts of *Hypericum calycinum* (St. John's wort) and especially the young seed pods contain a leucoanthocyanin yielding cyanidin. *Krameria* root, on the other hand, contains a leucoanthocyanin yielding pelargonidin, and *Quillia* shavings and *Musa sapientum* (banana) contain leucoanthocyanins yielding delphinidin. In the last case the leucoanthocyanin is contained in the fibres forming part of the meal. The fleshy part of the fruit was washed with alcohol and water; the residue, on boiling with alcoholic hydrogen chloride, afforded delphinidin chloride. Several of the materials tested (*e.g.*, *Krameria* and *Quillia*) have been found to contain much phosphate.

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