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FORMATION OF EPITHEAFLAVIC ACID AND ITS TRANSFORMATION TO THEARUBIGINS DURING TEA FERMENTATION

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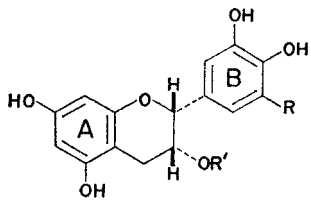
Abstract-The reactions of (-) epicatechin (I) and (-) epicatecbin gallate (II) with gallic acid in a model tea fermentation system were studied. The primary oxidation products formed from the oxidation of (I) or (II) with gallic acid in short reaction periods were bright red condensation products named epitheaflavic acid (IX) and 3-galloyl epitheaflavic acid (X). (IX) and (X) appear to be identical to an unknown 'complex Q' reported earlier to be a trace substance in black tea extracts. Roth (I) and (II) were oxidized in the model tea fermentation system, but gallic acid was not oxidized by itself. Oxidation of (I) in this system did not produce any (IX) unless gallic acid was also present. Some deesteritication of (II) took place in the model tea fermentation system with the result that oxidation of (II) by this system produced some (IX) and some (X) besides other oxidation products. (IX) was not reactive in the model tea fermentation system by itself, but (IX) was rapidly transformed to thearubigins (acidic brown pigments found in black tea) when (I) was also present. These results furnish more information regarding the mechanism by which the thearubigins of black tea are formed, and they point out again the central role of tea flavanol oxidation catalysed by tea catechol oxidase in tea fermentation.

INTRODUCTION

BLACK tea of commerce is prepared[1-3] from freshly harvested, tender shoot tips (collectively called the flush) of the tea plant, ***Camellia sinensis*** (L.) 0. Kuntze, by a process that may be described[4,5] as a series of partially controlled biochemical steps. The main part of this process is an enzymic oxidation called tea fermentation, and the primary reactions taking place are oxidations of the tea flavanols, catalysed by the enzyme tea catechol oxidase. The major products of this oxidation are colored compounds which were separated into two groups and named theaflavins (TFs) and thearubigins (TRs) by Roberts *et al.*[4,6,7]

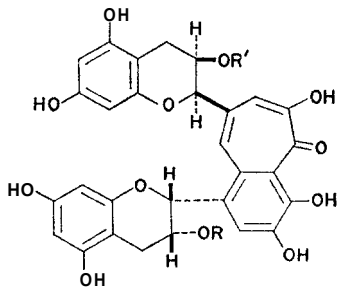
The TFs have been shown to be formed from the oxidative condensation of one molecule of (—) epicatechin, EC (I), or (—) epicatechin gallate, ECG (II); and one molecule of (—) epigallocatechin, EGC (III), or (-) epigallocatechin gallate, EGCG (IV).[8] The structures of the TFs (V, VI, VII, VIII) were determined by Takino *et al.*,[9] and by Brown *et al.*,[10]

[1] T. EDEN, ***Tea,*** 2nd Edn, Longmans Green, London (1965).
[2] E. HAINSWORTH, in ***Encyclopedia of Chemical Technology,*** 2nd Edn, Vol. 19, p. 743, Interscience, New York (1969).
[3] C. R. HARLER, ***Tea Manufacture,*** Oxford University Press, London (1963).
[4] E. A. H. ROBERTS, in ***Chemistry of Flavonoid Compounds*** (edited by T. A. GEISSMAN), p. ***468,*** Pergamon Press, London (1962).
[5] G. W. SANDERSON, in ***Recent Advances in Phytochemistry*** (edited by V. C. RUNECKLES), Vol. 5, to be published.
[6] E. A. H. ROBERTS, R. A. CARTWRIGHT and M. OLDSCHOOL, ***J. Sci. Food Agric. 8, 72 (1957).***
[7] E. A. H. ROBERTS and M. MYERS, ***J. Sci. Food Agric. 10, 167 (1959).***
[8] ***Y.*** TAKINO and H. IMAGAWA, ***Agric. Biol. Chem. 28,255 (1964).***
[9] ***Y.*** TAKINO, A. FERRETTI, V. FLANAGAN, M. A. GIANTURCO and M. VOGEL, ***Tetrahedron Letters 4019 (1965); 4024 (1966).***
[10] A. G. BROWN, C. P. FALSHAW, E. HASLEM, A. HOLMES and W. D. OLLIS, ***Tetrahedron Letters*** 1193 (1966).



I R = R′ = H
II R = H; R′ = G
III R = OH; R′ = H
IV R = OH; R′ = G

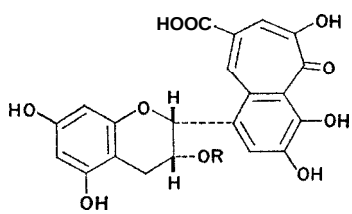
G = Galloyl (3,4,5-trihydroxybenzoyl)



V R = R′ = H
VI R = H; R′ = G
VII R = G; R′ = H
VIII R = R′ = G

G = Galloyl (3,4,5-trihydroxybenroyl)

and their configurations were determined more recently by Coxon *et al.*,[11] and by Bryce *et al.*[12] Roberts[4] suggested that the TRs were dicarboxylic acids formed by the further oxidation of TFs, but that there was no further condensation to form molecules larger than the TFs. However, more recent work by Nakagawa and Torii[13] and by Sanderson et *al.*[14] has shown that TRs can be formed through oxidation of tea flavanols in model tea fermentation systems in which TFs cannot be formed because the required flavanol pairs are not present. Further, Millin and Rustidge[15] have shown that the TRs have a wide range of molecular weights, i.e. 700–40,000, which would require considerable polymerization if they are formed by oxidative condensation of tea flavanols. These results[15] are reminiscent of the much earlier work of Bradfield and Penny[16] which also indicated that the TRs were polymeric substances. Finally, Brown *et al.*,[17] have shown that the TRs are proanthocyanidins providing further support for the idea that the TRs are formed by the oxidative polymerization of tea flavanols. In summary, many questions remain unanswered regarding the formation and the chemistry of the TRs.



IX R = H
X R = Galloyl (3,4,5-trihydroxybenzoyl)

[11] D. T. COXON, A. HOLMES, W. D. OLLIS and V. C. VORA, *Tetrahedron* Letters 5237 (1970).
[12] T. BRYCE, P. D. COLLIER, I. FOWLIS, P. E. THOMAS, D. FROST and C. K. WILKINS, *Tetrahedron Letters* 2789 (1970).
[13] M. NAKAGAWA and H. TORII, *Agric. Biol. Chem. 29. 278* (1965).
[14] G. W. SANDERSON, J. E. BERKOWITZ and H. Co, to be published.
[15] J. MILLIN and D. W. RUSTIDGE, *Process Biochem. 2. 9* (1967).
[16] A. E. BRADFIELD and M. PENNY, *J. Soc. Chemical Industry* 43, *306 (1944).*
[17] A. G. BROWN, W. B. EYTON, A. HOLMES and W. D. OLLIS, *Nature, Lond. 221,742 (1969); Phytochem. 8, 2333 (1969).*

We have continued the investigation of the products formed by the oxidation of various combinations of tea flavanols in model tea fermentation systems[14] and have noticed that systems containing ECG, or EC plus some other gallated catechin, always form a compound that appears to be the same as Roberts' substance Q.[4] The structure of a compound of this type formed by the co-oxidation of EC and gallic acid (GA) by potassium ferricyanide in dilute aqueous bicarbonate solutions was determined by Coxon et *al.*[18] and by Bryce *et al.*[12] This compound has been named epitheaflavic acid, ETA (IX), because of its structural parallel with TFs.[19] This structure established ETA as the product of the oxidative condensation of one molecule of EC and one molecule of GA. We report below on the formation of this compound during tea fermentation and the role it plays in the formation of the TRs.

RESULTS

Formation and Characterization of Epitheaflavic Acids

The oxidation of EC and GA in a model tea fermentation system containing a crude soluble tea (CST) enzymes preparation proceeds with fairly rapid color changes from greenish yellow (0 time) to brownish orange (15 min) to dark brown (40 min). A study by paper chromatography of the reaction products formed in this system reveals that several compounds are formed as a result of the oxidations taking place. In short oxidation periods ($\sim$ 15 min), the major oxidation product was a compound which we have identified as ETA (Fig. 1; Table 1, A).

When the substrates used in the model tea fermentation system were ECG and GA (Table 1, B), there were two major products after short oxidation periods, namely ETA and another compound which we believe to be 3-galloyl epitheaflavic acid, ETAG (X), for reasons explained below.

When EC was the only substrate in the model tea fermentation system (Table 1, C), TRs and the unidentified substances A and B were formed but ETA was not formed. On the other hand, when ECG was the substrate in the model tea fermentation system (Table 1, D), several oxidation products were formed which included both ETA and ETAG. It is noteworthy that GA was formed in this system as is usually found both in model tea fermentation systems whenever 3-galloyl catechins (i.e. II, IV) are present[13,14] and in regular tea fermentation.[10,14] In fact, the oxidation products produced by the model tea fermentation system acting on ECG alone closely resembled those produced from ECG plus GA as might be expected from a system which actively deesterifies gallated flavanols. There was no reaction when GA was the only substrate added to the model tea fermentation system (Table 1, E) showing once again that GA is not a substrate for tea catechol oxidase.[19,20]

Additional information regarding the nature of the reactions taking place in these model tea fermentation systems was obtained in respirometric studies. These studies showed that approximately one mole of CO_2 was given off for each two moles of O_2 consumed during the initial stages (up to $\sim$ 15 min) of oxidation in these model tea fermentation systems containing either EC and GA or ECG and GA (Fig. 1). When EC was the only substrate, no CO_2 evolution accompanied the oxidation process (Fig. 2). When ECG

[18] D. T. COXON, A. HOLMES and W. D. OLLIS, *Tetrahedron Letters 5247 (1970).*
[19] E. A. H. ROBERTS and D. J. WOOD, *Biochem. J. 47, 175 (1950).*
[20] R. P. F. GREGORY and D. S. BENDALL, *Biochem. J.* 101, 569 (1966).

TABLE 1. SUMMARY OF REACTION PRODUCTS* FOUND IN A MODEL TEA FERMENTATION SYSTEM OXIDIZING VARIOUSCOMBINATIONSOFTHEFOLLOWINGSUBSTRATES: EC, ECG, GA, AND ETA

Oxidation period (min)	Phenolic compounds present in reaction mixtures									
	EC (I)	ECG	(II)	GA	ETA (IX)	ETAG (X)	TRs	A†	B†	St‡
A. Subtrates = EC and GA										
0	++++		—	++++	—	—	—	—	—	—
10	++		—	++	+++	—	+	+	+	—
20	+			+	++	—	++	tr	tr	—
40	—		—	—	+	—	+++	—	—	—
B. Subtrates = ECG and GA										
0	—	++++	++++		—	—	—	—	—	—
10	+	++	++++		+	++	+	—	—	+
20	tr	+	++		+	+	++	—	—	tr
40	—		+		tr	tr	++	—	—	—
C. Substrate = EC only										
0	++++		—	—	—	—	—	—	—	—
20	++		—	—	—	—	+	tr	tr	—
D. Substrate = ECG only										
0	—	++++		—	—	—	—	—	—	—
20	+	+		+	tr	+	+++	—	—	+
E. Substrate = GA only										
0	—		—	++++	—	—	—	—	—	—
20	—			++++	—	—	—	—	—	—
F. Substrate = ETA only										
0	—		—	—	++++	—	—			
10	—		—	—	++++	—	—			
20	—		—	—	++++	—	—			
40	—		—	—	++++	—	—	—	—	—
G. Substrates = EC and ETA										
0	++++		—	—	++++	—	—	+	—	—
10	+		—	—			+++	+	tr	—
20	—		—	—	—	—	++++	tr	—	—
40	—		—	—	—	—	++++	tr	—	—

(See the Experimental section for conditions used.)

* Quantitations indicated are estimates of spot intensities on paper chromatograms. Key to abbreviations used: + + ++ = very dark spots, + ++ = dark spot, + + = medium spot, + = light spot, tr = trace spot, — = no spot.

† These oxidation products are unidentified.

‡ Streaking (St) in the area of R_f 0·40–0·70 (BAW) and 0–0·30 (2 % HOAc).

was the only substrate, there was less than one mole of CO_2 given off for each two moles of 0, consumed (Fig. 2). As expected, there was neither CO_2 given off nor O_2 consumed when GA was the only substrate (Fig. 2).

The requirement of a vicinal diphenolic grouping (as is found in the B-ring of either I or II) and a vicinal triphenolic grouping (as is found in GA) for the formation of the red colored compounds ETA and ETAG suggested that the reaction leading to their formation was analagous to the reaction leading to the formation of TF.[8,13,14] The following reactions summarize these suppositions :

(a) EC (I) + GA + 1 1/2 O_2 ⟶ ETA (IX) + CO,
(b) ECG (II) + GA + 1 1/2 0, ⟶ ETAG (X) + CO,
(c) EC + O_2 ⟶ Unknown A + Unknown B + TRs
(d) ECG + O_2 ⟶ Streaking + TRs
(d′) ECG ⟶ EC + GA ⟶ Reaction (a) + Reaction (b).

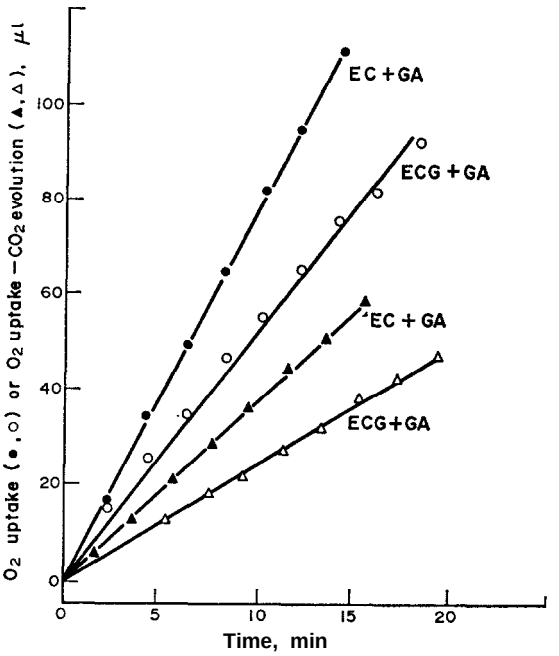


FIG. 1. GASEOUS EXCHANGE FOR A MODEL TEA FERMENTATION SYSTEM OXIDIZING EC AND GA AND ECG AND GA.

Further, since ETA and ETAG are the primary products of these reactions, it is clear that reactions (a) and (b) predominate over reactions (c) and (d). Note that reaction (d') produces the same result as reaction (a). The results of our respirometric studies (Figs. 1, 2) also support this explanation. Accordingly, structures (IX) and (X) were proposed for ETA and ETAG, respectively. Structure (IX) is identical to the structure proposed for a new compound named epitheaflavic acid'[8] which was prepared by co-oxidation of EC and GA by potassium ferricyanide in dilute bicarbonate solution.[12,18] Professor W. D. Ollis generously provided us with a sample of ETA which we found to be identical with our purified material.

It is noteworthy that Roberts[21] published the correct structure for ETA which he called 'substance Q'. However, he subsequently took a more cautious stand and described the structure of 'complex Q' in less definite terms in his later, more comprehensive review of tea chemistry.[4]

Dynamics of the Accumulation of ETA and ETAG

The dynamics of ETA accumulation in a model tea fermentation system containing EC and GA as substrates showed that ETA accumulates during the first part of the oxidation (i.e. up to ~ 15 min) after which the amount of ETA in the system begins to decrease, disappearing completely after oxidation periods of about 40 min (Table 1, A). As noted above, both ETA and ETAG accumulated in model tea fermentation systems containing ECG and GA as substrates. In this case (Table 1, B), both ETA and ETAG were found to

[21] E. A. H. ROBERTS, *Tea Quarterly 32,* 190 (1961).

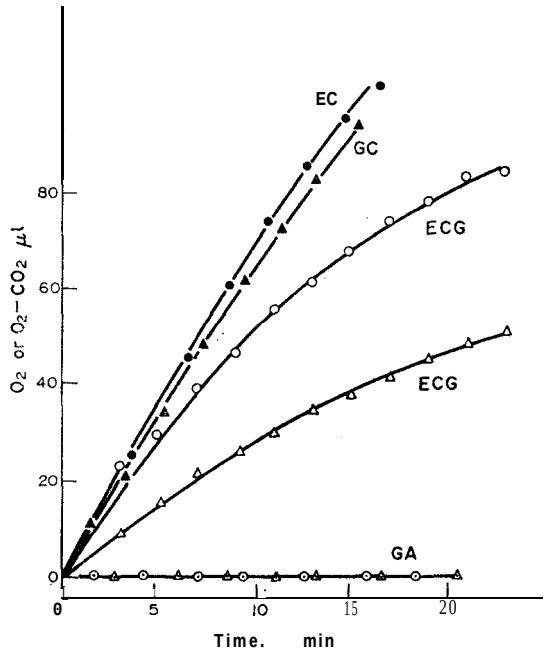


FIG. 2. GASEOUS EXCHANGE FOR A MODEL TEA FERMENTATION SYSTEM OXIDIZING EITHER EC, ECG, OR GA.

Oxygen uptake (circles) or oxygen uptake less carbon dioxide evolution (triangles).

accumulate during the first few minutes of the oxidation period and then to decrease while the TRs appeared to increase continuously until the free ECG was used up.

Transformation of ETA to TRs

In both the EC plus GA and the ECG plus GA model tea fermentation systems, the TRs appeared to accumulate as the oxidation progressed at the expense of the simpler polyphenolic compounds present, i.e. EC or ECG, GA, ETA or ETAG, etc. (Table 1).

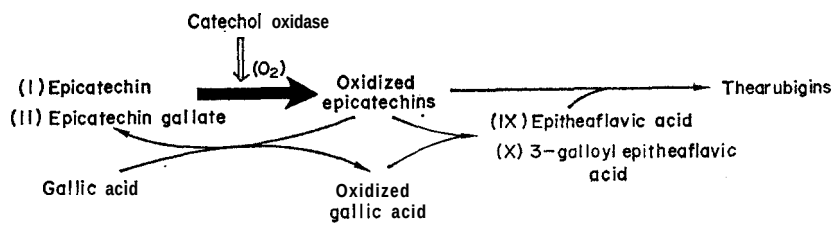


FIG. 3. FORMATION of ETA AND ETAG AND THEIR TRANSFORMATION TO TRs IN TEA FERMENTATION SYSTEMS.

The ability of the tea fermentation system to transform ETA to TRs was studied using a pure sample of ETA. These studies showed that ETA underwent no reaction when it was added to the model tea fermentation system by itself (Table 1, F). However, when EC was added to the model tea fermentation system along with ETA, EC and ETA both underwent rapid disappearance with the concomitant appearance of TRs (Table 1, G).

DISCUSSION

ETA (IX) and ETAG (X) resemble the TFs (V, VI, VII, VIII) in having a benzotropolone group that is apparently the chromophore which gives all of the compounds a bright red color. Both groups of compounds are formed by the oxidative condensation of a 3,4-diphenolic benzenoid ring (such as the B-ring of I or II) and a 3,4,5-triphenolic benzoid ring (such as the B-ring of III or IV or GA). An important difference is the fact that the tea catechol oxidase will catalyse the oxidation of both of the substrate molecules required for the formation of TFs but only one of the substrate molecules required for the formation of ETA and ETAG, namely the catechin molecule. However, oxidation of the GA molecule required for ETA or ETAG formation is readily affected by the oxidation of EC which is catalysed by the tea catechol oxidase. The results of this investigation also show clearly that ETA, and presumably ETAG, are converted to TRs by the tea fermentation system and that this transformation is dependent on, as is gallic acid oxidation, the tea catechol oxidase catalysed oxidation of tea flavanols. These results are summarized in Fig. 3.

ETA and ETAG can now be added to the list of TRs precursors which already includes all of the individual tea flavanols.[13,14] The suggestion[4] that TFs are transformed to TRs during tea fermentation has not yet been demonstrated, but it now appears most likely that this reaction does in fact take place under the same conditions that cause the transformation of ETA. A Figure presented in the introduction to a paper by Wood and Robert? indicates that these researchers appreciated the dependence of this transformation on being coupled to tea flavanol oxidation. Also, the high reactivity of ETA in the oxidizing tea fermentation system shown in this investigation explains why ETA and ETAG are normally only found as trace substances in black tea.[4]

It is noteworthy that the catechol oxidase catalysed oxidation of tea flavanols appears to be the driving force for many of the reactions taking place during tea fermentation. Examples of reactions which have been shown to fall in this category are the oxidative degradation of amino acids[23] and carotenoid compounds[24] which take place during tea fermentation and which are important in the formation of black tea aroma. The existing evidence suggests that this oxidative mechanism plays a most important role in tea fermentation. The inter-relationships which exist between the several reactions taking place during tea fermentation have been discussed recently.[5]

EXPERIMENTAL

Materials. Fresh green tea leaf was air-freighted to us[23] and kept at -40" for use when required. EC was purchased from Pierce Chemical Company. ECG and CST enzymes were prepared from the fresh-frozen tea leaves as described previously.[23]

Model tea fermentation systems. In studies of the dynamics of substrate oxidation, the reaction mixtures consisted of substrates, i.e. EC, ECG, and GA (each at 2·5 mM concentration) in 23 ml of citrate-phosphate buffer (0·1 M, pH 5.4) plus 2·0 ml CST enzymes added at the beginning of the reaction period. Oxidations were carried out at 30" with enough shaking to insure thorough and uniform aeration. Reactions were stopped by adding 20% trichloroacetic acid (TCA) (0·8/2·0 ml) and cooling to 0" to ensure complete precipitation of the protein. The samples were centrifuged for 15 min at 12,000 *g* at 4". The supernatants were decanted, and the precipitates extracted with 0·5 ml acetone. The acetone extracts were combined with the original supernatants for chromatography.

In respirometric studies, the reaction mixtures consisted of 3·0 ml of substrate (i.e. EC, ECG, and GA; each at 2·0 mM concentration) in phosphate-citrate buffer (0·1 M, pH 54) with and without 0·1 ml 10% NaOH on paper fans in the center well. All reactions were carried out at 30". After equilibrating for 20 min,

[22] D. J. WOOD and E. A. H. ROBERTS, *J. Sci. Food Agric.* 15, 19 (1964).
[23] H. Co and G. W. SANDERSON *J. Food Sci. 35, 160 (1970).*
[24] *G.* W. SANDERSON, H. Co and J. G. GONZALEZ, *J. Food Sci., 36,231 (1971).*

CST enzymes were added from the side arm and gaseous exchange was measured using a Gilson differential respirometer.

Chromatography of reaction mixtures. Aliquots were chromatographed 2-D on Whatman No. 1 paper with Butanol-acetic acid-water (4: 1 : 2.2) (BAW) and 2 % HOAc spots were visualized under UV and with $FeCl_3$–$K_3Fe(CN)_6$.

Enzymic synthesis andpreparation of ETA and ETAG. Mixtures of either EC and GA or ECG and GA (2·5 mM each) in 500 ml of citrate-phosphate (-0.1 M, pH 5·4) were incubated with 50 ml of CST enzymes for 15 min (time for maximum ETA accumulation) at 30". The reaction mixtures were then extracted successively with 500 ml EtOAc and iso-butyl methyl ketone until the organic layer was colorless. The combined organic extract was washed, dried, and taken to dryness under reduced pressure.

The dried extract was dissolved in 5 ml of 60% acetone, and applied to a column (2.5 x *45* cm) of Sephadex LH-20. The column was eluted with 60% aqueous acetone, giving two main fractions, a dark green-brown fraction and a bright orange fraction (mainly ETA or ETAG). The eluant compositions were then determined by paper chromatography. The fractions containing ETA were combined. ETA was characterized by comparison with authentic material by co-chromatography, NMR and IR.

Enzymic oxidation of ETA. A *25* mg sample of ETA was dissolved in 25 ml of citrate-phosphate buffer (-0.1 M, pH 5.4). The solution was split and about 10 mg of EC was added to one portion. The oxidations were started by adding 1·0 ml of the CST enzymes to each of the reaction mixtures. The reactions were carried out at 30" with shaking. Determination of reaction mixture constituents was carried out by paper chromatography.