Cacao Polyphenolic Substances

1. FRACTIONATION OF THE FRESH BEAN

By W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Extremely little is known about the polyphenolic substances of cacao. Numerous analyses have been made but few isolations. To obtain a rational idea of the changes taking place during the fermentation process in the preparation of the bean for the market, the chemistry of the polyphenolic substances present must be placed on a sounder basis. This is only possible by the isolation of the different substances from the fresh bean.

A catechin was isolated by Adam, Hardy & Nierenstein (1931) and by Freudenberg, Cox & Braun (1932), who showed it to be (–)-epicatechin. It is claimed (Adam, 1928) that the epicatechin is present at least in part as a catechin-caffeine complex. Various crude 'tannin' preparations have been isolated (Adam, 1928; Mosimann, 1944). Other substances have been shown to be present by qualitative reactions. The purple colouring matter is due to anthocyanins (Laurence, Price, Robinson & Robinson, 1938) having the colour and solubility reactions of a cyanidin-3-glycoside. Leucoanthocyanins are also present (Knapp & Hearne, 1939).

Numerous proximate methods of determining the 'tannin' of cacao have been developed, few of which have led to any understanding of the substances present. The sum of these studies has shown, however, that the material is a complex mixture of fractions of different solubility. Extractions have been made with ethyl acetate, water (Adam, 1928), neutral buffer, sodium hydroxide, acetic acid (Hallas, 1939), 40% acetone (Duthie, 1938), and dilute hydrochloric acid (Hallas, 1949). The greatest amount determined is about 15% of the dried bean (Hallas, 1939). Of this about two-thirds can be extracted with aqueous acetone, alcohols, or dilute acids, and roughly comprises the fraction studied here. Hallas (1949) has shown that the anthocyanins and leucoanthocyanins in the fresh purple bean are nearly completely soluble in ethanol.

If any success is to be gained in the separation of what is probably a complex mixture of polymers of different degrees of condensation new methods are obviously required. Bradfield, Penney & Wright (1947) and Bradfield & Penney (1948) have had considerable success in separating the simpler polyphenolic substances in tea by partition chro-

matography on silica gel. White (1949) has shown that the complexity of quebracho tannin extract can be gauged by paper chromatography. Bate-Smith (1949) has demonstrated that anthocyanins and flavones and many similar substances are separable by paper chromatography. A preliminary note (Forsyth, 1949) has been published on the paper chromatography of extracts of cacao beans.

METHODS AND RESULTS

Paper chromatography

Twenty fresh beans were removed from the pod, peeled, and immediately dropped into 100 ml. HCl (0.3N). The acid suspension of cotyledons was homogenized in a Waring Blendor for 3 min. After standing for 15 min. at room temperature (25°) the homogenate was filtered and the extract (20 μ l.) spotted on the chromatogram.

The most useful solvent for development was found to be amyl alcohol-acetic acid-water in the proportions 4:1:5. *n*-Butanol-acetic acid (Partridge, 1948) gave similar results, but with cacao extract the spots were not so well separated although they moved much faster. Butanol-acetic acid was used to confirm the identity of the separated substances under the rigid conditions specified by Bate-Smith & Westall (1950). The amyl alcohol-acetic acid chromatograms were run on Whatman no. 1 paper for 18 hr. at 25°. The substances were detected by visible colour and by spraying with ammoniacal AgNO₃. Aniline hydrogen oxalate (Partridge, 1949) was used to detect sugars.

Table 1. *Paper chromatogram of a 0.3N-HCl extract of fresh Forastero cacao beans*

(Solvent, amyl alcohol-acetic acid-water.)		
No. of spot	<i>R_F</i>	Probable nature
7	0.40	Epicatechin
6	0.22	Leucoanthocyanidin
5	0.16	Cyanidin-anthocyanin I
4	0.12	—
3	0.08	Cyanidin-anthocyanin II
2	0.04	—
1	0.0-0.02	Cyanidin diglycoside leucoanthocyanin

A typical chromatogram showing the main polyphenolic components of an HCl (0.3N) extract of fresh Forastero (purple) beans is shown in Table 1. These substances were invariably present in fresh Forastero beans from many different pods and were the major components.

By extracting the spots from large numbers of papers evidence has been produced (Forsyth, 1949), using Robinson

& Robinson's (1931, 1932) tests, that the components are as stated in the table. Isolation is necessary for better characterization.

As well as the two mobile anthocyanins there was also a trace of anthocyanin not extracted with amyl alcohol (spot no. 1) which had the properties of a cyanidin diglycoside. It hydrolysed to cyanidin and had the solubility of a diglycoside (R_F butanol-acetic 0.17).

Comparison of different beans. Twelve individual beans from the same Imperial College Selection 1 (I.C.S. 1) pod were extracted. They gave identical chromatograms. Twelve individual beans from twelve different I.C.S. 1 pods were extracted. They gave identical chromatograms. Beans of Criollo (non-pigmented) varieties give the same spots except that the anthocyanins are absent. The leucoanthocyanin was present in relatively greater quantity.

The following species and varieties used in chocolate manufacture have been examined and the same spots are present in all the purple beans and only the anthocyanin pigments are missing in the white beans: Red Venezuelan, Nicaragua Criollo, Ecuador Nacional, Trinitario, Calabacillo, *Theobroma pentagona*, *Th. leiocarpa* (Cheesman, 1944). The associated species *Th. bicolor* which is not used contained none of these compounds.

When white, purple-bordered, faint-purple, medium, strong and very strong purple beans were extracted the two anthocyanins were either both absent or both present in the same relative proportion.

Isolation

Imperial College Selection 1 pods were used. It was first necessary to determine what pretreatments of the beans to aid extraction were safe. It was found that fresh beans could be washed in an adapted clothes-washing machine to remove the pulp completely in 2 hr. and then could be heat-killed in boiling water for 5 min. and dried in the oven at 70° in 12 hr. without change in the acid-soluble compounds. In such heat-killed beans the enzymes were completely destroyed. The dried beans were readily peeled and the butter was partly removed by blending with three successive lots of light petroleum (90–100°, 1 ml./g.) at 60°. This procedure removed over 90% of the butter giving a readily extracted stable residue which on extraction gave a chromatogram qualitatively identical to that obtained from fresh beans.

The anthocyanins

Preparation of a concentrate. Attempts to concentrate the anthocyanins via the picrates were unsuccessful, even at concentrations where picric acid crystallized out the pigments remained in the mother liquors. The anthocyanins could, however, be readily concentrated through their lead salts. The method was basically that of Scott-Moncrieff (1930).

Wax-free cacao (100 g.) was blended with 500 ml. 0.3N-methanolic HCl and left overnight. The extract was filtered off and the process repeated. To the combined extract was added a saturated aqueous solution of lead acetate. The lead chloride which first precipitated was removed and then excess lead acetate added to bring down the pigments as a dark-blue precipitate. After standing overnight the precipitate was filtered off, washed with methanol, and dissolved in the minimum amount of glacial acetic acid (about 100 ml.). This extract was then treated with 2 vol. of ether.

The blue lead salt precipitate was washed with ether, dried and ground. The powder was extracted with 25 ml. methanol containing 5% (v/v) HCl and the crude glycosides thrown down as a bright-red gummy mass by addition of 12 vol. ether. The pigments were further purified by extraction with ethyl acetate.

The preliminary purification could also be done simply by ethyl acetate extraction. Wax-free cacao (100 g.) was extracted with 2 × 500 ml. 0.3N-methanolic HCl as before. The extract was diluted with 2 vol. ether and 4 vol. light petroleum and the crude anthocyanin precipitate was washed successively with light petroleum and ether. It was then extracted exhaustively with ethyl acetate to remove impurities soluble in ethyl acetate.

The simplest method of extraction is with methanol containing 10% acetic acid. After filtration the methanol is removed *in vacuo* and the anthocyanins precipitated by addition of a large excess of ether to the acetic acid. The solid is then washed with ethyl acetate.

Chromatography of the anthocyanin concentrate on columns

Chromatography on alumina, silica gel or kieselguhr did not give good results. The most satisfactory chromatograms were obtained on columns of cellulose pulp (Solka flock 200 mesh, Johnsen, Jorgensen and Wettre Ltd., London). The material, purified as described by Campbell, Work & Mellanby (1951), was packed dry in columns (25 × 3.7 cm.) and washed well first with the aqueous and then with the solvent layer of amyl alcohol-acetic acid.

The anthocyanin concentrate prepared by any of the above methods was taken up in the organic solvent layer of amyl alcohol-acetic acid and added to the column and developed with the same solvent (500 ml.). The column was sucked dry, extruded, and the two individual anthocyanin bands cut out separately and, after being allowed to dry at room temperature, were eluted with methanol containing 0.5% (w/v) HCl. Paper chromatograms of the extracts showed the anthocyanins uncontaminated with any substances reacting with Na_2CO_3 , FeCl_3 , or ammoniacal AgNO_3 .

The anthocyanins could be precipitated as bright-red gums by the addition of a very large excess of ether to the methanolic HCl extracts from the column. Attempts at crystallization of the small amount of either gum from methanol, ethanol or water containing various amounts of HCl have been unsuccessful. The anthocyanins can be precipitated from methanol solution with ether as dark-purple amorphous powders. These two powders are chromatographically homogeneous and both give cyanidin on hydrolysis with 20% (w/v) HCl, confirmed by paper chromatography in *n*-butanol-2N-HCl (Bate-Smith, 1949) $R_F=0.70$ and by colour reactions (Robinson & Robinson, 1931). Anthocyanin I is present in about three times the concentration of anthocyanin II as judged by the intensity of the spots.

Anthocyanin I. The R_F value under the standard conditions of Bate-Smith (1949) in butanol-acetic acid is 0.41.

The amorphous powder (10 mg.) was hydrolysed for 30 min. in 0.5N- H_2SO_4 (0.5 ml.) at 100° in a sealed capsule. The hydrolysate was neutralized with BaCO_3 , centrifuged and concentrated *in vacuo*. The residue was extracted with methanol and the methanol concentrated to 0.2 ml. This solution gave positive reactions with benzidine and orcinol tests. Paper chromatography showed that two sugars were

present. Spraying with selective reagents (Forsyth, 1948; Partridge, 1948) showed the two spots to be an aldohexose and an aldopentose. The two sugars could not be separated from glucose and arabinose when chromatographed in butanol-acetic acid, phenol (Partridge, 1948), or ethyl acetate-pyridine (Jermyn & Isherwood, 1949).

The pentose was readily separated from added xylose, ribose and lyxose, and the aldohexose readily separated from added galactose and mannose. Quantitative determination of the proportions of the two sugars in the chromatograms (Flood, Hirst & Jones, 1947) showed them to be present in the ratio of 1:1.

That the anthocyanin should be a simple 'cyanidin-glucose-arabinose' with an R_F of 0.41 is very unlikely when compared with the known cyanidin anthocyanins (Table 3). It is most probably acylated. This was confirmed by a brief alkaline hydrolysis for 30 sec. (Robinson & Robinson, 1931). The acyl group was removed and the anthocyanin now had an R_F of 0.35 which is in closer agreement with expectations on theoretical grounds.

Anthocyanin II. The R_F value under standard conditions in butanol-acetic acid is 0.33. The amorphous powder (10 mg.) was hydrolysed with 2N-H₂SO₄ and treated as was anthocyanin I. Chromatography of the sugar solution showed only a single aldohexose to be present which was not separable from glucose in the three solvent mixtures, but was readily separated from galactose or mannose. The anthocyanin was unaffected by brief alkaline hydrolysis, i.e. it is a simple cyanidin monoglucoside.

Non-mobile fraction

On paper chromatography of an HCl or methanolic HCl extract with amyl alcohol-acetic acid much of the material remains on the starting line. This spot contains a trace of an anthocyanin, but also considerable material, which gives cyanidin on boiling with conc. HCl, and reacts with ammoniacal AgNO₃.

An anthocyanin concentrate was prepared from wax-free cacao (100 g.) by methanolic-HCl extraction and ether-light petroleum precipitation as previously described. On

extracting the anthocyanins from the concentrate with amyl alcohol-acetic acid a considerable residue was left. This residue was dissolved in 0.3N-methanolic HCl (250 ml.) and reprecipitated with ether (2 vol.) three times. The product was then washed with ether and dried to give a brick-red residue which on paper chromatography remained on the starting line.

On boiling with 20% (w/v) HCl a coloration due to liberated cyanidin (R_F in butanol-2N-HCl, 0.69) was produced. Hydrolysis with 2N-H₂SO₄ followed by neutralization with BaCO₃ gave a sugar solution, shown by chromatography to contain glucose and arabinose in the proportion of roughly 3:1.

The ethyl acetate fraction

Wax-free cacao (100 g.) was extracted with two successive litre lots of 0.3N-HCl. The filtered extract was shaken with three successive 500 ml. lots of ethyl acetate. The ethyl acetate was dried (Na₂SO₄), the solvent removed under reduced pressure (nitrogen bubbler) and the residue transferred in boiling CHCl₃ to a sintered-glass funnel, washed with CHCl₃ and dried. About 3 g. of a light-tan coloured amorphous powder were obtained containing the substances which give 'AgNO₃-positive' spots on the paper chromatograms. The extraction with CHCl₃ had no effect on the pattern of any of the spots, showing that by this method of extraction no catechin-caffeine complex was present.

The powder was taken up in a small volume of the top layer of the amyl alcohol-acetic acid-water mixture and transferred to a cellulose pulp column (12.5 × 3.7 cm.) and the column developed with the same solvent. The eluate was collected in 2 ml. lots and spotted on paper chromatograms. Suitable cuts were made and similar fractions combined. Each fraction was then taken to dryness *in vacuo* and the residue washed with CHCl₃. The results are shown in Table 2.

Cut A. Attempts to obtain any crystalline products from cut A were unsuccessful. On paper chromatography a series of fast-moving brown and yellow streaks were observed. No spot was detected on spraying with aqueous NaCN or vanillin-HCl (Bradfield *et al.* 1947), or with acetic acid-

Table 2. Fractionation of the ethyl-acetate solubles (3 g.) on a cellulose column with amyl alcohol-acetic acid

Cut	Vol. eluate (ml.)	Dry wt. (mg.)	Components provisionally identified	R_F		
				Amyl alcohol-acetic acid	Butanol-acetic acid	Phenol-water
O	40	—	—	—	—	—
A	34	90	<i>o</i> -Hydroxyphenols absent	0.75-0.95	—	—
B	48	1028	(-)-Epicatechin	0.40	0.65	0.48
			Catechin	0.50	0.74	0.36
C	26	304	(-)-Epicatechin	—	—	—
			Gallocatechin	0.27	0.57	0.25
D	78	578	(-)-Epicatechin	—	—	—
			Gallocatechin	—	—	—
			Epigallocatechin	0.22	0.48	0.32
			Leucoanthocyanidin	0.19	0.40	—
E	98	169	Leucoanthocyanidin	—	—	—
			Epigallocatechin	—	—	—
			Spot no. 4 (Table 1)	0.12	0.44	0.21
F	144	84	Spot no. 4 (Table 1)	—	—	—
			Trace of anthocyanin I	—	—	—
G	200	22	Spot no. 2 (Table 1)	0.04	0.39	0.12

ammonium molybdate (Quastel, 1931), i.e. no catechins or other *o*-hydroxyphenols were present.

Cut B. *Cut B* consisted mainly of the major catechin (spot no. 7 in Table 1) with traces of another compound. Both substances were catechins since they could be detected by spraying with ammoniacal AgNO_3 , FeCl_3 , Na_2CO_3 , ammonium molybdate, NaCN and vanillin-HCl solutions.

The powder from *cut B* crystallized from water. The major catechin was the more insoluble and the material was recrystallized till the crystals were chromatographically homogeneous. In all 722 mg. of colourless short prisms were obtained, m.p. 237° (uncorr.); $[\alpha]_D^{20} - 58^\circ$ ($c = 2$ in aqueous acetone, 1/1). (Found: C, 49.6; H, 6.2. Calc. for $\text{C}_{16}\text{H}_{14}\text{O}_6 \cdot 4\text{H}_2\text{O}$: C, 49.7; H, 6.1.) The acetyl derivative was prepared, m.p. 151° .

The pure crystalline material was responsible for spot no. 7 on the chromatograms. It had the R_F values shown in Table 2. It gives an intense green colour with ferric salts and a heavy reddish brown precipitate with boiling HCl. All the above properties show the substance to be (–)-epicatechin. This has previously been isolated from cacao by ether extraction (Freudenberg *et al.* 1932). A preparation isolated in the conventional manner showed identical properties with the above.

The mother liquors from the crystallization of (–)-epicatechin were combined. A large excess of (–)-epicatechin was still present. The catechins could be purified and partly separated by simply adding the aqueous solution to a column of wet cellulose pulp (12.5×3.7 cm.) and eluting with water. After 30 ml. of blank eluate had passed through 34 ml. was collected containing only coloured impurities followed by 120 ml. of a mixture of catechins and then 130 ml. containing only (–)-epicatechin. The catechin fractions were exhaustively extracted with ether and the ether evaporated. (–)-Epicatechin (67 mg.) and 72 mg. of the mixture were obtained. The mixture gave on crystallization from water mainly short prisms with a few long needles. Paper chromatography suggested that (–)-epicatechin and the other catechin were present in the mixture in the ratio 4/1 as judged from the intensity of the spots. Thus the maximum possible yield of the minor catechin would be 14.5 mg. Isolation in the presence of excess (–)-epicatechin was not successful. Incubation of the material with a dried preparation of mycelium of *Aspergillus niger* for 12 days failed to liberate any gallic acid and the chromatogram was unchanged. The same preparation readily split an authentic sample of (–)-epigallocatechin gallate (Bradfield *et al.* 1947). The substance is not, therefore, a catechin gallate ester. It was also stable to mild acid hydrolysis. From R_F values (Bradfield & Bate-Smith, 1950; Wender & Gage, 1949) the substance is provisionally identified as catechin itself. The rotation is not known.

Cut C. Paper chromatography showed that *cut C* consisted almost entirely of (–)-epicatechin. On crystallization from water 234 mg. of (–)-epicatechin were obtained. The mother liquor on concentration showed traces of another catechin which gave all the catechin reactions and was not a gallate ester. It could not be separated by paper chromatography from an authentic sample of (±)-gallo catechin. The substance was obviously present in too small quantity for isolation to be possible.

Cut D. From *cut D* 578 mg. of a light-brown powder was collected. On refluxing with 20% (w/v) HCl, or better ethanolic HCl, cyanidin is produced, confirmed by paper chromatography in butanol-2*N*-HCl and by colour tests.

The powder was very soluble in water. On paper chromatography of the solution, it was seen to be a mixture of (–)-epicatechin, gallo catechin, and spot no. 6 of Table 1. The powder was dissolved in 5 ml. methanol and precipitated with 45 ml. ether. The ether-soluble fraction was evaporated to give 145 mg. of a colourless solid. This material did not give cyanidin on treatment with HCl. Paper chromatography showed (–)-epicatechin, gallo catechin, and a third spot giving all the catechin reactions to be present. The mixture has not been separated. The chromatogram was unaffected by tannase. The unknown catechin could not be separated from (–)-epigallocatechin on chromatography. The three catechins were judged to be present in the proportion 3:1:3 from the intensity of the spots.

The ether-insoluble fraction was a light-brown amorphous powder (400 mg.) which could not be obtained crystalline and which darkened on heating, but did not melt below 300° . This material gave cyanidin on treatment with strong acid, but no sugar could be detected on milder hydrolysis. It did not give an immediate spot with ammoniacal AgNO_3 but only on standing.

Cut E. *Cut E* was treated as was *cut D*, 65 mg. were ether-soluble. The ether-insoluble material was the leucocyanidin as before (100 mg.). The ether-soluble material was a mixture of epigallocatechin and spot no. 4 of Table 1.

Cut F. *Cut F* consisted mainly of spot no. 4 in Table 1. It gave the usual catechin colour reactions and was unaffected by tannase. No crystalline products could be obtained.

Cut G. *Cut G* contained very little material which had the properties of spot no. 2 in Table 1. It gave the catechin colour reactions, but was unaffected by tannase.

DISCUSSION

The fresh cacao bean is seen to contain a very complicated mixture of polyphenolic substances. At least eleven different compounds can be distinguished by chromatography. The similarity between widely different varieties is striking.

The two main anthocyanins have been shown to contain respectively, glucose and arabinose, and glucose, we would therefore expect them to be cyanidin-3-arabinoglucoside and cyanidin-3-glucoside, i.e. chrysanthemin. However, the second anthocyanin is certainly not chrysanthemin (solubility of picrate), and an examination of colour reactions would suggest that the sugars are linked to a different hydroxyl (Robertson & Robinson, 1929).

To settle this point it would be necessary to isolate the pigments in large yield and compare them with synthetic preparations. Although up to 50% of the naturally occurring cyanidin glycosides may be of the 'pentose' glycoside (including methyl pentose) form (Robinson & Robinson, 1931, 1932) it is seldom known which pentose is present, and it is usually assumed that the pentose is rhamnose. An exception is sambucyanin (Nolan & Casey, 1931), which contains an aldopentose and glucose. Arabinose does not appear to have been identified previously in a cyanidin glycoside, although

Table 3. Comparison of cacao, and known cyanidin anthocyanins

Anthocyanin	Sugars	R_F Butanol- acetic acid	Colour at pH 8	Sugar linkage
Mekocyanin	Gentiobiose	0.29	Brown-red	3
Cacao anthocyanin II	Glucose	0.33	Blue-violet	?
Chrysanthemin	Glucose	0.33	Brown-red	3
Mildly hydrolysed anthocyanin I	Arabinose-glucose	0.35	Blue-violet	?
Antirrhinin	Rhamnose-glucose	0.37	Brown-cherry	3
Cacao anthocyanin I	Arabinose-glucose	0.41	Blue-violet	?
Cyanidin	—	0.69	Blue-violet	—

(in butanol/
2N-HCl)

vicianose is a common constituent of other types of glycosides. The available information is summarized in Table 3.

At least two compounds of the leucoanthocyanin class are present, both based on cyanidin. One would appear to be a leucocyanidin and the other (no. 1) a leucocyanidin glycoside. The second is probably a mixture of different glycosides based on both arabinose and glucose. Attempts are being made to isolate the leucocyanidin in large yield for structural investigation.

At least six of the polyphenols have the properties of catechins of which the only one present in quantity (at least 40 % of the total ethyl acetate-soluble 'tannins') is the previously isolated (—)-epicatechin. No evidence of gallates was encountered. A comparison of the catechins isolated from cacao and tea (Bradfield *et al.* 1947; Bradfield & Penney, 1948) has led to the provisional identification of catechin, galocatechin, and epigallocatechin, by chromatography. The other two catechins are unidentified.

SUMMARY

1. The polyphenolic components in extracts of fresh cacao beans have been examined by paper strip chromatography.

2. At least eleven polyphenols are present and have been separated on columns of cellulose pulp.

3. In Forastero (purple) beans there are two main anthocyanins with also a trace of a third pigment, with the properties of a cyanidin diglycoside. The two main pigments are a cyanidin monoglycoside and a cyanidin pentose glycoside, the sugar radicals being respectively glucose and glucose-arabinose.

4. A sugar-free leucocyanidin and a mixture of leucocyanidin glycosides are also present.

5. There are at least six catechin-like substances of which epicatechin is the main component. From chromatographic comparison, catechin, epigallocatechin and galocatechin, would appear to be present.

6. The chemical similarity between different species and varieties of beans used in chocolate manufacture is striking.

I am indebted to Dr A. E. Bradfield for supplying the samples of galocatechin, epigallocatechin, and epigallocatechin gallate which were used in the chromatographic work, to Dr A. C. Thaysen, Director, for his interest in this work, and to the Colonial Products Research Council for permission to publish.

REFERENCES

- Adam, W. B. (1928). *Analyst*, **53**, 369.
 Adam, W. B., Hardy, F. & Nierenstein, M. (1931). *J. Amer. chem. Soc.* **53**, 727.
 Bate-Smith, E. C. (1949). *Biochem. Soc. Symp.* no. 3, p. 62.
 Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. Biophys. Acta*, **4**, 427.
 Bradfield, A. E. & Bate-Smith, E. C. (1950). *Biochim. Biophys. Acta*, **4**, 441.
 Bradfield, A. E. & Penney, M. (1948). *J. chem. Soc.* p. 2249.
 Bradfield, A. E., Penney, M. & Wright, W. B. (1947). *J. chem. Soc.* p. 32.
 Campbell, P. N., Work, T. S. & Mellanby, E. (1951). *Biochem. J.* **48**, 106.
 Cheesman, E. E. (1944). *Trop. Agriculture, Trin.*, **21**, 144.
 Duthie, D. W. (1938). *Analyst*, **63**, 27.
 Flood, A. E., Hirst, E. L. & Jones, J. K. N. (1947). *Nature, Lond.*, **160**, 86.
 Forsyth, W. G. C. (1948). *Nature, Lond.*, **161**, 239.
 Forsyth, W. G. C. (1949). *Nature, Lond.*, **164**, 25.
 Freudenberg, K., Cox, R. F. B. & Braun, E. (1932). *J. Amer. chem. Soc.* **54**, 1913.
 Hallas, C. A. (1939). *Res. Rec.*, no. 70. British Association of Research for the Cacao, Chocolate, Sugar Confectionery and Jam Trades.
 Hallas, C. A. (1949). *Res. Rep.* no. 13. British Food Manufacturing Industries Research Association.
 Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
 Knapp, A. W. & Hearne, J. F. (1939). *Analyst*, **64**, 475.

- Laurence, W. J. C., Price, J. R., Robinson, G. M. & Robinson, R. (1938). *Biochem. J.* **32**, 1661.
- Mosimann, C. (1944). *Mitt. Lebensm. Hyg., Bern.* **35**, 241.
- Nolan, T. J. & Casey, H. M. T. (1931). *Proc. R. Irish Acad.* **40**, 56.
- Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
- Partridge, S. M. (1949). *Biochem. Soc. Symp.* no. 3, p. 52.
- Quastel, J. H. (1931). *Analyst*, **56**, 311.
- Robertson, A. & Robinson, R. (1929). *Biochem. J.* **23**, 35.
- Robinson, G. M. & Robinson, R. (1931). *Biochem. J.* **25**, 1687.
- Robinson, G. M. & Robinson, R. (1932). *Biochem. J.* **26**, 1647.
- Scott-Moncrieff, R. (1930). *Biochem. J.* **24**, 753.
- Wender, S. H. & Gage, T. B. (1949). *Science*, **109**, 287.
- White, T. (1949). *J. Soc. Leath. Tr. Chem.* **33**, 39.

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2. CHANGES DURING FERMENTATION

By W. G. C. FORSYTH

Colonial Microbiological Research Institute, Trinidad, British West Indies

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Fresh cacao beans are prepared for marketing by heaping in boxes so that their covering of mucilaginous pulp can be broken down through the action of yeasts and acetic acid bacteria (Knapp, 1937). After this fermentation the beans are sundried. During both processes various changes take place in the cotyledons of the beans, which result in a product suitable for the manufacture of chocolate. The most striking chemical changes taking place during processing of fresh cacao beans are the alterations in the polyphenolic substances of the beans, the simpler polyphenols yielding insoluble complexes (Hallas, 1939, 1949).

From analogy with tea fermentation, it has generally been assumed (Knapp, 1937) that the initial change in the cacao bean fermentation is one of oxidation by atmospheric oxygen activated by a polyphenol oxidase system. Oxidases are certainly present in abundance (Brill, 1915; Ciferri, 1931).

In previous work in this series (Forsyth, 1949, 1952) it has been shown that the polyphenols extracted from fresh *Forastero* cacao with dilute acid consist mainly of catechin and cyanidin compounds. Both anthocyanins and leucoanthocyanins are present. Of the catechins, epicatechin is present in by far the greater proportion.

By quantitative paper chromatography of extracts of beans removed from the fermentation heap throughout the process, the destruction and conversion of the main polyphenols has now been followed. The changes during artificial treatments of the beans have also been investigated.

METHODS

Commercially fermented beans. Twenty beans were taken at random from a fermentation 'sweat box'. This number is required to give a representative sample. The beans were

peeled and the cotyledons (about 20 g.) immediately extracted with 0.3N-HCl as described in Part 1 (Forsyth, 1952).

Chromatographic estimation of polyphenols. The extract (0.2 ml.) was streaked across the starting line (16 cm.) of a Whatman no. 1 paper sheet 20 cm. wide. Two lanes at the

Fig. 1. Quantitative paper chromatography of 0.3N-HCl extract of fresh cacao beans. L_1 , complex leucocyanidins; L_2 , leucocyanidin; A_1 , cyanidin monoglucoside; A_2 , cyanidin arabinoglucoside; C , epicatechin.

side were used to spot on marker solutions of fresh bean extract. The sheets were then chromatographed with amyl alcohol-acetic acid-water for 18 hr. (Forsyth, 1949, 1952) by descending chromatography. Before drying the paper the anthocyanin pigments were cut out and extracted to pre-