Cacao Polyphenolic Substances

4. THE ANTHOCYANIN PIGMENTS*

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The purple colouring matter of cacao cotyledons has been shown to consist of two cyanidin glycosides (Forsyth, 1952). In that study the pigments were obtained only as amorphous powders, now known to have been grossly contaminated with colourless glucosides. The anthocyanin which moves the faster on chromatography was incorrectly considered to contain both glucose and arabinose, and the slower anthocyanin to contain glucose alone.

Better methods of extraction and purification have now yielded crystalline pigments shown by comparison with synthetic specimens to be 3-α-L-arabinosidyl cyanidin chloride and 3-β-D-galactosidyl cyanidin chloride.

EXPERIMENTAL

Synthetic cyanidin-3-monoglycosides. 3-β-D-Glucosidyl cyanidin chloride (chrysanthemin), 3-β-D-galactosidyl cyanidin chloride (idaein), and 3-β-D-xylosidyl cyanidin chloride were synthesized by the classical route (MacDowell, Robinson & Todd, 1934).

In contrast to the findings of MacDowell *et al.* (1934), no difficulty was experienced in crystallizing the xyloside as chloride nor was it found to be exceptionally unstable to acid. It crystallized as long needles from 5% ethanolic HCl [all alcoholic HCl percentages are for dry HCl gas in the alcohol (w/w)]. (Found on anhydrous pigment: xylose, 33·0. Calc. for monopentoside, 33·0%.)

3-α-L-*Arabinosidyl cyanidin chloride*, which had not hitherto been prepared, is readily synthesized by the usual route, with methods identical with those successful for the preparation of the xyloside. The picrate crystallizes as small dark needles from saturated aqueous picric acid solution at 0°. The chloride is formed by dissolving in 5% methanolic HCl and precipitating with ether. On dissolving this precipitate in 0·05N aqueous HCl and adding an equal volume of 5% ethanolic HCl the arabinosidyl cyanidin chloride crystallizes almost immediately. It is readily recrystallized from the same solvents as almost black prisms, with a green glance when dry. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 53·6; H, 4·1. Calc. for $C_{20}H_{19}O_{10}Cl$: C, 52·8; H, 4·2%.)

Cacao pigments. The beans used were from the clone Imperial College Selection no. 1. Freshly dried cacao cotyledons (1 kg.) are extracted with 0·1N-HCl (8 l.), the extract is saturated with NaCl, and the ethyl acetate-soluble and saline-insoluble phenols are removed, as previously described (Forsyth, 1955). The saline extract remaining is then shaken with *n*-butanol (¼ vol.) and the butanol concentrated under reduced pressure to about 200 ml. The NaCl is removed by filtration and the pigments are precipitated by dilution with 5 vol. of light petroleum (80–100°). The deep-red precipitate is filtered, washed with light petroleum and dried *in vacuo*.

A cellulose-pulp column (Forsyth, 1952) of 40 cm. × 3·7 cm. is of sufficient size to give two well-separated pigment bands when 2 g. of the crude pigments is chromatographed with the top layer of a pentanol–acetic acid–water (4:1:5, by vol.) mixture.

The column is extruded, the individual bands are excised and the pigments separately eluted with methanol. The methanol extracts are concentrated to small volume under reduced pressure and precipitated by dilution with light petroleum (80–100°). The crude pigments are dissolved in a minimal volume of saturated aqueous picric acid and crystallized overnight at 0°. The picrates are centrifuged off and washed with saturated aqueous picric acid, then with ether. They are converted into the chlorides on dissolving in 5% methanolic HCl and precipitating with ether. The chlorides are crystallized by dissolving in 0·05N aqueous HCl, adding an equal volume of 5% ethanolic HCl and allowing to stand at 0°. They can be purified by repeated recrystallization in this manner.

Sugar estimation. The glycosides are hydrolysed with 2N-H_2SO_4 for 30 min. at 100°. The glycosidic sugars present in the cacao bean, i.e. glucose, galactose, arabinose and xylose, are best separated on paper with the top layer of an ethyl acetate–pyridine–water (3:1:3, by vol.) mixture as solvent. They are quantitatively estimated on the paper by a modification of the method of Shu (1950), in which the sugars are not eluted from the paper but paper blanks are used. An internal standard (Flood, Hirst & Jones, 1947) is used.

RESULTS AND DISCUSSION

The faster-moving anthocyanin (3-α-L-*arabinosidyl cyanidin chloride*). When first crystallized this pigment gave on hydrolysis glucose and xylose as well as the main sugar. Recrystallization, however, gave a product yielding only a single sugar. Xylose was the more persistent contaminant, but after four crystallizations it was not generally detectable in the hydrolysate. The single sugar could not be separated from added arabinose in several solvents. The anthocyanidin from the hydrolysis of the pure pigment has the crystalline form, R_F values (Bate-Smith, 1949, 1954), colour reactions (Robinson & Robinson, 1931; Robertson & Robinson, 1929),

* Part 3: Forsyth (1955).

visible- (Scott-Moncrieff, 1930) and ultraviolet-absorption spectra of cyanidin obtained by the hydrolysis of the synthetic 3-α-L-arabinosidyl cyanidin chloride.

As isolated the anthocyanin contained 1 mol. prop. of water of crystallization. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 52·6; H, 3·9; Cl, 7·9. Calc. for $C_{20}H_{19}O_{10}Cl$: C, 52·8; H, 4·2; Cl, 7·8 %.)

The arabinose was estimated with rhamnose as an internal standard. [Found (calc. on anhydrous pigments): fast anthocyanin, 33·9, 34·1, 35·0, 35·5; synthetic 3-α-L-arabinosidyl cyanidin chloride, 34·0. Calc. for monopentoside, 33·0 %.]

The slower-moving anthocyanidin (3-β-D-galactosidyl cyanidin chloride). When first crystallized this pigment gave on hydrolysis glucose and arabinose together with galactose. Several crystallizations were required before the product gave only a single sugar on hydrolysis. The single sugar could not be separated from added galactose in several solvents. The anthocyanidin was cyanidin, as before, but on first crystallizing from the hydrolysed anthocyanin solution its crystalline form was different from that of the cyanidin from the synthetic arabinoside, but identical with that from the synthetic galactoside. As isolated the anthocyanin contained 2·5 mol.prop. of water of crystallization. (After drying for 3 hr. at 100° *in vacuo*. Found: C, 51·6; H, 4·3; Cl, 7·7. Calc. for $C_{21}H_{21}O_{11}Cl$: C, 52·0; H, 4·3; Cl, 7·3 %.)

The galactose was estimated with arabinose as internal standard. [Found (calc. on anhydrous pigment): slow anthocyanin, 37·4, 38·1; synthetic 3-β-D-galactosidyl cyanidin chloride, 37·6. Calc. for monohexoside, 37·2 %.]

Comparison with synthetic 3-glycosidyl cyanidin chlorides. The two isolated cacao monoglycosides have the sugar in the 3-position since they give identical colour changes as acid-base indicators (Robertson & Robinson, 1929) and have ultraviolet and visible spectra very similar to those of the four synthetic 3-glycosides, which themselves give identical colour reactions and have spectra closely similar to one another. The pentosides are readily distinguished from the hexosides by their R_F values in *n*-butanol–acetic acid (Bate-Smith & Westall, 1950): galactoside, 0·33; slower cacao anthocyanin, 0·33; glucoside, 0·34; arabinoside, 0·41; faster cacao anthocyanin, 0·41; xyloside, 0·43.

On paper chromatography mixtures of the arabinoside and xyloside or galactoside and glucoside (Robinson & Smith, 1955) separate sufficiently (although not into two discrete spots) to show that in such cases two compounds are present. If the glucosidyl and xylosidyl cyanidins are present they can be detected in the presence of the galactosidyl and arabinosidyl compounds. They are not detected in extracts of the cacao bean. The colour intensity of equal concentrations of the aglycone, pentoside and hexoside are in the ratio 1:0·62:0·52, the isolated pigments having the same intensity as the corresponding synthetic compounds. The slower-moving anthocyanin has the crystalline form, both as picrate and chloride, of the corresponding 3-β-D-galactosidyl cyanidin salts, the faster-moving anthocyanin that of the 3-α-L-arabinosidyl cyanidin salts. A few specks of 3-β-D-galactosidyl cyanidin chloride dusted on a saturated ethanolic HCl solution of the slower compound causes immediate crystallization; the same occurs when the faster compound is dusted with 3-α-L-arabinosidyl cyanidin chloride.

Confirmation by enzymic hydrolysis. During the curing of the cacao bean to produce the basic raw material of the chocolate industry the two anthocyanin pigments are destroyed. The first stage is enzymic hydrolysis of the glycosides (Forsyth & Quesnel, unpublished work). A glycosidase preparation of considerable activity and specificity can be prepared from the fresh bean.

Freshly dried cacao cotyledons are fractionated to separate the pigmented cells by sedimentation in light petroleum as described by Brown (1954). The

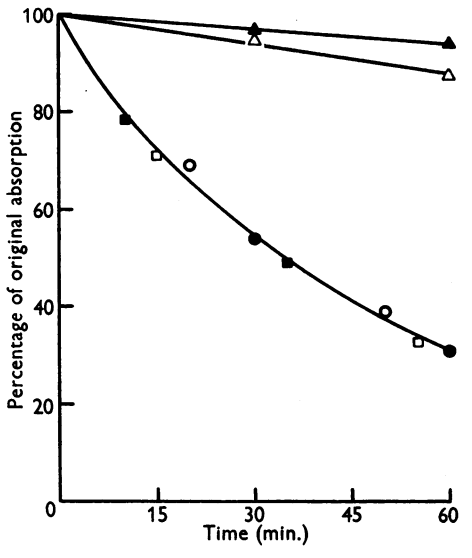

Fig. 1. Enzymic hydrolysis of cyanidin-3-glycosides with cacao-bean preparation (see text) at 44° and pH 4·0 in the absence of air. Anthocyanin [1 mg. in 50 % (v/v) ethanol (0·2 ml.)] was present in the top of the Thunberg tube; 2 ml. of enzyme preparation (pH 4·0) was in the bottom. The tubes were evacuated, allowed to equilibrate at 44°, and the contents were mixed. After reaction, the volume was made up to 50 ml. with 0·2 N ethanolic HCl and the absorption read with yellow-green filter, max. transmission 540 mμ. ▲, Xyloside; △, glucoside; ■, galactoside; □, slow-moving cacao anthocyanin; ●, arabinoside; ○, fast-moving cacao anthocyanin.

lighter fraction, mainly free from pigmented cells, is washed with 50 % (v/v) aqueous acetone at 0° until free from phenols, and then with 80 %, and finally anhydrous, acetone to dry it. The acetone-dried white powder (25 g.) is extracted with 0·2M-Na barbital buffer (500 ml.), pH 8·0, in a blender, after first passing it as a paste through a triple-roll mill. After centrifuging, the extract is adjusted to pH 4·0 with 0·2N-HCl, filtered, and 2 vol. of ethanol added to the acid filtrate at 0°. The resultant precipitate is centrifuged off and dissolved in 50 ml. of McIlvaine buffer, pH 4. It is stable at 0° for at least 4 weeks. The activity of such a preparation towards the anthocyanins is shown in Fig. 1. It is necessary to use anaerobic conditions to preclude polyphenol oxidase activity. The hydrolysis is most readily followed by colorimetry. A Spekker photoelectric absorptiometer was used with a 0·5 cm. cell and a yellow-green filter (no. 7 of Kodak set H 769; peak transmission 540 mμ.). Under the conditions used, the resultant cyanidin is mainly, but not entirely, in the colourless pseudo-base form. This accounts for the residual adsorption (Fig. 1), although the sugars are liberated stoicheiometrically as confirmed by direct quantitative chromatography.

It would appear that β-D-galactopyranosyl activity is the major glycosidase function present and that the isolated pigments are indeed 3-β-D-galactosidyl cyanidin chloride and the corresponding 3-α-L-arabinosidyl cyanidin chloride of closely similar structure.

Huang (1956) has used a similar technique with a fungal β-glucosidase to confirm that the anthocyanin pigment of the blackberry is a β-glucoside.

Confirmation by infrared spectra. The infrared spectra of all the 3-monoglycosides were determined with the KBr pellet technique (Li & Wagenknecht, 1956). The results of this analysis confirmed that the slower-moving cacao anthocyanin was indeed the 3-β-D-galactosidyl cyanidin chloride and the faster one the 3-α-L-arabinosidyl cyanidin chloride.

SUMMARY

The anthocyanin pigments of the cacao bean are 3-β-D-galactosidyl and 3-α-L-arabinosidyl cyanidin salts.

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Manganese Oxidation in the Pea Plant (*Pisum sativum* L.) Grown Under Conditions of Manganese Toxicity

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Manganese is known to be an essential micronutrient of plants. It has frequently been suggested that its physiological effects may, at least in part, be due to its capacity for valency change. Kenten & Mann (1949, 1950) showed that Mn^{2+} ions at physiological concentrations are oxidized by peroxidase systems and suggested that *in vivo* manganese is involved in a cycle of oxidation and reduction which may be partly responsible for the stimulating effects of manganese on plant respiration shown by Lundegårdh (1939). But there is as yet no direct evidence that manganese is oxidized in plants grown under normal conditions of manganese supply. If the oxidation of manganese does in fact occur *in vivo* under such conditions, then, as suggested by Kenten & Mann (1952*a*), it might be expected that the oxidation product would be reduced by plant metabolites as rapidly as it was