Cacao Polyphenolic Substances

5. THE STRUCTURE OF CACAO 'LEUCOCYANIDIN 1'

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Forsyth (1955) showed that the fresh cacao bean contains three 'leucocyanidin' compounds, which were termed L1, L2 and L3 in order of decreasing concentration in the bean. We have previously shown (Forsyth & Roberts, 1958) that the cacao leucocyanidin 1 molecule is built up of two C₁₅ flavan units, one of which is (-)epicatechin, and the other a residue which yields cyanidin on treatment with acid. Structure (I) was tentatively suggested as the most likely structure for the whole molecule. Recent work by Gramshaw, Johnson & King (1958), taken in conjunction with the results reported here, show that (I) is incorrect, and (II) is the most probable structure of cacao leucocyanidin.

(I)

(II)

MATERIALS AND METHODS

Imperial College selection clone no. 1 cacao beans were used throughout. Pulp was removed from the fresh beans by agitation in a household washing machine, and the pulp-

free beans were spread out to dry in the sun for 3-4 days. Drying was essential, as attempts to extract the polyphenols from fresh beans were hindered by polyphenol-oxidase activity.

Isolation of leucocyanidin 1

Dry beans (1 kg.) were blended in small lots in a Waring Blender with portions of 2 l. of 80% ethanol. After blending the suspension was filtered through sintered glass. The bean powder was returned to the Blender and the extraction repeated twice.

The combined dark-purple filtrates (6 l.) were evaporated *in vacuo* on a water bath to approx. 500 ml. This solution was extracted with an equal volume of light petroleum (100-120°), and then with a half volume of ether to remove any residual petroleum, which would interfere with subsequent steps in the isolation. The aqueous solution was then subjected to an ethyl acetate-water counter-current extraction and subsequent chromatography as described by Forsyth (1955). Leucocyanidin 1 was obtained as a white amorphous powder with m.p. 197-203° (decomp.); $[\alpha]_D^{25} + 40^\circ$ in acetone (c, 10). U.v. max., 277 m μ ($E_{1\%}^{1\text{cm}}$, 185) (Found: C, 60.6; H, 4.6; OMe, 0. C₂₀H₂₄O₁₅ requires C, 60.6; H, 4.4%). The compound was homogeneous on chromatograms in water, butanol-acetic acid-water (4:1:5) and *m*-cresol-acetic acid-water (50:2:48) and moved as one round spot on electrophoresis in 0.05 M-boric acid-sodium borate buffer, pH 7.6.

Methylation of leucocyanidin 1

Methylation was carried out in two ways, either by the action of dimethyl sulphate in acetone in the presence of anhydrous potassium carbonate, or, better, with diazo-methane.

Dimethyl sulphate-potassium carbonate method. Leucocyanidin 1 (1 g.) was dissolved in acetone (100 ml.) and anhydrous potassium carbonate (10 g.) and dimethyl sulphate (2 ml.) were added. The solution was heated under reflux. After 1 hr. a sample of the solution was removed and tested for the presence of phenols with the ferric chloride-potassium ferricyanide reagent of Barton, Evans & Gardner (1952). Dimethyl sulphate (0.5 ml.) was again added and refluxing continued for a further 1 hr. The test was then repeated. This procedure was continued until the test for phenols was negative (4 hr.; total dimethyl sulphate added: 3.5 ml.). The solution was filtered and the potassium salts were washed with acetone (50 ml.). A few drops of concentrated aqueous ammonia solution were added to the filtrate to destroy any residual dimethyl sulphate. The solution, after dilution with water, was extracted with chloroform (3 × 50 ml.) and the extract dried (sodium sulphate). The extract was chromatographed on silica-

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treated paper (Roberts, 1958) and the products were detected with a solution of vanillin in conc. hydrochloric acid. Three compounds were usually revealed: 1, the major spot of the methylated leucocyanidin; 2, a minor spot with R_F identical with that of 3':4':5:7-tetra-*O*-methyl-epicatechin; 3, a trace of a substance of high R_F , which turned red on exposure to cold conc. hydrochloric acid. The relative amount of the minor constituents was found to vary depending on the presence of traces of water or sulphuric acid in the reagents used. It was frequently necessary to purify the methylated leucocyanidin 1 by chromatography on a silica-gel column. This was achieved as follows.

Silica gel (Hopkin and Williams Ltd., M.F.C. grade; 20 g.) was shaken with Celite 545 (Johns-Manville; 10 g.) and the mixture packed into a column 6 in. $\times$ 1 in. The methylated leucocyanidin solution was concentrated *in vacuo* to approx. 5 ml. and 15 ml. of benzene was added. The solution was applied to the column and the chromatogram developed with 25% (v/v) of chloroform in benzene (300 ml.). This solvent eluted the impurities, and the methylated leucocyanidin could then be eluted with chloroform. The chloroform solution on evaporation to dryness yielded up to 700 mg. of methylated leucocyanidin 1 as an amorphous solid. Solution of this solid in hot ethanol and cooling deposited the methylated leucocyanidin as a white powder m.p. 132° (decomp.) [$\alpha_D^{25} + 75^\circ$ in acetone (c, 2.5) [Found: C, 64.4; H, 6.0; OMe, 34.6. Mol.wt. (Rast) 710, 725. $C_{30}H_{18}O_8(OMe)_8$ requires C, 64.6; H, 6.0; OMe, 35.1%. Mol.wt. 706].

The compound was insoluble in dilute aqueous sodium ferric chloride solution and gave no colour with ethanolic ferric chloride solution.

The infrared spectrum of the compound, by comparison with the spectrum of 3':4':5:7-tetra-*O*-methyl(-)epicatechin, shows the presence of two (probably adjacent) hydroxyl groups per molecule, but no significant carbonyl absorption.

Methylation with diazomethane. A solution of diazomethane in ether was prepared from nitrosomethylurea (10 g.) and distilled. One-half of the ethereal distillate was added to a solution of leucocyanidin 1 (1 g.) in methanol (100 ml.) at 0°. The solution was kept at 0° for 24 hr. The other half of the diazomethane was then added and the solution kept at 0° for a further 48 hr. A test with the ferric chloride-potassium ferriocyanide reagent indicated that all the phenolic groups had then been methylated.

After evaporation to 25 ml. *in vacuo*, water was added and the mixture cooled to 0° and filtered. The amorphous product was dissolved in hot ethanol and cooled, yielding a white powder, m.p. 132° (decomp.), identical with the material isolated from the dimethyl sulphate-potassium carbonate preparation.

Acetylated leucocyanidin 1

This compound was prepared in the usual way by the action of acetic anhydride and pyridine at room temperature for 48 hr. The reaction mixture was poured into ice-water, stirred and the product isolated by filtration. Acetylated leucocyanidin 1 crystallized from ether at -60°, m.p. 135°, as colourless prisms [Found: C, 59.4; H, 4.7; Ac, 36.65, 36.4. Mol.wt. 1100 (Rast) 1150 (ebullioscopic in chloroform). $C_{30}H_{18}O_{13}(Ac)_8$ requires C, 59.3; H, 4.5; Ac, 36.9%. Mol.wt. 930]. The previously reported value of 42.1% of acetyl (Forsyth & Roberts, 1958) has

now been shown to be erroneous. This result was probably due to the use of alkaline conditions of hydrolysis during the determination, which in this class of compound frequently yields excessively high values.

Attempted acetylation of methylated leucocyanidin 1. Acetylation of methylated leucocyanidin 1 was attempted by the above method. The product, a white amorphous solid, m.p. 120-130°, contained no acetyl group.

RESULTS

Degradations of leucocyanidin 1

Production of cyanidin. Treatment of leucocyanidin 1 in strongly acid solution (2N) at the boiling point produced an intense red, which was extracted into butanol. A sample of this butanol solution spotted on a paper chromatogram and developed in Forestal solvent (acetic acid-water-conc. hydrochloric acid, 30:10:3) showed a spot (R_F 0.50) identical in colour with, and not separated from, a sample of synthetic cyanidin.

Leucocyanidin 1 (50 mg.) was dissolved in butanol containing 20% (v/v) of conc. hydrochloric acid (15 ml.) and the solution heated for 30 min. in a sealed tube at 100°. The solution was then washed with an equal volume of water, and the butanol layer was applied to a column of cellulose which had been equilibrated with the upper phase of a butanol-2N-hydrochloric acid mixture (1:1, v/v). Development of the chromatogram with the same solvent washed down a fast-moving brown band, followed closely by a broad red band. The red band was collected and concentrated *in vacuo* on a water bath. Chromatography of this solution on paper chromatograms developed in butanol-2N-hydrochloric acid or Forestal solvent showed the presence of a single spot, indistinguishable in R_F and colour from a sample of synthetic cyanidin chloride [R_F (Forestal solvent), 0.50; R_F (butanol-2N-hydrochloric acid), 0.70]. The absorption spectrum of the solution between 400 and 600 m μ was identical with that of the synthetic cyanidin chloride (λ_{max} 540 m μ) in the same solvent.

No attempt was made to determine the yield of product on a dry-weight basis, as previous experience indicated that acid eluents from cellulose columns frequently contain colourless cellulosic impurities in appreciable amounts. The yields of cyanidin obtainable from leucocyanidin 1 have been studied by making use of the cyanidin absorption maximum at 540 m μ . The visible absorption spectrum of acid-hydrolysed leucocyanidin 1 exhibits two absorption maxima, a minor one at 460 m μ and the major peak at 540 m μ (cf. Raudnitz, 1958). (It is likely that the peak at 460 m μ is due to a catechin polymer, as treatment of epicatechin in strongly acid solution produces a

substance having an absorption maximum at this wavelength; cf. Swain & Hillis, 1959.) The maximum yield of cyanidin which could be obtained was 14%, based on the weight of leucocyanidin 1. This was achieved by use of a mixture of butanol-water-conc. hydrochloric acid (7:2:1), heated for 30 min. at 100° in a sealed tube. The yield of cyanidin was not significantly affected by the use of an inert atmosphere.

Isolation of (-)epicatechin

Hydrolysis in 0.1N-hydrochloric acid. Forsyth (1953) has discussed some quantitative aspects of this hydrolysis. Leucocyanidin 1 (500 mg.) dissolved in 0.1N-hydrochloric acid (25 ml.) was boiled for 5 min., cooled and passed through a diagonal five-funnel ethyl acetate-water counter-current extraction (25 ml. phases) (Bush & Densen, 1948). After separation, paper chromatography of each of the ten fractions showed that epicatechin was present in the ethyl acetate layers and the aqueous layers contained leucocyanidin 1 and more complex products. The ethyl acetate layers were bulked, dried (sodium sulphate) and evaporated to dryness *in vacuo*. The solid residue was dissolved in water (10 ml.) and extracted with ether (10 × 10 ml.). The combined ether extracts were dried (Na₂SO₄) and taken to dryness *in vacuo*. The solid residue (about 10 mg.) was crystallized from water (1 ml.), yielding 3 mg. of colourless crystals with m.p. 232–234° (decomp.). The mixed m.p. with an authentic sample of (-)epicatechin was 231–235° (decomp.).

Hydrolysis in sulphurous acid. Leucocyanidin 1 (1 g.) in water (50 ml.) was heated under reflux in a stream of sulphur dioxide for 2½ hr. Paper chromatography of the hydrolysis mixture showed that this treatment had split the molecule into halves, one of which had *R_F* values corresponding with epicatechin, and the other had *R_F* (water) 0.90, *R_F* (butanol-acetic acid-water) 0.20. This substance is referred to as substance A below. The two products were separated as follows. The cooled solution was applied to a column (40 cm. × 3 cm.) of washed cellulose powder (Solka Floc) and the chromatogram was developed with water. Fractions (10 ml.) were collected and examined by paper chromatography. Those fractions containing substance A and those containing epicatechin were separately combined, frozen and evaporated to dryness in a vacuum desiccator over conc. sulphuric acid.

The solid residue from the solution of substance A (459 mg.) could not be crystallized. A sodium-fusion test for elements indicated the presence of sulphur and a test with cold sodium bicarbonate solution showed that the substance was acidic. By analogy with lignin chemistry the substance was

thought to be a sulphonic acid. Treatment of the substance with boiling 20% hydrochloric acid in butanol gave a purple solution, which was shown by chromatography in Forestal solvent to contain a small quantity of cyanidin chloride together with a pigment moving as a purple spot *R_F* 0.8. Substance A was not investigated further.

The solid residue from the epicatechin solution (303 mg.) was dissolved in acetone (25 ml.) and the solution filtered to remove a small amount of cellulose impurity. Evaporation of the acetone and crystallization of the residue from water gave (-)epicatechin as needles, m.p. 229°; [α]_D²⁵ -36° in acetone (c, 2.5) (155 mg.). The mixed melting point with an authentic sample of (-)epicatechin was 229–238°.

(-)epiCatechin was submitted to the hydrolysis described above and recovered chemically unchanged in 90% yield. After recrystallization from water it had m.p. 235–240°; [α]_D²⁵ -46° in acetone (c, 2).

Stability of leucocyanidin 1 in aqueous solution

To portions (5 ml.) of leucocyanidin 1 solution (1 mg./ml.) were added 0.05M-buffer solutions (5 ml.) in the range pH 4.0–10.0. After the solutions had been allowed to stand at room temperature for 18 hr., samples (2 ml.) were taken and conc. hydrochloric acid (1 ml.) and butanol (7 ml.) were added to each one. The solutions were then heated in sealed tubes at 100° for 30 min., to convert the

Fig. 1. Yields of cyanidin obtained by acid hydrolysis of leucocyanidin 1 after it had been standing for 18 hr. in buffer solutions.

leucocyanidin into cyanidin. Determinations of extinction of the solutions at $540\text{ m}\mu$ gave a measure of the leucocyanidin remaining. The results are plotted in Fig. 1. It can be seen that outside the range pH 6.0–7.0 leucocyanidin 1 is unstable, decomposing to compounds which do not give cyanidin on treatment with acid. The pH of the fresh cacao cotyledon is 6.5.

Degradations of methylated leucocyanidin 1

Hydrolysis in acid solutions. Methylated leucocyanidin 1 (600 mg.) was dissolved in 0.5N-hydrochloric acid in 50% ethanol (60 ml.) and boiled under reflux for 30 min. The red solution was poured into water and exhaustively extracted with chloroform, then with a small volume of ether. The combined organic layers were shaken with 2N-hydrochloric acid (50 ml.) and the red aqueous layer was added to the aqueous residue from the extraction.

The organic layer was dried (sodium sulphate) and neutralized (sodium bicarbonate) (fraction A). The combined aqueous layers were extracted with butanol, and the butanol extract was concentrated to small volume *in vacuo* (fraction B).

Fraction A was evaporated to dryness *in vacuo* and the residue was dissolved in 25% (v/v) chloroform in benzene (10 ml.). This solution was applied to a silica-gel-Celite column (6 in. $\times$ 1 in.), and the chromatogram developed with the same solvent. The eluted fractions were chromatographed on silica-treated paper, and those containing tetra-*O*-methyl*epicatechin* were bulked (100 ml.). Evaporation of this solution *in vacuo* gave 101 mg. of a gum which crystallized on rubbing with methanol. Recrystallization from methanol gave colourless needles, m.p. 142° ; $[\alpha]_D^{25} = -51.5^\circ$ in acetone (c, 1). The m.p. was unchanged after admixture with an equal amount of authentic 3':4':5:7-tetra-*O*-methyl(-)*epicatechin*. After elution of the methylated *epicatechin*, the solvent was changed to chloroform and a fraction (150 ml.) obtained containing unchanged methylated leucocyanidin 1. Evaporation of this solution gave a white amorphous powder (173 mg.), m.p. $127\text{--}132^\circ$, which was undepressed by admixture with a sample of the original compound.

Fraction B was absorbed on the centre of a circle of Whatman Seed Paper, 15 cm. in diameter (previously washed with ethanol), and chromatographed with the butanol-2N-hydrochloric acid solvent by the Rutter (1950) technique. The red band, R_F 0.5–0.6, was cut out and the pigment eluted from the paper with boiling ethanol containing a few drops of conc. hydrochloric acid. The pigment obtained was not separated by chromatography in butanol-2N-hydrochloric acid or butanol-acetic acid-water from an authentic sample of 3':4':5:7-tetra-*O*-methylcyanidin [synthesized by

the method of Gramshaw *et al.* (1958)]. The visible absorption spectrum of the solution was indistinguishable from that of the synthetic tetra-methylcyanidin in the same solvent (λ_{max} $530\text{ m}\mu$).

Attempted hydrolysis in alkaline solution. Methylated leucocyanidin 1 (10 mg.) was boiled in 0.5N-sodium hydroxide in aqueous 50% ethanol (10 ml.) for 30 min. The solution was then diluted with water and extracted with chloroform. The chloroform solution was chromatographed together with control samples of methylated leucocyanidin 1 and methylated *epicatechin* on silica-treated paper with ether as the developing solvent. Compounds were detected with a solution of vanillin in conc. hydrochloric acid. No methylated *epicatechin* was present in the hydrolysis mixture. The only detectable compounds were unchanged methylated leucocyanidin 1 and a trace of a compound with R_F identical with that of the product obtained by methylation of cacao leucocyanidin 2. This result indicated that leucocyanidin 1 and leucocyanidin 2 may be stereoisomers. The structural relationship of the two compounds is being investigated.

Oxidation with periodic acid. Methylated leucocyanidin 1 (77 mg.) was dissolved in aqueous 50% methanol (99 ml.) and 0.5M-periodic acid (1 ml.) was added. A blank solution containing only periodic acid was prepared at the same time. Samples (5 ml.) were taken from each solution at specified time intervals and the residual periodic acid was determined in the usual way by titration with standard sodium arsenite solution. No periodic acid was utilized during 3 hr. After 10 days in the dark at room temperature, approx. 0.5 mole of periodic acid had been consumed/mole of methylated leucocyanidin 1.

Oxidation with lead tetra-acetate. A similar experiment to the above was carried out with lead tetra-acetate in acetic acid as the oxidant. It was found that methylated leucocyanidin 1 rapidly consumed the oxidant to constant value (1 mole/mole of methylated leucocyanidin). This result does not necessarily indicate glycol cleavage, as subsequent experiments showed that 3':4':5:7-tetra-*O*-methyl*epicatechin* is oxidized under the same conditions, with the consumption of 0.5 mole of oxidant.

DISCUSSION

The identification of 3':4':5:7-tetra-*O*-methylcyanidin and 3':4':5:7-tetra-*O*-methyl(-)*epicatechin* as the sole products of acid hydrolysis of methylated leucocyanidin 1, taken with the analytical figures and molecular weights obtained for the leucocyanidin and its derivatives, indicate that the leucocyanidin 1 molecule is built up from two C_{15} flavan units. One of the units is (-)*epicatechin* and the other a residue which yields cyanidin on

treatment with acid. The ease of hydrolysis of leucocyanidin 1 and its methyl ether in acid solution, and the stability of the methyl ether in alkaline solution, indicate the possibility of an acetal type of linkage between the halves of the molecule. As the products of hydrolysis of methylated leucocyanidin 1 possess no free phenolic groups, and the general instability of the molecule eliminates the possibility of C-C bond between the units, the linkage must be concerned with the alcoholic hydroxyl groups in the halves. Consideration of all the above-mentioned factors, and allowing an ether bond as a possible mode of linkage, there are seven possible structures for the molecule. Of these, four (including I) possess a hydroxyl group at C₂. Recent work by Gramshaw *et al.* (1958) shows that a hydroxyl group in this position exists largely as the keto form (i.e. as the dihydrochalcone) and exhibits pronounced absorption in the carbonyl region of the infrared spectrum. Since methylated leucocyanidin 1 has no absorption in this region, structures of this type can be eliminated. Further, (IV) may be eliminated on the grounds that a compound of this structure would yield dihydroquercetin on hydrolysis in dilute acid, a product which is not obtained from leucocyanidin 1. Decision between structures (II) and (III) must be made on the basis of reactivity of the hydroxyl groups. These groups in (III) are secondary and apparently not sterically hindered, and would therefore be expected to show the normal reactions of such hydroxyl groups: i.e. they would be readily acetylated, and easily

cleaved by periodic acid oxidation. As the hydroxyl groups in methylated leucocyanidin 1 undergo these reactions with difficulty, if at all, (II) must be accepted as the most likely structural formula for leucocyanidin 1. The lack of reactivity of the C₄ hydroxyl group is presumably due to steric hindrance by the *epicatechin* molecule.

We realize that linkage of two large units by a semiketal band in a stable molecule is unusual. The main property of this class of compound is, however, its instability. We have been unable to detect even a transient ketonic group on hydrolysis, but it is well known that the pseudo base of cyanidin is entirely in the enolic form. Structure (III) would be a more stable molecule and cannot be completely discarded. Until the absolute configuration of (-)-*epicatechin* is settled it is impossible to determine whether the *epicatechin* group could hinder both hydroxyl groups in this structure.

SUMMARY

1. The chief products of acid hydrolysis of cacao leucocyanidin 1 are (-)-*epicatechin* and cyanidin. When the non-phenolic octa-*O*-methyl ether of leucocyanidin 1 is used as starting material, 3':4':5:7-tetra-*O*-methylepicatechin and 3':4':5:7-tetra-*O*-methylcyanidin are obtained. These facts, taken with the molecular weights and analytical figures obtained for the leucocyanidin and its derivatives, indicate that the leucocyanidin 1 molecule is built up of two C₁₅ flavan units.

2. Consideration of the general chemical and physical properties of the compound leads to two possible structures in which the two C₁₅ residues are joined by acetal-type links between their alcoholic hydroxyl groups. No decision is made between these structures.

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