*THE NATURE OF THE PHENOLIC OXIDATION PRODUCTS IN MANUFACTURED BLACK TEA

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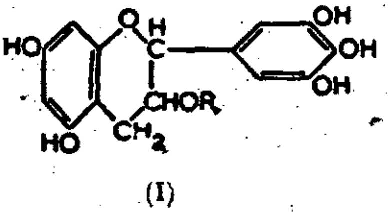
Dr. E. A. H. Roberts, of the Indian Tea Association (London), was recently asked to contribute a review of his work to the Biokhimija Chainogo Proizvodstva (Biochemistry of Tea Production, Seventh Series) published by the U.S.S.R. Academy of Sciences. We are indebted to the Indian Tea Association (London) for permission to publish the original English version of this article in the Tea Quarterly.

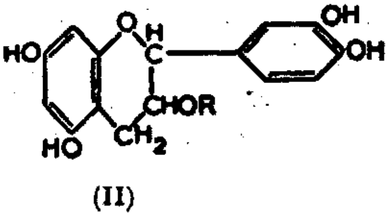
It has long been realised that the fermentation process in black tea manufacture is essentially an enzymic oxidation of polyphenolic substances, but it is only comparatively recently that the nature of the substrates undergoing oxidation has been adequately established. In this paper it is proposed to outline our present knowledge of the phenolic substances occurring in the freshly plucked tea shoots, to indicate which of these substances undergo oxidation during fermentation and to discuss the nature of the enzymic oxidation products which occur in black tea.

The phenolic substances in plucked tea-shoots

Our detailed knowledge of the phenolic substances in tea had to await the development of partition chromatography. Using this technique Bradfield and his collaborators (1,2), and Zaprometov (3) isolated several flavanols from green tea. These were identified by Bradfield as l-gallocatechin (I, R=H), dl-gallocatechin (I, R=H), l-*epi*catechin (II, R=H), dl-catechin (II, R=H), l-gallocatechin gallate (I, R=galloyl), a gallocatechin gallate (I, R=galloyl) and l-*epi*catechin gallate (II, R=galloyl). Later Bradfield & Bate-Smith (4) concluded that l-gallocatechin and its gallate should be regarded as l-*epi*gallocatechin and its gallate, a view which is now generally accepted.



(I)



(II)

The gallocatechin gallate isolated by Bradfield *et al* from green tea is not usually found in freshly plucked tea-shoots in North East India, and in the author's opinion (5) is an artefact produced by epimerisation of l-*epi*gallocatechin gallate during the course of manufacture of green tea. It also seems probable that some epimerisation of l-*epi*gallocatechin and l-*epi*catechin occurs during the manufacture of green tea with formation of l-gallocatechin and l-catechin respectively. These epimerisations probably account for the isolation of the dl-isomers from green tea, for in the unprocessed shoots plucked in North East India d-gallocatechin and d-catechin are present unmixed with the l-isomers.

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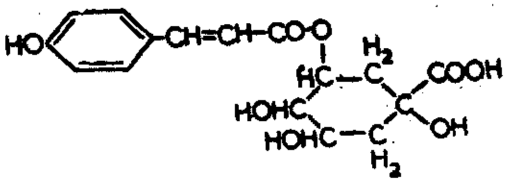
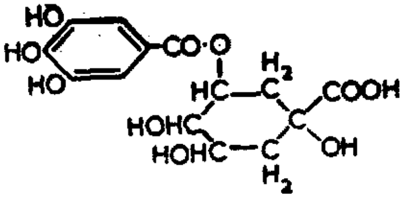


(III)

3-p-Coumarylquinic adide

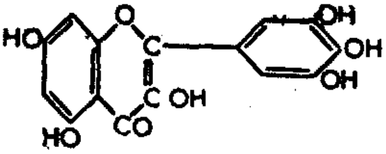


(IV)

3-galloylquinic acid

(Theogallin?)

Paper chromatographic studies (8-11) have revealed the presence in tea shoots of many more phenolic substances in addition to the flavanols already mentioned. These include chlorogenic and *neo*chlorogenic acids, together with the corresponding *p*-coumarylquinic acids (III). Theogallin (IV), which ranks as one of the major constituents of tea, also belongs to this class of depsides, and previous views that it was a galloyl-quinic acid (12) have now been fully confirmed (13) although it has not yet been definitely established that the galloyl group is attached to the 3-position of the quinic acid moiety.



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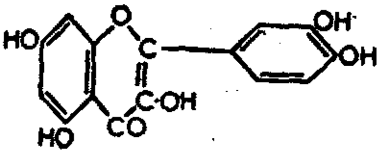

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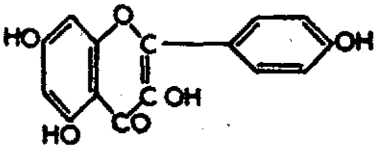
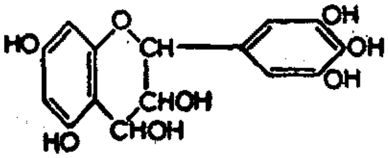


(VII)



(VIII)

In addition to the above depsides a number of flavonols have been detected. Those identified with reasonable certainty include the 3-glucoside and 3-rhamnoglucoside of myricetin, isoquercitrin, rutin, the 3-glucoside and 3-rhamnoglucoside of kaempferol, and, in China varieties, the 3-rhamnodiglucosides of quercetin and kaempferol. Traces of myricitrin, quercitrin, and the aglycones, myricetin (V), quercetin (VI) and kaempferol (VII) have also been detected. Some teas contain leuco-anthocyanins with leuco-delphinidins (VIII) predominating over leuco-cyanidins and occasionally an uncharacterised substance IC, is found which yields gallic acid on hydrolysis. Some of the above polyphenolic substances have considerable taxonomic significance (14).

Paths of oxidation

It has been established that the oxidation of all the above flavanols, the leuco-anthocyanins, chlorogenic acid and myricetin is catalysed by the tea oxidase. It may be taken as probable that *neo*chlorogenic acid also undergoes direct enzymic oxidation. The remaining phenolic substances found in the freshly plucked shoots are not oxidised in the presence of the tea oxidase, but many of them undergo a coupled oxidation in the presence of a suitable carrier. Thus myricitrin and theogallin are oxidised enzymically when d-catechin or l-*epi*catechin is present as a carrier. The gallocatechins are unable to function as carriers for these oxidations.

The enzymic oxidation products of one substrate, (*e.g.* l-*epi*gallocatechin gallate) may undergo further oxidation if a carrier of higher oxidation-reduction potential (*e.g.* l-*epi*catechin) is added to the system. Simultaneous oxidation of two substrates, (*e.g.* l-*epi*gallocatechin and l-*epi*gallocatechin gallate) may also yield products differing from those obtained by the oxidation of either substrate oxidised singly. It will be seen, therefore, that the oxidation of the mixture of phenolic substances in the tea shoots is likely to result in a mixture of end-products of some complexity.

The possibilities, however, are not exhausted as coupled oxidations of non-polyphenolic substances must also be considered. Any ascorbic acid present in the freshly plucked shoots will be oxidised by ortho-quinones during fermentation, but the end-products of this oxidation are unlikely to be of any great significance. On the other hand coupled oxidations of amino-acids may be of importance in view of the relatively high content of amino-acids in tea, particularly after the withering process.

It has already been demonstrated that enzymic oxidation of pyrocatechol in the presence of amino-acids leads to a condensation of the resultant o-benzquinone with amino-acids followed by coupled oxidation of the amino-acid to ammonia and the corresponding keto-acid (15). The mechanism of these chemical changes has also been considered in some detail (16). The proposed mechanism is summarised in Figure 1.

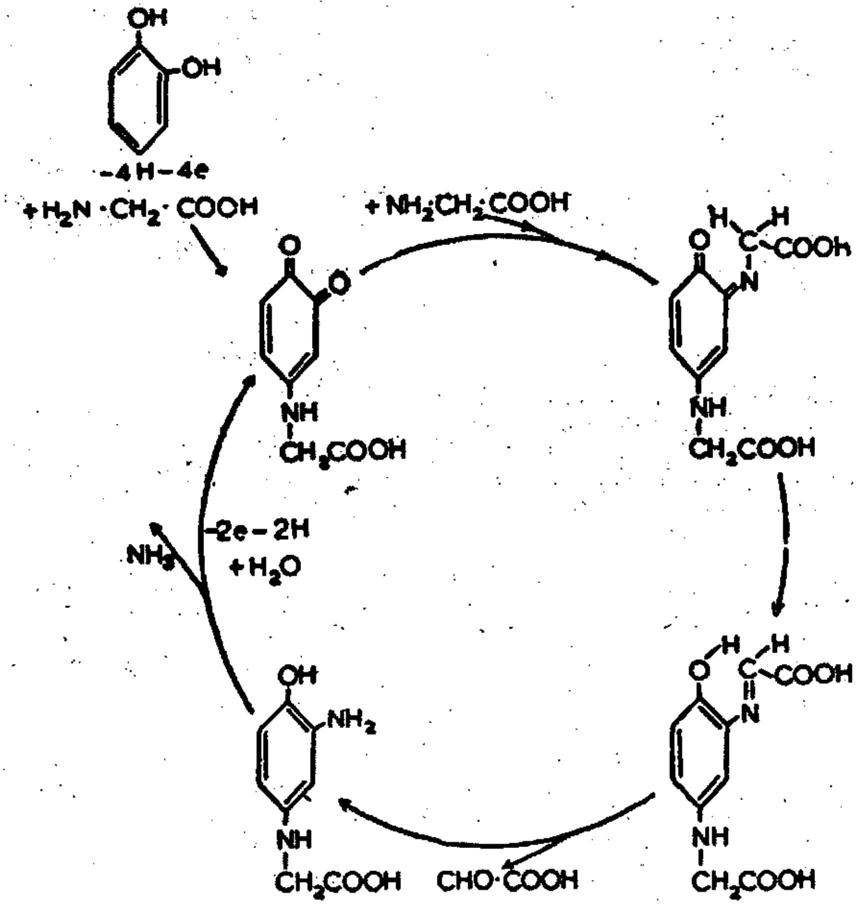
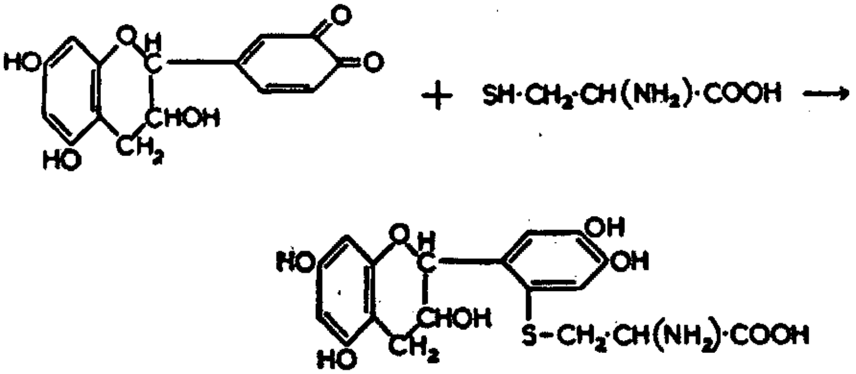


Fig. 1

It does not appear that catechins or gallocatechins can replace pyrocatechol in the above reaction scheme, for manometric studies (17) have shown that addition of amino-acids to systems composed of the tea oxidase and its substrates is without significant effect upon the rate of oxygen uptake or the total uptake realised. On the other hand Popov (18) has demonstrated that a conversion takes place of amino-acid into ammonia, carbon dioxide and the corresponding aldehyde. Only about 1% of the total amino-acid seems to undergo this conversion which accounts for the failure to demonstrate this change by manometric methods. Although the extent of amino-acid oxidation is rather limited it is considered probable that the resultant aldehydes are present in sufficient concentration to make an appreciable contribution to the liquor characters of the black tea.

Although limited coupled oxidation of amino-acids has been established there is as yet no evidence of the formation of condensation products between amino-acids and flavanol oxidation products. However it has been shown that the ortho-quinones of tea flavanols can condense with thiol groups in substances such as cysteine and glutathione (19). On oxidation of a mixture of d-catechin and cysteine the condensation presumably takes place according to the following reaction after



which oxidation of the pyrocatechol group probably takes place with the production of highly coloured end-products. Although changes of this nature have been demonstrated to take place *in vitro*, water-soluble products of this type have not been detected as yet in black tea. It is possible that orthoquinones produced in tea fermentation may become linked to thiol groups in proteins in this fashion thus accounting for the substantial proportion of polyphenolic matter which becomes water-insoluble as a result of fermentation.

Phenolic oxidation products in black tea

Two-way paper chromatographic examinations of black tea extracts (20) have shown that the main products of polyphenol oxidation show up as brown streaks of zero R_F in 2% acetic acid. The material responsible for these streaks has been separated into different fractions, but all of these fractions have very similar absorption spectra (21) and are sufficiently acidic to be extracted from solutions in organic solvents by aqueous sodium bicarbonate. These substances are referred to collectively as thearubigins (22)—the brown substances of tea—the name implies no greater extent of uniformity than is found with the melanins.

In addition to the thearubigins, five other substances were established, by paper chromatographic methods, to occur in tea in moderately high concentration. Two of these substances, originally referred to as substances X and Y (20), have now been isolated from tea (23), and it has been estalished that Y is a gallic acid ester of X (24). It has been proposed that X and Y should be known as theaflavin and theaflavin gallate respectively; the names reflect their origin and colour. The three other substances detected (A, B and C), are colourless and it has been shown that A is probably a digallic, and B a gallic acid ester of C.

Four other substances have been detected in trace amounts, substances P, Q, R and Z. Apart from P it is unusual to be able to detect these substances unless the extracts have been subjected to further fractionation. P is probably an anthocyanidin, and Q and R have colours similar to those of the theaflavins, but these three substances make very little contribution to the total colour of a tea extract. Substance Z is colourless, and has a fluorescence very similar to that of ellagic acid, although its R_F values distinguish it from the latter substance.

The nature of the phenolic oxidation products in tea

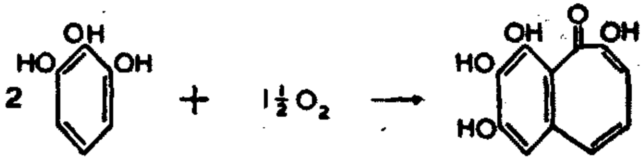
Examination of the paper chromatograms of black tea extracts showed that all the phenolic substances detected in the freshly plucked shoots were present in black tea also, and that the only substances which appeared to have undergone any substantial reduction during manufacture were l-*epi*gallocatechin and its gallate. The oxidation-reduction potentials of these substances are lower than those of the other oxidisable polyphenols occurring in unfermented tea (25) so that it is very likely that these should be the only substrates oxidised.

A study of the enzymic oxidation of individual tea substrates (26) has shown that l-*epi*gallocatechin gallate yields theaflavin gallate, substance A and gallic acid as the main products of reaction, together with smaller amounts of substances P, Q and Z. Substance C was found to be the main oxidation product obtained from l-*epi*gallocatechin, and when a mixture of these two substrates was oxidised the products also included theaflavin and substance B. Some substance Q was obtained on the enzymic oxidation of l-*epi*catechin gallate, but otherwise, none of the above products were obtained as a result of enzymic oxidation of flavanols other than l-*epi*gallocatechin and its gallate.

Apart therefore from the thearubigins, whose origin will be considered later, these experiments establish that the main products of tea fermentation are derived from two substrates only, l-*epi*gallocatechin and its gallate. In these experiments no free amino-acids were present in the reaction systems. It follows therefore that none of the reaction products could have been formed as a result of condensations of polyphenolic substances with amino-acids.

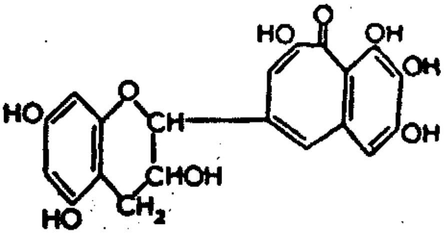
Despite the complex mixture of phenolic substances occurring in the freshly plucked tea-shoot it is clear that most of the oxidation products arise from two only of these substances. This considerable simplification of the problem justifies an attempt to deduce the nature of the end-products of oxidation. The first mechanisms to be considered were those in which oxidation to the ortho-quinone was followed by condensation of the ortho-quinone group with a phloroglucinol ring. Such mechanisms have already been proposed by Kursanov, Dshemuchadze and Zaprometov (27), and by Hathway and Seakins (28).

Support for such mechanisms was obtained when it was shown that the end-products of pyrocatechol oxidation (29, 30) were completely changed on addition of phloroglucinol to the system (31). It was clear that o-benzquinone condensed preferentially with phloroglucinol rather than with another molecule of o-benz quinone. However parallel experiments with pyrogallol showed that the latter substance behaved in quite a different manner from pyrocatechol. The main product of enzymic oxidation of pyrogallol is purpurogallin whether phloroglucinol



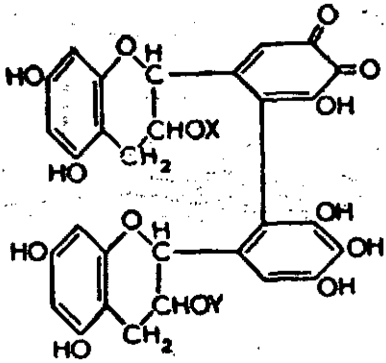
is added to the system or not, and in contrast to the experiments with pyrocatechol, condensation products of phloroglucinol with pyrogallol are only produced in trace amounts. It would therefore appear that pyrogallol groups do not resemble pyrocatechol groups in condensing readily into phloroglucinol rings, and that gallocatechins might differ from catechins in their oxidative condensations.

This view was supported by the finding that l-*epi*gallocatechin showed no tendency to combine with phloroglucinol when oxidised enzymically. On the other hand oxidation of a mixture of l-*epi*gallocatechin and pyrogallol yielded, in addition to the oxidation products of the individual substrates, a substance whose R_F values, colour reactions and absorption spectrum were consistent with its formulation as (IX).



(IX)

As l-*epi*gallocatechin underwent an oxidative condensation with pyrogallol but not with phloroglucinol it was concluded (32) that the most likely condensation to take place after enzymic oxidation of l-*epi*gallocatechin would be between two oxidised pyrogallol groups with the formation of an intermediate dimer of structure (X).

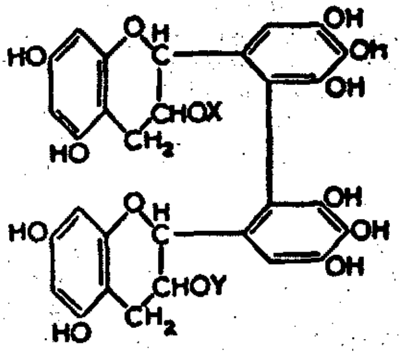


(X)

In tea fermentation the formation of three such intermediates would be expected, one arising from the condensation of two molecules of l-*epi*gallocatechin gallate (X=Y=galloyl), a second from two molecules of l-*epi*gallocatechin (X=Y=H), and a third from one molecule each of the two substrates (X=galloyl, Y=H).

At first sight it would seem that there is nothing to prevent further polymerisations of this type, but a study of three dimensional molecular models showed that steric hindrance effectively bars condensations beyond the dimer stage. With further polymerisation excluded it appeared likely that the next stages in the fermentation sequence of reactions would be either oxidations or reductions of the intermediate dimers (33).

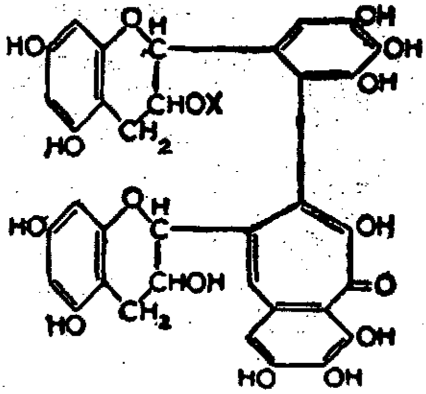
Reduction of these intermediates, with flavanol molecules functioning as hydrogen donors, would result in the formation of bisflavanols of structure (XI).



(XI)

It is suggested that substances A, B and C are bisflavanols of this structure, with A a digallate (X=Y=galloyl), and B a gallate (X=galloyl, Y=H) of C.

In considering possible oxidation mechanisms of the intermediates a special case arises when the intermediate contains at least one galloyl group. Oxidation of a galloyl group could be followed by condensation with the ortho-quinone group already present to form a benztropolone. This mechanism would lead to the formation of the two substances of structure (XII) which are tentatively identified with theaflavin (substance X) and theaflavin gallate (substance Y).

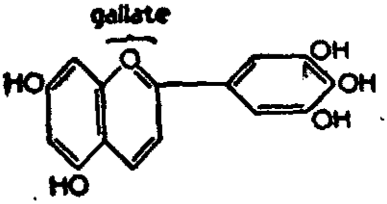


(XII)

As, according to this mechanism, one galloyl group is incorporated into the benztropolone residue, oxidation of two molecules of l-*epi*gallocatechin gallate would yield a product containing one galloyl group only (X=galloyl), and with mixed substrates the end-product (X=H) would have no free galloyl group. Oxidation of l-*epi*gallocatechin would not yield a product of this type as the intermediate contains no galloyl group. The formation during tea-fermentation of only two different theaflavins, as opposed to three bisflavanols, is therefore understandable on the above hypothesis.

Degradation studies of theaflavin and theaflavin gallate are not yet sufficiently advanced for these structures to have received confirmation. Molecular formulae and absorption spectra data (23) accord well with these structures, but at present they can only be considered as working hypotheses.

As far as the trace constituents of black tea are concerned it has been shown (21) that substance P is probably the anthocyanidin (XIII) bearing the same relation to delphinidin that luteolinidin does to cyanidin. It is probable that substance R has structure (IX) and that substance Q is a mixture of purpurogallincarboxylic acid (XIV), the 3-galloyl ester of R, and another benztropolone (XV) produced by the oxidative condensation of l-*epi*catechin with gallic acid (19, 24). Substance Z may have the structure (XVI) which would account for the similarity of its properties to ellagic acid. (33).



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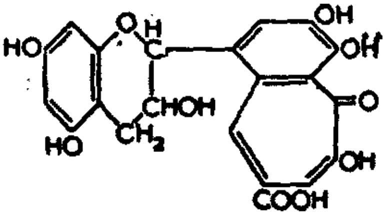

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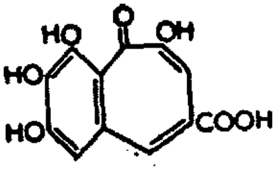

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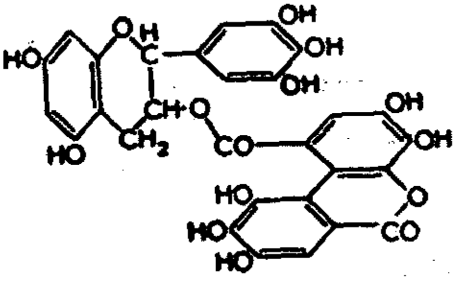


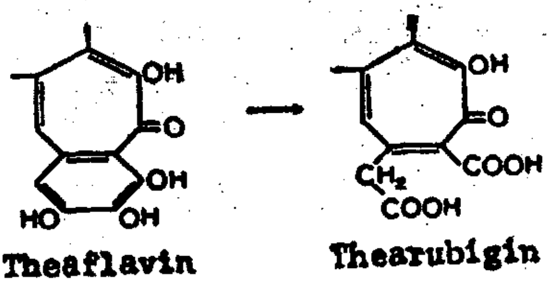
(XVI)

This brings us to a consideration of the nature of the thearubigins. The thearubigins account for from 10 to 15% of the total dry matter in Assam black teas, and must be derived largely from l-*epi*gallocatechin and its gallate, although recent results have suggested that l-*epi*catechin gallate may have become incorporated into some of the thearubigin molecules. It is doubtful whether any of the thearubigin fractions isolated from Assam teas contain nitrogen, so that these fractions probably contain no products formed by condensations of phenolic substances with amino-acids.

Methods have been developed for the colorimetric estimation of theaflavins and thearubigins (22), and with the help of these methods it has been shown that the thearubigins increase in concentration during fermentation at the expense of the theaflavins. This is an indication that theaflavins may be intermediates in thearubigin formation. More recently (19) it has been established that l-*epi*-catechin, d-catechin and l-*epi*catechin gallate can function as carriers for coupled oxidations both of theaflavin and theaflavin gallate, and that the products of these coupled oxidations behave similarly to thearubigins on paper chromatograms. Similar coupled oxidations of the bisflavanols have also been demonstrated.

In the enzymic oxidations of l-*epi*gallocatechin and its gallate the theaflavins and bisflavanols are end-products and undergo no appreciable oxidation. However the fermenting tea-leaf contains suitable carriers, in the shape of the catechins and l-*epi*catechin gallate, and it seems probable that coupled oxidations of this type occur during fermentation. There are therefore good reasons for believing that the thearubigins are oxidation products of theaflavins and bisflavanols. This dual origin would also account for the presence in Assam black teas of two main groups of thearubigins, the S I and S II fractions (20).

At present one can only speculate on the nature of the oxidation products formed, but molecular weight estimations (20, 27) show that oxidation to the thearubigin stage is unlikely to be accompanied by any further polymerisation. The acidity of thearubigins is probably due to carboxyl groups produced by the opening of pyrogallol rings. One way in which this could occur is exemplified below.



Summary and Conclusions

1. It is established that the oxidations taking place during tea-fermentation are largely confined to those affecting l-*epi*gallocatechin and its gallate.

2. The condensations of the gallocatechins after enzymic oxidation follow a different course from those of the catechins and a link between two pyrogallol groups to form a dimer is postulated. Polymerisation beyond the dimer stage is unlikely on steric grounds.

3. Three different dimers can be produced from the two substrates according to the above hypothesis. Reduction of these dimers is considered to result in the formation of the corresponding bisflavanols, identified tentatively with substances A, B and C.

4. It is suggested that oxidation of the dimers containing at least one galloyl group results in the formation of purpurogallin derivatives, identified tentatively with theaflavin and theaflavin gallate.

5. Coupled oxidations of theaflavins and bisflavanols, with catechins or l-*epi*-catechin gallate as carriers, is suggested to result in the formation of the thearubigins, the main products of tea fermentation.

6. Alternative paths of oxidation and oxidative condensation lead to the production of the trace substances P, Q, R and Z, for which possible structures are suggested.

7. Interaction of polyphenolic oxidation products with amino-acids has not led to the production of substances detectable by paper chromatography. On the other hand a limited coupled oxidation of amino-acids to aldehydes is established, and the resultant aldehydes are probably of significance in determining the aroma of tea.

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