THE PHENOLIC SUBSTANCES OF MANUFACTURED TEA. VII.*—The Preparation of Individual Flavanols

By E. A. H. ROBERTS and (MRS.) M. MYERS

Methods are described for the preparation from dried green tea-leaf of (−)-epigallocatechin, (+)-gallocatechin, (−)-epigallocatechin gallate and (−)-epicatechin gallate. The isolation of (+)-gallocatechin confirms earlier predictions that this isomer, and not the racemate, would be found in unprocessed tea-leaf.

Introduction

Studies of enzymic oxidations of individual flavanols and mixed substrate systems, reported in Parts IV and VIII of this series,[1, 2] have added much to our understanding of the mechanism of tea fermentation. The methods described by Bradfield and his co-workers[3, 4] for the preparation of individual flavanols are not suitable for large-scale work, and this paper describes alternative methods which employ Craig counter-current distributions and column chromatography on Magnesol–Celite and silica gel.

Experimental

Material

Shoots consisting of two leaves and the terminal apex were plucked from the clonal source 14/5/18 under cultivation at Tocklai, Assam. The plucked shoots were dried in a blast of hot air in a firing machine used for the drying of black tea.

Identifications by paper chromatography

With the solvent combination butanol–acetic acid–water (4 : 1 : 2·2) followed by 2% acetic acid, each of the six flavanols occurring in tea-leaf has a well-defined position on a paper chromatogram.[5, 6] This position, especially if related to that of gallic acid, has proved a reliable means of identifying the flavanols under consideration. Two-way paper chromatography has also proved useful in assessing the degree of purification attained by the methods to be described below. Identifications of the individual flavanols have also been confirmed by the use of specific spot tests. While all reducing polyphenols give a deep blue colour with ferric chloride and potassium ferricyanide, the reactions with vanillin and tetrazotised benzidine are more specific and distinguish the flavanols from gallic acid and substances yielding gallic acid and non-polyphenolic material on hydrolysis. The fluorescence in ultra-violet light, and colour reactions with ferric salts, potassium cyanide and ethylenediamine are also diagnostic.[6] Colours with 0·5N-NaOH are quite distinctive (Table I).

Table I

Colour sequences observed on spraying flavanols with 0·5N-NaOH

(+)-Gallocatechin and (−)-Epigallocatechin	Yellow-brown, slowly fading until almost colourless
(+)-Catechin and (−)-Epicatechin	Orange-brown, slowly intensifying
(−)-Epigallocatechin gallate	Pinkish-brown, slowly fading, but some residual colour
(−)-Epicatechin gallate	Pinkish-brown, deepening, finally orange-brown

Determination of phenolic nuclei

Pyrocatechol and pyrogallol groups were determined by the rather approximate method of Kursanov & Zaprometov as modified by King & White.[7] The phloroglucinol group was estimated by the method described by Swain & Hillis.[8]

Löwenthal titrations

For a Löwenthal titration of flavanols a convenient quantity to use was 20–40 mg. If necessary one-tenth of these quantities could be used, and the titration carried out with 0·02N-permanganate from a 10-ml. burette. On the larger scale 0·04N-permanganate was used for the titration with indigocarmine as an internal indicator, as described by Barua & Roberts.[9] No blank was carried out for non-tans as the non-tan fraction in tea-leaf has been shown to

* Part VI: *J. Sci. Fd Agric.*, 1959, **10**, 176

consist of flavanols not precipitated by gelatin.[10] When pyrogallol and gallic acid were titrated sharp end-points were obtained with titres of 6·1 equivalents, agreeing well with values obtained by Williams.[11] Substances containing pyrogallol groups gave equally sharp end-points, but with pyrocatechol derivatives the end-points were much less sharp and the titration values recorded were distinctly higher than those found by Williams.

Results

Preliminary separation of flavanols

The preliminary extraction of dried green leaf with methanol and the subsequent precipitation by chloroform has already been described.[6, 12] The chloroform precipitate was dissolved in water and extracted at least six times with equal volumes of ethyl acetate. The united ethyl acetate extracts were evaporated to dryness under reduced pressure. The yield of mixed flavanols was 10 g. from 150 g. of dried green leaf. Paper chromatograms showed the product to contain the six flavanols expected, together with smaller amounts of the monoglucosides of kaempferol, quercetin and myricetin,[13] and traces of leucoanthocyanins,[14] chlorogenic acids,[15] and *p*-coumarylquinic acids.[16]

This mixture was fractionated in a 50-tube Craig distribution train, each tube having a lower-phase capacity of 60 ml. The mixture was dissolved in a mixture of ethyl acetate (60 ml.) and water (60 ml.) and added to tube 0. Tubes 1–49 each contained 60 ml. of water, saturated with ethyl acetate. Fifty fundamental transfers were carried out, 60 ml. of ethyl acetate, saturated with water, being added to tube 0 after each transfer. After the fiftieth transfer the upper phase of tube 49 was collected as Fraction 1. Twenty further transfers were carried out, fresh ethyl acetate being added to tube 0 and the upper phase withdrawn from tube 49 after each transfer. Transfers were then continued without addition of fresh ethyl acetate to tube 0, until, in all, 70 ethyl acetate layers (Fractions 1–70) had been collected. The 50 aqueous layers left in the distribution chain constituted Fractions W 0–W 49.

When emulsions proved troublesome the contents of the leading tube were discarded, so long as there had already been at least ten fundamental transfers. It was sometimes necessary to discard the leading fraction more than once. The leading fractions were always very deeply coloured, so that their rejection involved no loss of eventual yield. At the end of the distribution, colour was most apparent in Fractions 1–5 and W 0–W 4, but none of the fractions was completely free from coloured impurities.

The 120 fractions were examined by one-way paper chromatography (butanol–acetic acid–water). Fractions of similar composition were pooled, and the combined extracts evaporated to dryness under reduced pressure. The compositions of these pooled fractions, as revealed by two-way paper chromatography, are illustrated in Table II.

Table II

Composition of pooled fractions after Craig distribution of tea-leaf flavanols

Fraction	Yield g.	Substances detected by paper chromatography
1–5	1·45	Mainly (−)-epicatechin gallate and (−)-epigallocatechin gallate. Traces of a catechin gallate, a gallocatechin gallate and kaempferol-3-glucoside. Some orange-coloured oxidation products
6–10	1·93	Mainly (−)-epigallocatechin gallate, with traces of (−)-epicatechin gallate, (+)-catechin and kaempferol-3-glucoside
11–21	1·13	(−)-Epigallocatechin gallate, (+)-catechin, (−)-epicatechin and traces of (+)-gallocatechin
22–33	0·24	(+)-Gallocatechin with some (−)-epicatechin and traces of (−)-epigallocatechin and *iso*quercitrin. Also an uncharacterised polyphenol of R_F values 0·45 and 0 in butanol–acetic acid–water and 2% acetic acid
34–48	0·28	(+)-Gallocatechin and (−)-epigallocatechin with some isoquercitrin and traces of leucoanthocyanins
49–70	0·48	Mainly(−)-epigallocatechin, with a little (+)-gallocatechin and traces of leucoanthocyanins
W 40–W 49	0·08	(−)-Epigallocatechin and traces of myricetin-3-glucoside and leuco-anthocyanins
W 30–W 39	0·03	(−)-Epigallocatechin, some myricetin-3-glucoside and traces of leucoanthocyanins and chlorogenic acids
W 0–W 29	0·02	Small amounts of (−)-epigallocatechin, myricetin-3-glucoside, rutin, kaempferol-3-rhamnoglucoside, leucoanthocyanins, chlorogenic acids, *p*-coumarylquinic acids and theogallin

Preparation of (−)-epigallocatechin

Fractions rich in (−)-epigallocatechin (e.g., fractions 49–70 and W30– W 49 in Table II) from four separate Craig distributions were combined, and the mixture fractionated in a 50-tube Craig distribution chain in exactly the same way as described for mixed flavanols. Fractions 45–70 (yield 1·6 g.) were shown by paper chromatograms to consist almost entirely of (−)-epigallocatechin together with traces of myricetin-3-glucoside and leucoanthocyanins, and small amounts of brown oxidation products. After recrystallisation from water the product had m.p. 206–208° (decomp.) and $[\alpha]_D^{17}$ −56·3° (c = 1·243 in ethanol). Bradfield *et al.* recorded m.p. 217–218° and $[\alpha]_D^{22}$ −60°.[3] The percentages of pyrogallol and phloroglucinol found were 38·7% and 39·4%, respectively ($C_{15}H_{14}O_7$ requires 41·2% in each case). If a molecular weight of 306 be assumed, the Löwenthal titration was 6·4 equivalents per mole, corresponding with one pyrogallol group in the molecule. The product was identical in its R_F values and all spot reactions with the (−)-epigallocatechin isolated from green tea by Bradfield *et al.*[3]

Preparation of (+)-gallocatechin

Fractions rich in (+)-gallocatechin (e.g., Fractions 22–48 in Table II) from four separate Craig distributions were combined and the mixture (2·0 g.) fractionated in a 50-tube Craig distribution chain exactly as with (−)-epigallocatechin. (+)-Gallocatechin was detected in Fractions 22–45 and was the main constituent of the combined Fractions 26–39 (0·59 g.). Further purification was effected on a silica gel column following the procedure described by Bradfield *et al.*,[3] but the relatively low solubility of the crude (+)-gallocatechin proved to be a complication and only 0·13 g. of (+)-gallocatechin was obtained free from (−)-epigallocatechin. This was further purified by recrystallisation from water. The product had m.p. 180° and $[\alpha]_D^{20}$ +13·2° (c = 1·388 in 50% acetone). The (+)-gallocatechin isolated by Mayer from the bark of oak and sweet chestnut had m.p. 186–189° and $[\alpha]_D$ +14·7°.[17] Micro-analysis gave C 50·8% and H 5·5% ($C_{15}H_{14}O_7,2H_2O$ requires C 52·6% ; H 5·3%). The product was identical in its R_F values and spot reactions with the (+)-gallocatechin isolated by Mayer.[17] It was also identical chromatographically with the substance of higher R_F in 2% acetic acid contained in the (±)-gallocatechin isolated by Bradfield *et al.* from green tea.[3]

Preparation of (−)-epigallocatechin gallate

Fractions 6–10 (Table II) represent a moderately pure preparation of (−)-epigallocatechin gallate. Such fractions were dissolved in the minimum quantity of ether (containing a few drops of ethyl acetate) and applied to a column of Magnesol–Celite (13 × 2 cm.) prepared as described by Pearl & Dickey.[18] The column was eluted with peroxide-free ether, saturated with water, and fractions were collected as soon as the eluate gave a positive test for polyphenols. Fractions were analysed by one-way paper chromatography ; both butanol–acetic acid–water and 2% acetic acid were used as solvents. Nearly all of the (−)-epicatechin gallate, (+)-catechin and (−)-epicatechin were found in the first 45 ml. of the eluate collected after the first breakthrough of the polyphenols. Subsequent fractions (255 ml.) were combined and evaporated to dryness under reduced pressure (yield 0·97 g.).

Paper chromatograms indicated very slight contamination with (−)-epicatechin and (+)-catechin ; the main constituent was identical in its R_F values and spot reactions with the (−)-epigallocatechin gallate isolated from green tea by Bradfield & Penney.[4] Its rotation $[\alpha]_D^{17}$ −151° (c = 1·234 in ethanol) was a little lower than that previously recorded (−179°).[4] The percentages of pyrogallol and phloroglucinol found were 48·3% and 28·3%, respectively ($C_{22}H_{18}O_{11}$ requires 55·0% and 27·5%). As already reported the value for $\lambda_{max.}$ at 278 mμ is approximately equal to the sum of the corresponding values for (−)-epigallocatechin and gallic acid.[19] The Löwenthal titre amounted to 12·1 equivalents per mole for a molecular weight of 458, as required for one pyrogallol and one gallic acid group. Trimethylgallic acid (m.p. and mixed m.p. 164°) was obtained by methylation with diazomethane and subsequent alkaline hydrolysis.

Preparation of (−)-epicatechin gallate

(−)-Epicatechin gallate is concentrated in Fractions 1–5 (Table II). Four such fractions were combined, and fractionated further in a 20-tube Craig distribution train, with ether and

water as the two phases. After 30 fundamental transfers, with withdrawal of the ether layers when they reached tube 19, transfers were continued without adding ether to tube 0. Eventually the material was distributed between 30 ethereal and 20 aqueous phases. Each of these fractions was analysed by one-way paper chromatography. Pooled fractions of similar composition were evaporated to dryness under reduced pressure. The composition of the pooled fractions, as determined by two-way paper chromatography, is given in Table III.

Table III

Composition of fractions obtained after Craig distribution of crude (—)-epicatechin gallate

Fraction	Yield, g.	Substances detected by paper chromatography
1–3	?	Deeply coloured oxidation products
4–9	0·29	(—)-Epicatechin gallate with small amounts of catechin gallate and gallocatechin gallate ; colour rather yellow
10–18	0·86	(—)-Epicatechin gallate with some gallocatechin gallate
19–28	0·30	(—)-Epicatechin gallate, gallocatechin gallate, and (—)-epigallocatechin gallate
29–30	1·77	(—)-Epigallocatechin gallate
+W 5–W 19		

Fractions 1–9 were rejected owing to their high content of oxidation products. Fractions 29, 30 and W 5–W 19 consisted of almost pure (—)-epigallocatechin gallate. The (—)-epicatechin gallate was concentrated in Fractions 10–18. The latter were dissolved in the minimum quantity of ether, containing a few drops of ethyl acetate, and purified on a column of Magnesol–Celite in exactly the same way as described for (—)-epigallocatechin gallate. The first fractions collected, after the initial break-through of the polyphenols (total volume 135 ml.), were shown by paper chromatography to contain (—)-epicatechin gallate alone (yield 0·46 g.). Intermediate fractions (total volume 60 ml.) were shown to contain a mixture of (—)-epicatechin gallate and a gallocatechin gallate, and the final fractions (total volume 120 ml.) contained a gallocatechin gallate (40 mg.) identical chromatographically with the substance 2A described by Bradfield & Penney.[4]

The (—)-epicatechin gallate was identical in its R_F values and spot reactions with the product isolated by Bradfield & Penney.[4] Its rotation $[\alpha]_D^{17}$ −155° (c = 1·230 in ethanol) was rather lower than that previously recorded (−190°).[4] The percentages of pyrocatechol, pyrogallol and phloroglucinol found were 17·4%, 26·6% and 24·4% respectively ($C_{22}H_{18}O_{10}$ requires 24·8%, 28·3% and 28·3% respectively). Good yields of trimethylgallic acid (m.p. and mixed m.p. 164°) were obtained by methylation with diazomethane and subsequent alkaline hydrolysis.

Discussion

Before this work was undertaken it was accepted that the investigations of Tsujimura,[20] Oshima,[21] and Bradfield and his collaborators[3, 4, 22] had established the presence in green tea and in unfermented tea-leaf of (—)-epicatechin, (—)-epigallocatechin and their 3-galloyl esters. The analytical data presented above are not meant to provide further evidence in favour of these views and should be taken as confirmations of the identifications made by paper chromatography

The preparations of tea oxidase substrates [(—)-epicatechin and (+)-catechin are more conveniently obtained from alternative sources] have proved entirely suitable for studies of substrate oxidation,[1, 2] but it is clear that the (—)-epigallocatechin, (—)-epigallocatechin gallate and (—)-epicatechin gallate are less pure than the substances isolated by Bradfield and his collaborators from green tea. This is considered to be due to contamination by oxidation products introduced during the drying of the tea-leaf.

When freshly plucked leaf is dried in the equipment used for the firing of tea some time necessarily elapses before the temperature of the leaf reaches a level at which the oxidising enzymes are inactivated. When the temperature of the leaf reaches 50° the semi-permeability of the vacuolar membrane is lost and the contents of the vacuole diffuse into the cytoplasm. Until further temperature increases destroy the enzyme there will be a period in which flavanols will undergo enzymic oxidation.[23] The oxidation products, so formed, are not completely separable from flavanols by Craig distributions and the flavanols prepared from dried green leaf are therefore less pure than those obtained from green tea.

The use of green tea as a source of flavanols introduces another complication. In green tea manufacture[24] the leaf is first steamed, after which it is subjected to relatively prolonged rolling at temperatures of up to 70°. Such conditions would favour the epimeric changes

(−)-epicatechin → (−)-catechin . . . . . (1)

(−)-epigallocatechin → (−)-gallocatechin . . . . . (2)

Instead, therefore, of isolating (+)-gallocatechin from green tea, one would expect to obtain a mixture of (+)- and (−)-gallocatechin. Mayer obtained such a mixture from green tea with a rotation of +3°.[25] It is probable that Bradfield obtained a similar mixture, but as he recrystallised his product it is not surprising that the racemate was obtained. Therefore although Bradfield's characterisation as a gallocatechin is accepted,[3] it is not necessarily true that green tea contains this racemic mixture, but can contain other mixtures of the (−)- and (+)-forms as indicated by Mayer's findings.

From purely paper chromatographic evidence it was previously concluded that unprocessed or dried green tea leaf contained the (+)-isomer, and the isolation from dried green leaf of a gallocatechin with rotation +13·2° is considered to provide the necessary confirmation of this earlier deduction. It follows that if (+)-gallocatechin is required it must be prepared from unprocessed or dried leaf, and not from green tea.

Acknowledgments

The authors were indebted to the late Dr. A. E. Bradfield for samples of (−)-epigallocatechin, (−)-epigallocatechin gallate, (±)-gallocatechin, a gallocatechin gallate (substance 2A) and (−)-epicatechin gallate. Thanks are also due to Dr. W. Mayer for a sample of (+)-gallocatechin. This paper is published with the permission of the Indian Tea Association (London).

Indian Tea Association Chemical Laboratory
Butler's Wharf
London, S.E.1

Received 29 June, 1959

References

[1] Roberts, E. A. H., & Myers, M., *J. Sci. Fd Agric.*, 1959, **10,** 167
[2] Roberts, E. A. H., & Myers, M., *J. Sci. Fd Agric.*, 1960, **11,** 158
[3] Bradfield, A. E., Penney, M., & Wright, W. B., *J. chem. Soc.*, 1947, p. 32
[4] Bradfield, A. E., & Penney, M., *J. chem. Soc.*, 1948, p. 2249
[5] Roberts, E. A. H., & Wood, D. J., *Biochem. J.*, 1953, **53,** 332
[6] Cartwright, R. A., & Roberts, E. A. H., *J. Sci. Fd Agric.*, 1954, **5,** 593
[7] King, H. G. C., & White, T., Symposium Soc. Leather Trades' Chemists (Cambridge), 1956, p. 31
[8] Swain, T., & Hillis, W. E., *J. Sci. Fd Agric.*, 1959, **10,** 63
[9] Barua, D. N., & Roberts, E. A. H., *Biochem. J.*, 1940, **34,** 1524
[10] Roberts, E. A. H., & Wood, D. J., *Biochem. J.*, 1951, **49,** 414
[11] Williams, A. H., *Annu. Rep. agric. hort. Res. Sta. Bristol,* 1952, p. 223
[12] Roberts, E. A. H., & Myers, M., *J. Sci. Fd Agric.*, 1958, **9,** 701
[13] Roberts, E. A. H., Cartwright, R. A., & Wood, D. J., *J. Sci. Fd Agric.*, 1956, **7,** 637
[14] Roberts, E. A. H., Cartwright, R. A., & Wood, D. J., *J. Sci. Fd Agric.*, 1956, **7,** 253
[15] Roberts, E. A. H., *Chem. & Ind.*, 1956, p. 985
[16] Cartwright, R. A., Roberts, E. A. H., Flood, A. E., & Williams, A. H., *Chem. & Ind.*, 1955, p. 1062
[17] Mayer, W., Symposium Soc. Leather Trades' Chemists (Cambridge), 1956, p. 127
[18] Pearl, I. A., & Dickey, E. E., *J. Amer. chem. Soc.*, 1951, **73,** 863
[19] Roberts, E. A. H., & Williams, D. M., *J. Sci. Fd Agric.*, 1958, **9,** 217
[20] Tsujimura, M., *Sci. Pap. Inst. phys. chem. Res., Tokyo,* 1929, **10,** 253; 1930, **14,** 63; 1931, **15,** 155; 1934, **24,** 149; 1935, **26,** 186
[21] Oshima, Y., & Goma, T., *J. agric. chem. Soc., Japan,* 1933, **9,** 948; Oshima, Y., *ibid.*, 1936, **12,** 103
[22] Bradfield, A. E., & Bate-Smith, E. C., *Biochim. biophys. Acta,* 1950, **4,** 427
[23] Roberts, E. A. H., *Nature, Lond.*, 1941, **148,** 285; *Biochem. J.*, 1941, **35,** 1289
[24] Leonard, W. H., & Roberts, R., 'Tea in Japan', Natural Resources Section, General Headquarters, Supreme Commander for the Allied Powers, Report Number 125, 1949, Washington, U.S.A.
[25] Mayer, W., personal communication