Specialities (Syntex) Co. Inc., New York City, for corticosterone and hydrocortisone; and to Dr W. J. Tindall of Organon Laboratories Ltd. for deoxycorticosterone. Dr K. Hallas-Møller of the Novo Terapeutisk Laboratorium, Copenhagen, kindly gave us generous supplies of crystalline insulin free from glucagon. The technical assistance of Messrs S. C. Watson and B. W. E. Peaple is much appreciated, and we are indebted to Miss May Beard for the care of the sheep.

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Cacao Polyphenolic Substances

3. SEPARATION AND ESTIMATION ON PAPER CHROMATOGRAMS*

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(*Received* 8 *October* 1954)

Previous work on the chromatography of cacao polyphenols was carried out on one-way chromatograms with amyl alcohol–acetic acid, *n*-butanol–acetic acid, or phenol (Forsyth, 1952*a*). It was shown that the main polyphenols present could be grouped as cyanidin glycosides, catechins and the so-called 'leucoanthocyanins'. The introduction of water as a solvent for the separation of polyphenols on a paper chromatogram (Roberts & Wood, 1953) has greatly assisted the study of the cacao polyphenols, especially the ill-defined 'leucoanthocyanin' group. A previous attempt (Forsyth, 1952*b*) to estimate the polyphenols on the chromatograms has been greatly improved. Williams (1953) has applied a similar method to the tannin of cider.

MATERIALS AND METHODS

Imperial College of Tropical Agriculture Selection Clone no. 1 beans were used throughout.

Dehydrated cotyledons. Great care must be taken in the drying of cacoa beans because of the powerful oxidase systems present. Fortunately the enzymes are spatially separated from the polyphenols in the fresh bean. Dehydrated bean powder can therefore be prepared (Forsyth, 1952*b*) with the phenolic components unchanged (Forsyth & Rombouts, 1952).

Polyphenol storage cells. The cells containing the polyphenols were separated from powdered dried cotyledons by sedimentation in light petroleum (b.p. 80–100°) by the method of Brown (1954). It is preferable to use a long shallow trough, tilted at a slight angle with the light petroleum flowing gently over the powder. In this way a heavy fraction containing only purple cells (97% soluble in 70% (v/v) aqueous acetone) was obtained. These cells were free from polyphenol oxidase which could be recovered from a lighter fraction (Forsyth & Quesnel, unpublished.)

Ethyl acetate soluble polyphenols. Freshly dried cacao cotyledons (1 kg.) were blended in small lots in a Waring Blendor with parts of 8 l. of 0·1 N-HCl cooled to 0°. After blending, the bulk of the 0·1 N-HCl, cooled to 0°, was added and the suspension stirred for 15 min. The suspension was then filtered through muslin and cleared in a Sharples centrifuge. The acid extract (*ca.* 6 l.) was saturated with NaCl and stirred for 15 min. with ethyl acetate (3 l.). The mixture was separated by passing through a Sharples centrifugal separator and the aqueous layer again treated with ethyl acetate (1·5 l.). The combined ethyl acetate extracts were dried over Na_2SO_4 (150 g.) containing $NaHCO_3$ (50 g.). Because of the known instability of the leucocyanidins in acid solution (Forsyth, 1953) no delay was warranted up to this stage. Thereafter the ethyl acetate

* Part 2: Forsyth (1952*b*).

extract was left overnight to dry and then concentrated under reduced pressure (nitrogen bubbler) to about 250 ml. and the polyphenols precipitated by dilution with $CHCl_3$ (6 vol.). The light tan precipitate was collected on a sintered glass funnel, washed with $CHCl_3$, and dried *in vacuo* (yield about 15 g.). No effort was made to extract completely the polyphenols from the bean powder. Much greater volumes of solvents would have been required and, with the increased time, degradation would have taken place.

Catechin and leucocyanidin fractions. The ethyl acetate-soluble polyphenols (10 g.) were passed through a 10-funnel diagonal countercurrent separation (Bush & Densen, 1948) with 250 ml. lots of ethyl acetate and 250 ml. lots of water previously mutually saturated. In this separation 10 funnels with final complete separation of the phases yielded 20 fractions. The catechins were mainly confined to the ethyl acetate fractions (2–10) and the leucocyanidins to the aqueous fractions (11–19). The extremes 1 and 20 contained much brown pigment and were discarded. Fractions 2–10 were combined, dried, concentrated, and precipitated as before to give a catechin rich fraction (6·2 g.). Fractions 11–19 were combined, saturated with salt, extracted with ethyl acetate, dried, concentrated, and precipitated as before to give a leucocyanidin-rich fraction (1·9 g.).

(−)-epi*Catechin.* This was readily crystallized with water from the catechin-rich fraction (yield 4·3 g.), m.p. 237° (uncorr.); $[\alpha]_D^{20}$ −58° in 50% (v/v) aqueous acetone (*c*, 2) (Forsyth, 1952*a*).

Leucocyanidin 1. The leucocyanidin-rich fraction was accumulated and 3 g. was taken up in 30 ml. water and transferred to a cellulose pulp column (40 × 3 cm.) (Forsyth, 1952*a*) and the column developed with water. The eluate was collected in 5 ml. lots which were examined on paper chromatograms developed with water. The main leucocyanidin (L_1) moved as a single zone in advance of the other components in the first 150 ml. of polyphenol-containing eluate, and was collected, saturated with NaCl, extracted with ethyl acetate, concentrated, and precipitated as before. The colourless powder (2·1 g.) obtained was chromatographically homogeneous in water and butanol–acetic acid. No other phenolic substance is present.

Chromatographic technique. This was as described by Roberts & Wood (1953). Water was used as the first solvent and *n*-butanol–acetic acid–water (4:1:5) as the second. The phenols were detected with $FeCl_3$ and $K_3Fe(CN)_6$ (Barton, Evans & Gardner, 1952). The presence of leucocyanidins was confirmed by excising the spots from duplicate chromatograms and heating for 5 min. in *n*-butanol–HCl, sp.gr. 1·18 (4:1) in a boiling-water bath for liberation of cyanidin. In this way confusion with any coloration due to the polymerization of catechins was minimized. That cyanidin was actually produced was confirmed by chromatography in butanol–2N-HCl (Bate-Smith, 1949) and acetic acid–HCl–water (30:3:10) (Bate-Smith, 1954) against a synthetic sample.

Quantitative paper chromatography. The extract (0·1 ml.) containing a suitable concentration (*ca.* 1%) of the polyphenols was streaked across the starting line of a Whatman no. 1 paper strip 8 cm. wide. The paper was developed with water by downward movement until the solvent front was 30 cm. from the starting line (4 hr. at 25°). The paper when dry was cut into horizontal 0·5 cm. strips and each strip titrated in 10 ml. 1% (w/v) H_2SO_4 with 0·01N-$KMnO_4$. The end-point was taken when the permanganate colour was stable for 1 min. Blank paper strips were used as a control in each set.

(−)-*epi*Catechin and leucocyanidin 1 were used as standards. Pure anhydrous (−)-*epi*catechin and chromatographically homogeneous leucocyanidin 1 required, when chromatographed as above, respectively 9·2 and 6·3 ml. of 0·01N-$KMnO_4$/mg. Assuming that the other catechins and leucocyanidins were oxidized to the same respective extents, these compounds were estimated and expressed in terms of (−)-*epi*catechin and leucocyanidin 1.

Total polyphenols. This estimation was based on the method of Hallas (1949). Dehydrated cotyledons (1 g.) or polyphenol storage cells (0·2 g.) were blended with 100 ml. 0·1N-HCl for 3 min. and after standing for 15 min. at 25° filtered through no. 1 Whatman paper. The various isolated polyphenol fractions (0·2 g.) were dissolved in water (100 ml.). To 50 ml. of the acid filtrates or aqueous solutions were added 50 ml. of Stiasny's reagent (5 vol. distilled water, 5 vol. conc. HCl, and 7·5 vol. 40% (w/v) formaldehyde solution), and the mixture allowed to stand overnight. It was then refluxed for 1 hr., filtered, washed, dried, and weighed. A correction for the amount of formaldehyde condensed was made. This point seems to have been formerly ignored. Catechol and (−)-*epi*catechin both give a value corresponding to a 138% yield of condensed product. All the polyphenols in fresh cacao cotyledons are soluble in dilute HCl.

RESULTS

Fig. 1 shows the polyphenolic components present in an acid extract of fresh beans. Acid extracts of dehydrated cotyledons and water, aqueous acetone,

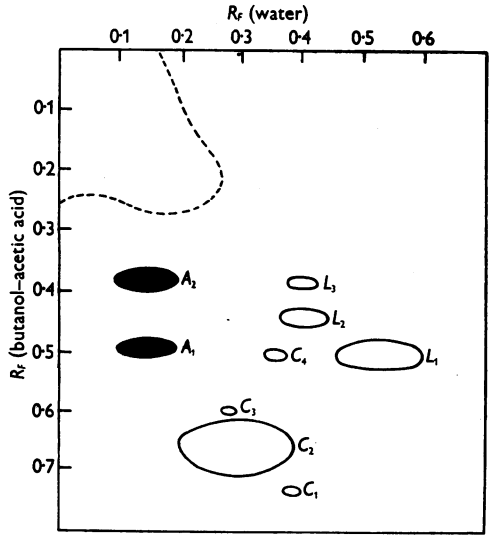

Fig. 1. Chromatogram of 0·1N-HCl extract of fresh cacao cotyledons (1 g./5 ml.) showing position of polyphenolic components. The chromatogram was run first from left to right with water as solvent and then downwards with *n*-butanol–acetic acid. Polyphenols detected with $FeCl_3$–$K_3Fe(CN)_6$. A_{1-2}, cyanidin glycosides; L_{1-3}, leucocyanidins; C_2, (−)-*epi*catechin; provisional identifications: C_1, (+)-catechin: C_3, (+)-gallocatechin; C_4, (−)-*epi*gallocatechin.

or acid extracts of the polyphenol storage cells gave identical results. It is possible to use water extracts of the storage cells since they lack polyphenol oxidase. Some complex materials are diffused over a wide area in the top left hand corner. They give a positive leucocyanidin reaction. The mobile components separated consist of two anthocyanin pigments, four catechins, and three leucocyanidins. Forsyth (1952*a*) showed by isolation that the most abundant catechin (C_2) was (−)-*epi*catechin. By partition chromatography on a cellulose column and by subsequent paper chromatography the other catechins were provisionally identified as catechin (C_1), gallocatechin (C_3), and *epi*gallocatechin (C_4). By utilizing chromatography with water which appears to separate the optical antipodes (Roberts & Wood, 1953), the three catechins were not separated from added (+)-catechin, (+)-gallocatechin, and (−)-*epi*gallocatechin, but the first two could be separated from (−)-catechin and (−)-gallocatechin produced by epimerization of the corresponding (−)-*epi* compounds. (+)-*epi*Gallocatechin was not available for comparison.

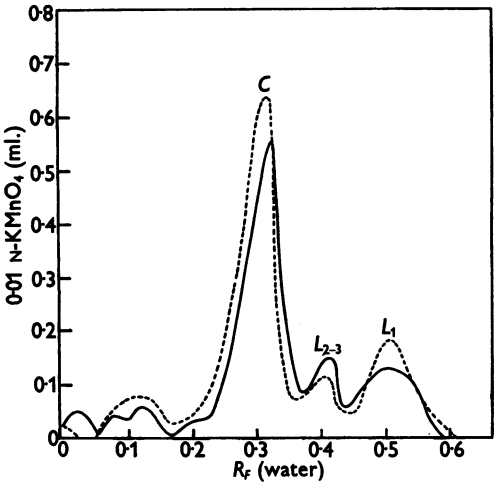

Fig. 2. Quantitative paper chromatograms with water as solvent. —, 1 mg. polyphenol storage cells in 70% aqueous acetone; - - -, 0·1 ml. 0·1 N-HCl extract of cacao cotyledons (1 g./5 ml.). Solvent front allowed to run for 30 cm. and then each 0·5 cm. horizontal strip titrated with 0·01 N-$KMnO_4$ (see text). Symbols as in Fig. 1.

The leucocyanidins gave all the colour reactions of catechins (Forsyth, 1952*a*) with aqueous NaCN, vanillin–HCl, acetic acid–ammonium molybdate, and ammoniacal $AgNO_3$. They are not glycosidic. The anthocyanins and L_3 were not extracted with ethyl acetate. L_2 is extracted with difficulty.

The results from two quantitative paper chromatograms are shown in Fig. 2. Of considerable interest is the fact that little permanganate-oxidizable material remains on the starting line. It is apparent that the bulk of the so-called cacao tannins consists of catechins and leucocyanidins.

The results of the countercurrent separation of the polyphenols soluble in ethyl acetate are shown in

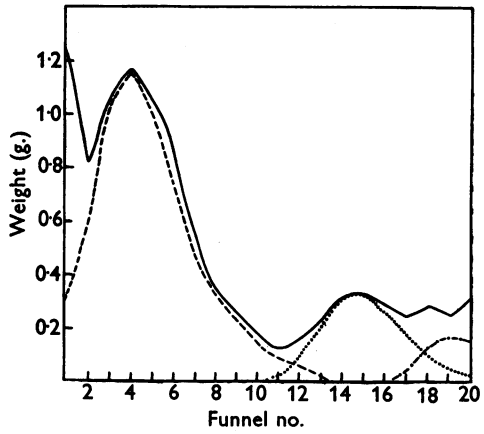

Fig. 3. Countercurrent separation of catechins and leucocyanidins from 10 g. of ethyl acetate-soluble polyphenols. Ten-funnel diagonal system (see text) using ethyl acetate–water. —, dry matter; - - -, catechins; ....., leucocyanidin 1; ·—·—, leucocyanidin 2.

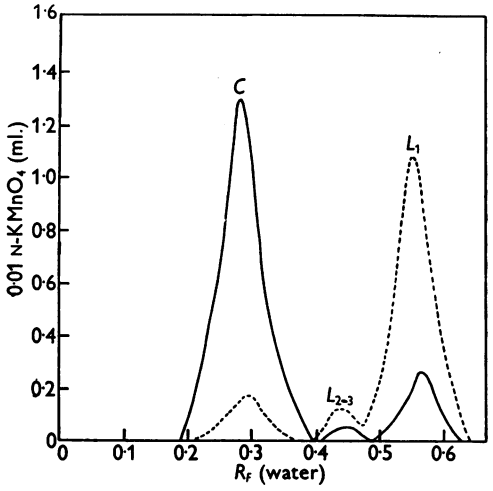

Fig. 4. Quantitative paper chromatograms with water as solvent. —, 1 mg. ethyl acetate-soluble cacao polyphenols; - - -, 1 mg. leucocyanidin concentrate. Conditions as in Fig. 2.

Fig. 3. The partition coefficient of the crude catechins was about 3·0, that of L_1 0·7 and of L_2 0·25. The results of the quantitative analysis of some fractions isolated during the separation are shown in Fig. 4. The leucocyanidin 1 gave all the colour

reactions of an *o*-hydroxyphenol of the catechin type (Forsyth, 1952*a*, 1953). Its structure is being investigated. By quantitative chromatography in *n*-butanol–acetic acid it was shown that 92 % of the catechins in the catechin rich fraction consisted of (−)-*epi*catechin.

DISCUSSION

Table 1 shows the percentages of the catechins and leucocyanidins calculated from Figs. 2 and 4. Because of incomplete extraction the quantities cannot be directly calculated from the results on the freshly dried powder but by estimating the amounts of the total phenols in the beans and in the extract used for chromatography it was calculated that the dry bean contained 3·0 % of catechins and 2·4 % of leucocyanidins. Alternatively, it was calculated from analysis of the polyphenol storage cells, which amount to 12 % of the whole by weight, that there were 3·0 % of catechins and 2·5 % of leucocyanidins in the freshly dried bean. The catechins and leucocyanidins account for about 70 % of the total polyphenols (Table 1). The cyanidin glycosides account for a further 4–5 % leaving about 25 % which is presumably present as complex 'tannin'. This material is being prepared in quantity for further study. It is non-mobile on the chromatograms, non-dialysable, is glycosidic, gives a strong leucocyanidin reaction, but a negligible titration with permanganate. Presumably the *o*-dihydroxy grouping is not available for reaction.

Table 1. *Polyphenols in cacao fractions*

Results for catechins and leucocyanidins calculated from Figs. 2 and 4 and expressed as percentage of the fraction.

Fraction	Total polyphenols	Catechins	Leucocyanidin 1	Leucocyanidin 2, 3
Dried cotyledons	7·8	3·0	1·6	0·8
Polyphenol storage cells	67·6	25	14	7
Ethyl acetate-soluble polyphenols	89·2	69	14	2
Leucocyanidin concentrate from countercurrent separation	97·3	8	76	5

SUMMARY

1. The application of two-way and quantitative paper chromatography to the polyphenols of the cacao bean is illustrated.

2. The bean contains four catechins (of which 92 % is (−)-*epi*catechin), as well as at least three leucocyanidin compounds and two cyanidin glycosides. Some 3·0 % of catechins and 2·5 % of leucocyanidins are present in the freshly dried bean.

3. Separation of the main leucocyanidin from the other polyphenols has been followed.

The author is indebted to Dr A. C. Thaysen, Director of the Colonial Microbiological Research Institute, for his interest, and to the Colonial Products Council for permission to present this paper.

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