Phytochemistry, 1972, Vol. 11, pp. 1243 to 1248. Pergamon Press. Printed in England.

IN VITRO OXIDATION OF FLAVANOLS FROM TEA LEAF

P. J. HILTON
Tea Research Foundation of Central Africa, P.O. Box 51, Mulanje, Malawi

(*Received* 24 *June* 1971, *in revised form* 15 *October* 1971)

Abstract—The six major flavanols present in fresh shoots of tea (*Camellia sinensis* L.) were extracted, purified, and oxidized *in vitro* with a soluble and partially purified polyphenol oxidase preparation, also extracted from fresh tea shoots. Besides different forms of theaflavin gallate, two species of theaflavin were observed. When the six flavanols were oxidized together, epigallocatechin limited total theaflavin production.

INTRODUCTION

THE IMPORTANCE of theaflavins in determining the quality of black tea was first demonstrated by Roberts.[1,2] He obtained correlations between theaflavin content and tea-tasters' scores for colour and 'briskness' (or astringency) of tea liquors,[3] and these observations have since been verified by direct tasting of purified theaflavin in aqueous solution.[4] In commercial samples of tea not exhibiting 'flavour' characteristics, the theaflavin content represents a high variance component of the selling price.[5]

Besides theaflavin itself, iso-theaflavin[6] and three theaflavin gallates[7–9] have been reported as occurring in black tea infusions, besides smaller proportions of theaflavic and epitheaflavic acids.[8] *In vitro* studies have indicated that theaflavin and its gallates are derived from oxidations of various pairs of the catechin flavanols found in the green tea leaf,[7,10–12] and it has been suggested that iso-theaflavin is formed in a similar manner.[6]

Green tea leaf contains 10–20% (w/w) of the six major catechins, while seldom more than 2% (w/w) total theaflavins is found in the manufactured tea. Planting material may be bred and selected for its catechin content, but it is important for the plant breeder to know which catechin is most likely to be limiting total theaflavin production during the manufacturing process.

This paper confirms the production of two theaflavins and at least two theaflavin gallates during an *in vitro* enzymic oxidation of pairs of purified catechins, and reports the

[1] E. A. H. ROBERTS and F. R. SMITH, *Analyst*, **86**, 94 (1961).
[2] E. A. H. ROBERTS and R. F. SMITH, *J. Sci. Food Agric.* **14**, 689 (1963).
[3] D. J. WOOD and E. A. H. ROBERTS, *J. Sci. Food Agric.* **15**, 19 (1964).
[4] D. J. MILLIN, D. J. CRISPIN and D. SWAINE, *J. Agric. Food Chem.* **17**, 717 (1969).
[5] P. J. HILTON and R. T. ELLIS, *J. Sci. Food Agric.* In press.
[6] D. T. COXON, A. HOLMES and W. D. OLLIS, *Tetrahedron Letters* **60**, 5241 (1970).
[7] T. BRYCE, P. D. COLLIER, I. FOWLIS, P. E. THOMAS, D. FROST and C. K. WILKINS, *Tetrahedron Letters* **32**, 2789 (1970).
[8] D. T. COXON, A. HOLMES, W. D. OLLIS and V. C. VORA, *Tetrahedron Letters* **60**, 5237 (1970).
[9] A. G. H. LEA and D. J. CRISPIN, *J. Chromatog.* **54**, 133 (1971).
[10] Y. TAKINO and H. IMAGAWA, *Agric. Biol. Chem. Tokyo* **27**, 64 (1963).
[11] Y. TAKINO, H. IMAGAWA, H. HORIKAWA and A. TANAKA, *Agric. Biol. Chem. Tokyo* **28**, 64 (1964).
[12] Y. TAKINO, T. OZAWA, H. SANADA, S. MASAKI and C. FUKUNAGA, *J. Agric. Chem. Soc. Japan* **45**, 176 (1971).

effect of varying the proportions of six substrate catechins upon the total theaflavin produced in the incubation.

RESULTS

No coloured products were formed as a result of incubation of any flavanols, or combination of flavanols, with the enzyme preparation which had been previously immersed in boiling water for 10 min.

During the course of enzymic oxidation of pairs of flavanols, theaflavin, theaflavin-3-gallate, and theaflavin-3'-gallate were formed in accordance with the scheme of theaflavin synthesis proposed by other workers.[8,11,12] Theaflavins were recognized by their extractibility in organic solvents, absorption spectra, positions on thin-layer chromatograms, and by chromatography on LH-20 Sephadex. TLC resolved the two theaflavin mono-gallates, theaflavin-3'-gallate moving faster than theaflavin-3-gallate in the organic solvent, but the technique was not sufficiently sensitive to resolve theaflavin-3,-3'-digallate, though this may well have been formed.

Oxidation of epicatechin together with gallocatechin resulted in a compound having the spectral characteristics of a theaflavin, but chromatographically distinct from the theaflavins mentioned above.

When any two flavanols were oxidized together in equimolar proportions, one was invariably consumed faster than the other. Information from all combinations of pairs indicated that flavanols were oxidized preferentially in the following order: epigallocatechin, epigallocatechin gallate, gallocatechin, epicatechin gallate, epicatechin, and catechin.

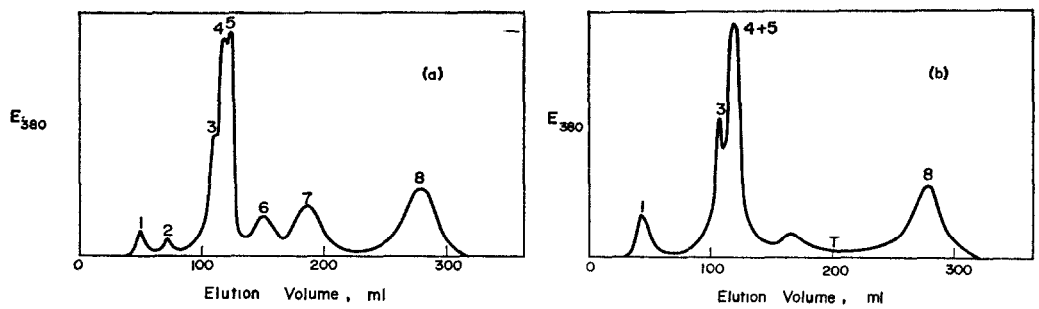

FIG. 1. ELUTION OF OXIDATION PRODUCTS OF FLAVANOLS FROM A COLUMN OF SEPHADEX LH-20 WITH 60%(v/v) AQUEOUS ACETONE.
(a) Aqueous infusion of black tea; (b) Mixture of six catechins after *in vitro* enzymic oxidation.

Incubation of a mixture of six catechins in proportions similar to those existing in the green leaf from which the catechin were originally extracted resulted in the production of two theaflavins and at least two theaflavin gallates. Thin layer chromatograms of the extract showed all the spots produced during incubations of individual and paired catechins. The same spots were visible on chromatograms of a hot aqueous infusion of black tea, although in this case the S_1 thearubigin fraction[13] was more in evidence. An elution profile of the oxidation products on alkylated Sephadex showed the absence of two well defined components which were observed in the black tea extract (Fig. 1).

All six catechins were consumed during this reaction, but at different rates: in general the galloyl catechins were oxidized faster than the simple catechins (Fig. 2).

[13] E. A. H. ROBERTS, R. A. CARTWRIGHT and M. OLDSCHOOL, *J. Sci. Food Agric.* **8**, 72 (1957).

To investigate which of the catechins were likely to be limiting total theaflavin production in the above incubation, the concentration of certain catechins was doubled by addition of individual catechins to the mixture. Results indicated that doubling the epigallocatechin content of the mixture resulted in a 64% increase in theaflavin production (Table 1). Doubling either the epigallocatechin gallate, or the epicatechin content of the mixture failed to increase theaflavin production, and in one case actually reduced it.

TABLE 1. ENZYMIC OXIDATION OF A MIXTURE OF SIX CATECHINS: EFFECT OF CATECHIN COMPOSITION UPON QUANTITIES OF THEAFLAVINS PRODUCED

Substrate	Total Theaflavins (mg)
51·3 mg control mix.	3·50
50·2 mg control mix. + 11·8 mg EGC	5·74
50·3 mg control mix. + 4·0 mg EC	3·31
50·6 mg control mix. + 21·0 mg EGCG	2·05

For details of incubation procedure and analyses see Experimental.

Quantitative assay of the catechins showed that increasing the initial concentrations of either epigallocatechin or its gallate lowered the initial rate of disappearance of epicatechin and its gallate; when most of the galloyl catechins had been consumed, the rate of disappearance of the simple catechins was increased. Increasing the concentration of epicatechin in the mixture did not increase the initial rate of consumption of this catechin: little epicatechin was consumed until most of the galloyl catechins had disappeared. Nor was the rate of oxidation of epigallocatechin or its gallate appreciably affected.

To compare this *in vitro* system with the actual factory 'fermentation' process, samples were taken from leaf undergoing conventional C.T.C. (cut, tear, curl) manufacture, and analysed for catechin content, total theaflavin content, and overall density of colour of the aqueous infusion. While there was little change in the level of the catechins during the initial rolling process, as soon as drastic cell damage was induced by the C.T.C. machine, the time-course of catechin disappearance was very similar to that experienced in the *in vitro* incubations (Fig. 3). During the course of the fermentation process, the total theaflavin content reached a peak and then began to fall. This peak appeared to correspond with the effective exhaustion of epigallocatechin. (Although epigallocatechin was detectable after this time, very little was consumed, and was presumably contained in cells undamaged in the manufacturing process.)

DISCUSSION

The theaflavin formed during the incubation of epicatechin with gallocatechin is presumably the iso-theaflavin reported as occurring in black tea by Coxon *et al.*,[6] who have suggested from that iso-theaflavin should be derived from these two flavanols.

Roberts and Myers[14] suggest that the gallocatechins were preferentially oxidized by

[14] E. A. H. ROBERTS and M. MYERS, *J. Sci. Food Agric.* **11**, 158 (1960).

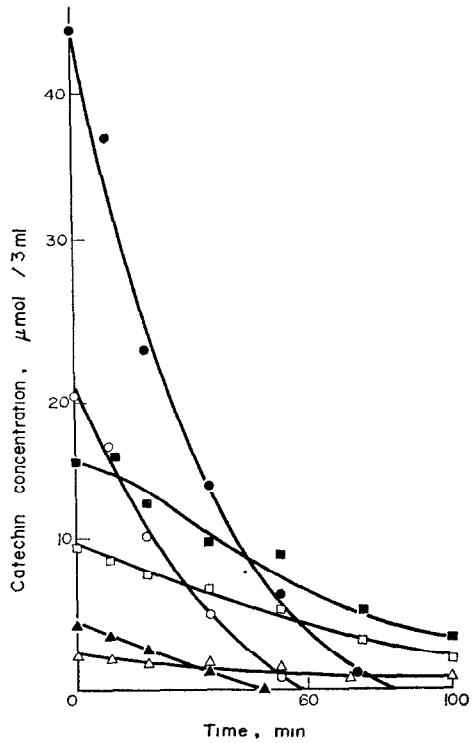

FIG. 2.

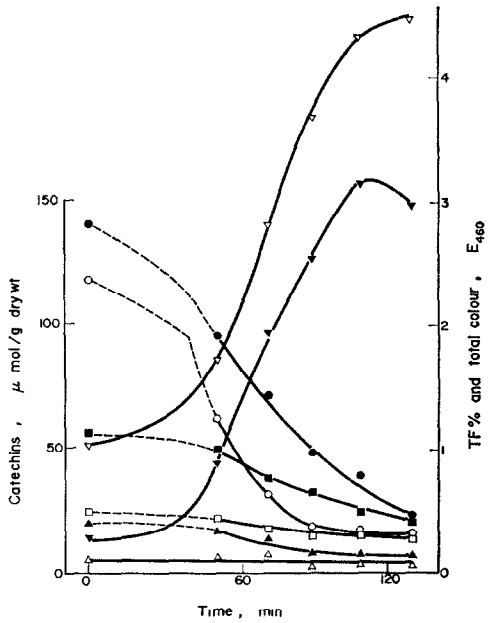

FIG. 3.

FIG. 2. DISAPPEARANCE OF CATECHINS DURING ENZYMIC OXIDATION OF A MIXTURE OF SIX CATECHINS. For conditions of incubation and analyses see experimental section. ○—epigallocatechin; ●—epigallocatechin gallate; □—epicatechin; ■—epicatechin gallate; △—catechin; ▲—gallocatechin.

FIG. 3. DISAPPEARANCE OF CATECHINS AND APPEARANCE OF COLOUR AND THEAFLAVINS DURING CONVENTIONAL MANUFACTURING PROCESS.
Shoots from a clonal tea population growing in Malawi were rolled for 40 min. before passing twice through a 'C.T.C.' (cut, tear, curl) machine, and allowed to 'ferment' thereafter in a stream of air at 25° and 95% relative humidity. ▼—Theaflavin; ▽—total colour. All other symbols as for Fig. 2.

virtue of their lower redox potentials. Results presented here (Fig. 2 and text) show that the six catechins under study may be arranged in the following order of ascending redox potential: epigallocatechin, epigallocatechin gallate, gallocatechin, epicatechin gallate, epicatechin, catechin. It is likely that the catechins will act as electron carriers for the preferential oxidation of the gallocatechins. However, none of these components can be perfect carriers, as some consumption of epicatechin and epicatechin gallate can be observed in all incubations studied, regardless of whether substances of lower redox potentials are present. Indeed, since the benzene ring of the theaflavin benzotropolene nucleus is derived from the dihydroxy catechins, production of theaflavins is dependent upon concurrent consumption of dihydroxy and trihydroxy catechins.

If catechin, epicatechin, or epicatechin gallate are acting as electron carriers, then in the presence of an excess of substances of lower redox potential, the system will become saturated with these molecules at catalytic rather than substrated concentrations. This would

explain why increasing the epicatechin component in a mixture of six catechins fails to increase the overall catechin oxidation rate, or the production of theaflavins.

Given that there is an initial and continuous slow consumption of dihydroxy flavanol carrier molecules, even in the presence of unoxidized substrates of lower redox potential, the oxidized carrier can undergo further independent reaction either with another molecule of its own species, such as the co-polymerization proposed for catechin,[15] or with another molecular species. If this new molecular species be a quinone derived from a trihydroxy flavanol, a theaflavin will be formed. The likelihood of this happening will depend on the concentration in the medium of trihydroxy flavanol-derived quinones, which will be a function of the concentration of the parent flavanol, and the life of the corresponding quinone before it undergoes further reaction. The quinone derived from epigallocatechin would be expected to have the longest life since epigallocatechin has the lowest redox potential of the six catechins under investigation, and evidence is presented here (Table 1) that its concentration is limiting in theaflavin production.

Brown *et al.*[16] identify the thearubigins as polymeric proanthocyanidins, and demonstrate that at least four of the six catechins investigated here are involved in thearubigin structure. However, incubation of the six catechins alone does not produce all the component of the thearubigin fraction observed in made tea extracts (Fig. 2). While conditions of oxidation in the fermenting tea leaf may be very different from the *in vitro* conditions produced here, the dynamics of catechin disappearance and theaflavin production are very similar, and is seems likely that substances other than the six catechins studied are substantially involved in the thearubigin complex.

EXPERIMENTAL

Purification of polyphenol oxidase. An acetone-dried powder was made from fresh tea shoots, and extracted with ascorbate buffer pH 10·2.[17] The solution was dialysed overnight against distilled water, and the precipitate extracted with 0·1 M citrate buffer pH 5·6. The solution was subjected to ammonium sulphate fractionation, and that fraction precipitating between 60% and 85% saturation at 23°C was dialysed overnight against 0·05 M NaOAc buffer pH 5·4 at 4°. The supernatant was applied to a column of DEAE-cellulose, and eluted with 0·05 M NaOAc buffer pH 5·4. The unretarded fraction was almost colourless, and was concentrated by selective filtration (Dynaflow pressure filter) using a membrane retaining molecules above a MW of 10 000. No flavonoid compounds were detectable on TLC chromatograms of the preparation, which when assayed by polarograph[17] showed an activity of 150 μM O_2 min^{-1} ml^{-1}.

Purification of flavanols. Flavanols were extracted from dried green tea shoots by the method of Vuataz *et al.*[18] as modified by Gregory and Bendall.[17] TLC showed the preparation to be virtually free from phenolic contaminants, and it was used as the basic mixture of six catechins in experiments. Epicatechin gallate, gallocatechin, epigallocatechin and its gallate were separated from this mixture by partition chromatography on cellulose,[18] while catechin and epicatechin were obtained commercially and recrystallized. All catechins were chromatographically pure.

Assay of flavanols. Where only qualitative assessment was required, TLC plates were sprayed with *bis*-diazotized benzidine solution.[19] Where quantitative analysis was necessary, the flavanols were located by fuming the TLC plates in I_2, removing spots, and assaying using the vanillin reaction.[20]

Assay of oxidation products. Catechin oxidation products were visible on TLC plates sprayed with benzidine, and were further analysed by chromatography on a column of sephadex LH-20.[21] Theaflavins were estimated from the integrated elution profile. By this method theaflavins from a black tea infusion could be estimated to a coefficient of variation of 2·8%.

[15] D. E. Hathaway and J. W. T. Seakins, *Biochem. J.* **67**, 239 (1957).
[16] A. G. Brown, W. B. Eyton, A. Holmes and W. D. Ollis, *Phytochem.* **8**, 2333 (1969).
[17] R. P. F. Gregory and D. S. Bendall, *Biochem. J.* **101**, 569 (1966).
[18] L. Vuataz, H. Brandenberger and R. Egli, *J. Chromatog.* **2**, 173 (1959).
[19] D. G. Roux and A. E. Maihs, *J. Chromatog.* **4**, 65 (1960).
[20] G. I. Forrest and D. S. Bendall, *Biochem. J.* **113**, 741 (1969).
[21] D. J. Crispin, R. N. Payne and D. Swaine, *J. Chromatog.* **37**, 118 (1968).

Theaflavins produced during the normal manufacturing process were extracted in a hot aqueous infusion of the 'fermenting' leaf. A suitable dilution of the infusion was extracted with an equal volume of *iso*-BuCOMe. To 2 ml *iso* BuCOMe extract were added 2 ml EtOH and 2 ml 2% (w/v) ethanolic flavognost (diphenyl boric acid ethanolamine complex, Heyl & Co., Berlin). After standing for 5 min the extinction at 600 nm was measured in a colourimeter. The method was highly reproducible (coefficient of variation < 1% for the same tea infusion) and in absence of any pure theaflavin standards, was calibrated against Roberts' method for theaflavin analysis,[1] with which it showed good correlation ($r = 0{\cdot}98$).

Incubation procedure. Incubations were performed in a Clark oxygen electrode chamber equilibrated at 30°. The catechins under investigation (10 mg quantities) were dissolved in 2·5 ml 0·05 M citrate buffer pH 5·6, and the reaction started by adding 0·5 ml of the enzyme preparation. The level of oxygen was kept at an arbitrary constant level (as measured by the chart recorder attached to the electrode) by bubbling warmed and moistened oxygen through the medium. Periodically, the rate of oxygen uptake was measured by cutting off the oxygen supply and closing the electrode chamber.

The reaction was monitored by TLC at intervals, using 5 μl samples, and stopped by heating when the rate of oxygen uptake was 10–15% of its initial value. Half the mixture was evaporated to dryness under vacuum and subsequently analysed by alkylated Sephadex chromatography as described above. The remainder was exhaustively extracted with EtOAc, and the absorption spectrum determined. Pilot incubations were run to determine the time at which theaflavin production was maximal, and subsequent incubations stopped at these times. However, theaflavin analysis in these experiments can only be regarded as being semi-quantitative, as the quantities of substrates available did not allow serial sampling, and only one determination per incubation was performed.

Acknowledgements—This work forms part of the content of a thesis for the degree of Doctor of Philosophy in the University of Durham. I am very grateful to The Tropical Products Institute for generous financial support, and indebted to The Botany Department of Durham University and The Tea Research Foundation of Central Africa for use of their respective laboratory facilities. I thank Professor D. Boulter and Dr. R. T. Ellis for their continued help and guidance.

Key Word Index— *Camellia sinensis*; Theaceae; tea leaf; catechin oxidation; theaflavins.