96. *Nitrogenous Anthocyanins. Part III.* Preliminary Experiments with Betanidin.*

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The existence of a class of nitrogenous anthocyanins was brought to light by Willstätter, who in collaboration with Schudel (G. Schudel, Dissert., Zürich, 1918, now a very scarce publication) isolated the pigment of *Beta vulgaris* in the form of a crystalline chloride, the composition of which was not fully determined. The substance was, however, found to contain nitrogen and it was shown to be a monoglycoside on the assumption of a molecular weight comparable with that of other anthocyanins. The method of isolation involved at one stage extraction of the colouring matter from an aqueous solution by means of a mixture of amyl alcohol and acetophenone containing dichloropicric acid, a process which is not free from the danger of attachment of some chloropicryl groups to the pigment molecules. Our attempts over a period of several years to find a simpler and more direct process have met with a very limited measure of success, largely owing to the small tendency to crystallise exhibited by the members of this series and the difficulty of freeing them from organic and inorganic impurities. Willstätter and Schudel (*loc. cit.*) found that betanin could not be hydrolysed to its aglucone by means of aqueous acids without decomposition, although they obtained an alkylated salt by using alcoholic hydrochloric acid. This has also been

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* Part I, J., 1932, 1439; Part II, J., 1933, 25. The title has been shortened in order to include investigations of natural products of the group.

our experience with specimens of betanin chloride isolated, in connection with another investigation, by adsorption on and elution from alumina. We have, however observed that hydrolysis occurs in a solution made by soaking sliced beet in distilled water, doubtless as the result of the action of a hydrolysing enzyme, and such solutions contain the nitrogenous anthocyanidin, which may be termed betanidin. A solution in 1% hydrochloric acid remains unchanged. After the addition of sodium chloride the colouring matter could be extracted by means of *iso*amyl alcohol and returned to a smaller volume of water after the extract had been mixed with light petroleum : this process was repeated. The aqueous solution was then evaporated under diminished pressure, and the residue extracted with alcohol. After repetition of this process the final residue was washed with water and dried in a vacuum. It was unfortunately not uncommon to sustain the loss of an entire batch from 56 lb. of beet on account of the sensitivity of the anthocyanidin to oxygen. One such loss occurred when an attempt was made to precipitate the substance from an alcoholic solution by the addition of ether. Ultimately we carried out all the operations in an atmosphere of hydrogen or nitrogen. The product was an amorphous, dark purple solid possessing a green reflex and giving 3·0% of ash on combustion. It had all the anticipated properties and was judged to be sufficiently pure to give some information on analysis. Our earlier experiences with synthetic aminoflavylium salts did not dispose us to anticipate close agreement with a theoretical value, especially that for chlorine. A frequent occurrence is the replacement of hydrogen chloride by water just as the reverse process occurs in the case of several anthocyanins. The analyses of different specimens showed fair consistency and on the assumption of a molecular weight of the same order as those of the usual anthocyanidins the molecule contains two atoms of nitrogen and loss of hydrogen chloride is indicated.

Methoxyl and methylimino-groups were not present. The carbon nitrogen ratio indicated $C_{20}N_2$ and all the results were in agreement with the formula $C_{20}H_{19-23}O_7N_2Cl,3H_2O$, in which about 30% of the hydrogen chloride was replaced by an equal weight of water. The specimens lost the equivalent of $3H_2O$ at 110° in a high vacuum, but anthocyanins are known that retain water of solvation under these conditions; hence it is uncertain that the molecule includes O_7; it may be O_6.

When hydrogen chloride was passed into a methyl-alcoholic solution of *betanidin chloride*, so as to boil the liquid, a definite change occurred in the properties of the colouring matter and analysis of the recovered salt showed that two methyl groups had been introduced. Other evidence showed that milder treatment suffices to bring about esterification and we are convinced that one of the esterified groups must be carboxyl.

The composition does not admit of the occurrence of two carboxyls unless we assume that there are only two hydroxyls in the flavylium nucleus, and from what we know of the aminohydroxyflavylium series the deep colour of betanidin could find no explanation on this basis. Therefore the formation of a second methoxyl is most probably due to etherification of a polyhydroxyphenolic section of the molecule, for example, a phloroglucinol or hydroxyphloroglucinol nucleus.

The composition and properties of betanidin chloride have led us to the speculation that it may be regarded as comprising a pentahydroxyflavylium nucleus and an ornithine residue condensed by loss of one molecule of water so as to leave four hydroxyls in the flavylium nucleus and to attach the more basic nitrogen of the ornithine directly to one of the benzene rings. Naturally, if we find that our specimens retained $1H_2O$ at 110°, there would be only three free hydroxyls in the flavylium nucleus (for example, cyanidin chloride, $C_{15}H_{11}O_6Cl$, plus ornithine, $C_5H_{12}O_2N_2$, minus $1H_2O$ gives $C_{20}H_{21}O_7N_2Cl$; or pelargonidin chloride, $C_{15}H_{11}O_5Cl$, plus $C_5H_{12}O_2N_2$ gives $C_{20}H_{23}O_7N_2Cl$ or $C_{20}H_{21}O_6N_2Cl,H_2O$). We have not obtained degradation products from betanidin, and the failure to detect even traces of phloroglucinol after fusion with alkali is perhaps significant. The investigation is being continued with the help of a process depending on adsorption of betanin on alumina.

EXPERIMENTAL.

Extraction and Fermentation of the Beet Pigment.—Of the several varieties of beet examined, that known as " Small globe " was rich in colouring matter and gave consistent results. Aqueous extracts of the sliced beet were acidified : the proportion that passed to butyl alcohol was very small indeed, even after saturation of the solution with salt; the distribution to *iso*amyl alcohol was still lower; and *cyclo*hexanol extracted no pigment whatever. A mixture of butyl alcohol and acetophenone (2 : 1), with or without picric acid, extracted a small part of the colouring matter, but not to a useful extent. A solution in 1% hydrochloric acid was bluer in tone than the aqueous solution and was unchanged after 15 days. Finely sliced beet covered with distilled water slowly evolved carbon dioxide after about 8 days, the liquid became slimy and slightly bluer, and the p_H fell from 6·1 to 4·1. The betanidin produced could now be extracted by means of butyl alcohol. After purification as described below, the distribution number, equilibrated butyl alcohol–0·5% hydrochloric acid, was found to be 82 (83), and that for equilibrated *iso*amyl alcohol–0·5% hydrochloric acid was 33 (35·5). In the presence of salt the ratios are more favourable; for example, the pigment is completely transferred from aqueous solution containing salt to *iso*amyl alcohol–acetophenone (2 : 1) or *cyclo*hexanone–*cyclo*hexanol (10 : 1). On the other hand, normal anthocyanidin behaviour was not exhibited, since the colouring matter was not extracted to any extent by ethyl acetate or by ether in the presence of picric acid from a solution in acidified brine.

In some cases the fermentation of the beet solution did not occur and hence we suspected that it was due to the growth of a micro-organism. That this is the case was proved by inoculation. A mixture (4 lb. of beet and 3 l. of water) after 2 days had p_H, 6·1; already fermented solution (25 c.c.) was then added. Fermentation proceeded with evolution of carbon dioxide. The p_H was 5·6 on the third day and 4·0 on the fifth; thereafter there was a slow increase of butyl alcohol-soluble pigment, a maximum being attained on the tenth day. After some further experiments on the effects of light and air the following process was adopted.

Finely sliced beet (56 lb.) and distilled water (45 l.) were placed in enamelled buckets and covered with toluene; after 9—11 days the hydrolytic process appeared to be complete (the solutions were tested each day in respect of p_H and formation of betanidin, and inoculation was usually unnecessary). The liquid was filtered by means of a large centrifuge, each 5 l. was mixed with concentrated hydrochloric acid (80 c.c.) and sodium chloride (1500 g.) and kept for ½ hour with occasional stirring, and the aqueous solution was then separated from the toluene by siphoning and extracted with *iso*amyl alcohol (2500 c.c.). The aqueous layer was again extracted with *iso*amyl alcohol (2500 c.c.), and this alcoholic layer used for the first extraction of another 5 l. of the aqueous solution. The *iso*amyl-alcoholic extract (30 l.) was mixed with an equal volume of light petroleum (b. p. 60—80°) and shaken with water (5 l.) in a tumbling barrel; the aqueous solution was separated, saturated with sodium chloride, and extracted with *iso*amyl alcohol (5 l.). This more concentrated solution was mixed with light petroleum (15 l.) and shaken thrice with water (300 c.c. each time.) The combined aqueous extracts were washed with benzene and concentrated to a small volume under diminished pressure of hydrogen at 30—40°. The solid was collected, drained from the small residual volume of liquid (which was added to another batch), and dried in a vacuum over potassium hydroxide. It was then extracted with the minimum quantity of absolute ethyl alcohol, filtered from sodium chloride (chiefly), diluted with one-third of its volume of water, and, after addition of concentrated hydrochloric acid (2 c.c.), evaporated to dryness below 40° under diminished pressure of hydrogen (or nitrogen). This material was again taken up in absolute ethyl alcohol, the solution evaporated to dryness as before, the residue triturated with water (5—10 c.c.), and the solid *betanidin chloride* (0·2 g. from 56 lb. of beet) collected and dried in a vacuum over potassium hydroxide [Found : C, 49·0; H, 5·1; N, 5·9; Cl, 5·0; loss at 110°, 11·7. $C_{20}H_{19}O_7N_2Cl,3H_2O$ requires C, 49·2; H, 4·9; N, 5·8; Cl, 7·3; $3H_2O$, 11·1%. Found in material dried at 110° to constant weight in a high vacuum : C, 53·8, 53·8; H, 5·2, 5·1; N, 6·4, 6·3; Cl, 5·0, 5·0; MeO, 0; MeN, trace; MeC(Kuhn–Roth), 0. $C_{20}H_{23}O_7N_2Cl$ requires C, 54·7; H, 5·2; N, 6·3; Cl, 8·1%]. The significance of the analytical data has been discussed (p. 447). Crystallisation of the salts of betanidin has not yet been effected, but is still being attempted.

An aqueous solution shows the following behaviour with reagents. Sodium carbonate—the colour becomes a little more red in tone (from reddish-purple) and then brown; concentrated hydrochloric acid—slightly bluer tone; ferric chloride—brown coloration and destruction of pigment; ammonia—colour discharged to yellowish-brown; sodium hydroxide—immediate formation of a yellow solution, after 30 seconds the original colour is largely restored on acidification, after 10 minutes the colouring matter cannot be regenerated; sodium nitrite—stable; nitrous acid—immediate destruction, forming a yellow solution; barium chloride, ferrous sulphate, stannous chloride—no visible reaction; lead acetate—solution decolorised (pale yellow) and a pale pink precipitate formed, from which the colour can be partly regenerated by treatment with hydrochloric acid; basic lead acetate—no colour change.

Esterification.—Treatment of betanidin chloride with hot propyl-alcoholic hydrogen chloride completely changed the properties of the pigment. It became bluer, much more soluble in organic solvents, and more stable. The esterified salt was completely extracted from aqueous acid solution by *iso*amyl alcohol.

Betanidin chloride (57 mg.) was dissolved in absolute methyl alcohol (*ca.* 10 c.c.), and hydrogen chloride passed through the purplish-red solution until the liquid boiled; it became markedly bluer in tone. After cooling to room temperature, hydrogen chloride was again passed, but there was no apparent further colour change. After 2 hours, the liquid was added to ether (700 c.c.) and kept for 3½ hours; the colouring matter was not completely precipitated; it was collected, dried (14 mg.) in a vacuum over potassium hydroxide, and obtained as a dark purplish-black, amorphous solid with a greenish-brown lustre (Found: loss at 110°, 7·1; MeO, 11·8. $C_{22}H_{25}O_7N_2Cl,2H_2O$ requires $2H_2O$, 6·9; 2MeO, 11·5%).

Betanidin chloride (0·5 g.) was heated at 90—95° with potassium hydroxide (8 g.) and water (3 c.c.); there was vigorous decomposition and evolution of a base with a methylamine-like odour. The temperature was raised to 200—210° during 5 minutes and maintained for 2 minutes. No product could be identified, but phloroglucinol was almost certainly absent. Evidence of the presence of a pyrrole derivative was obtained in the form of a positive reaction of one of the extracts with Ehrlich's reagent, and a fatty acid resembling butyric or valeric acid in odour was produced in small quantity.

Celosia plumosa.—The flowers were extracted with 1% hydrochloric acid. The reactions of the bluish-red solution closely resembled those of a solution of betanin. The aqueous extract did not undergo fermentation with the formation of an anthocyanidin. The pigment will, it is hoped, be studied by application of the alumina method.

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