Available online at www.sciencedirect.com
SCIENCE DIRECT®
PERGAMON
Phytochemistry 64 (2003) 61–73
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Review

Thoughts on thearubigins

Edwin Haslam*

Department of Chemistry, Dainton Building, University of Sheffield, Sheffield S3 7HF, UK

Received 21 February 2003; received in revised form 22 May 2003

Dedicated to the memory of Professor Jeffrey B. Harborne.

Abstract

The chemistry underlying the changes which occur during tea leaf fermentation is reviewed and used as a basis for proposals for the structure of thearubigins, the major pigments of black teas.

Keywords: Tea leaf fermentation; Polyphenoloxidase; Phenolic flavan-3-ols; Theaflavins; Benztropolones; Theasinensins; Thearubigins

Contents

1. Introduction 61

2. Substrates and enzymes 63

3. Pathways of oxidation 64
   3.1. Enzymic browning 65
   3.2. Theaflavins and other benztropolones 65
   3.3. Theasinensin formation 66

4. Thearubigins 67
   4.1. Nomenclature and isolation 67
   4.2. Chemistry 67
   4.3. Thearubigins—structural speculations 68

Acknowledgements 72

References 72

1. Introduction

Jeffrey Harborne's name is synonymous with the science of Phytochemistry and in particular with the chemical interactions between plants, animals and fungi. Two years ago his scientific achievements and his many contributions to the establishment and the running of this journal were warmly celebrated in a special issue of *Phytochemistry* (2001, volume 56, pp. 217–312). Quiet and unassuming, he was always happiest, I suspect, and enjoyed the greatest fulfilment when 'at the bench' or preparing a paper for publication. It was an honour to have known Jeffrey. It is an honour to be asked to contribute to this memorial issue of the journal he helped to create. Just two years on it is nevertheless one undertaken with great sadness.

* Present address: 7 Curie Mews, Exeter EX2 4TB, UK. Tel.: +44-1392-256-262.
E-mail address: edwin.haslam@btinternet.com (E. Haslam).

0031-9422/03/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/S0031-9422(03)00355-8

The Phytochemical Society (eventually to become the Phytochemical Society of Europe) had its origins in an informal association of chemists and biologists formed, principally at the instigation of E.C. Bate-Smith and Tony Swain, following a meeting of the Society of Leather Trades' Chemists in the University of Cambridge in the spring of 1956. A prominent member of The Plant Phenolics Group, as it quickly became, and an enthusiastic contributor to its early proceedings was E.A.H. Roberts (known affectionately as 'Tea' Roberts) whose pioneering work on the chemistry of tea, particularly its phenolic constituents, has proved to be seminal. Until his early untimely death it was carried out in the Dickensian environs of the laboratories of the Indian Tea Research Association situated on Butler's Wharf in the East India dock of the Port of London. Forty years on Butler's Wharf has been transformed for those aspiring to luxurious life styles but Roberts' ideas and unique perceptions, gained in those most unlikely surroundings, remain and have set the agenda for much of the subsequent work and developments in this area.

The young green shoots ('flush') of the tea plant (*Camellia sinensis* var *sinensis*, *Camellia sinensis* var *assamica*) contain high concentrations (from 10 to 25% dry weight) of phenolic flavan-3-ols (**1**–**6**), principally epicatechin (**1**) and epigallocatechin (**2**) and their corresponding 3-*O*-gallate esters (**3**,**4**), sequestered in the cytoplasmic vacuoles of the leaf cells. There are three principal types of manufactured tea, namely green (unfermented), oolong (partially fermented) and black (fully fermented), where the term 'fermented' refers to natural browning reactions induced by oxidative enzymes within the plant cell. Roberts identified two principal groups of phenolic pigments in black teas—the discrete orange-red theaflavins (which he correctly speculated contained a benztropolone nucleus) and the rusty red-brown thearubigins. Subsequent work has shown the four principal theaflavins to be readily separated and identified, and their mode of formation has been delineated. Further studies over the past forty years have added to the number of phenolic benztropolones and other minor pigments which are also formed during black tea fermentations. They generally constitute up to 2% of the dry weight of black tea and up to 6% of the solids extracted in the brewing of a cup of tea. On the other hand progress in identifying the chemical nature of the thearubigins has hardly advanced since Roberts' day. Notwithstanding the fact that up to 75% of the phenolic flavan-3-ol substrates may ultimately find their way into thearubigins, many of the characteristics of black tea infusions and many technical innovations aimed at making a more 'consumer friendly' tea are critically dependent on their presence. While accurate analysis of their concentrations in teas is difficult, they are believed to comprise up to 20% of the black tea leaf and up to 60% of the solids in a black tea infusion. Thus although they constitute the largest group of phenolic substances in black teas they are the least well understood of its components.

The generation of black tea polyphenols is a specific example of the phenomenon of 'enzymic browning', which can occur if the cell structure of a plant is damaged, degraded adventitiously by a predator, or as the tissue senesces. Phenolic metabolites once liberated into the general milieu of the cell cytoplasm then have the opportunity to display, often under the influence of oxidative enzymes, their chemical promiscuity and reactivity with other cytoplasmic constituents, and in particular proteins. In some instances, browning caused by accidental damage during harvesting, storage and

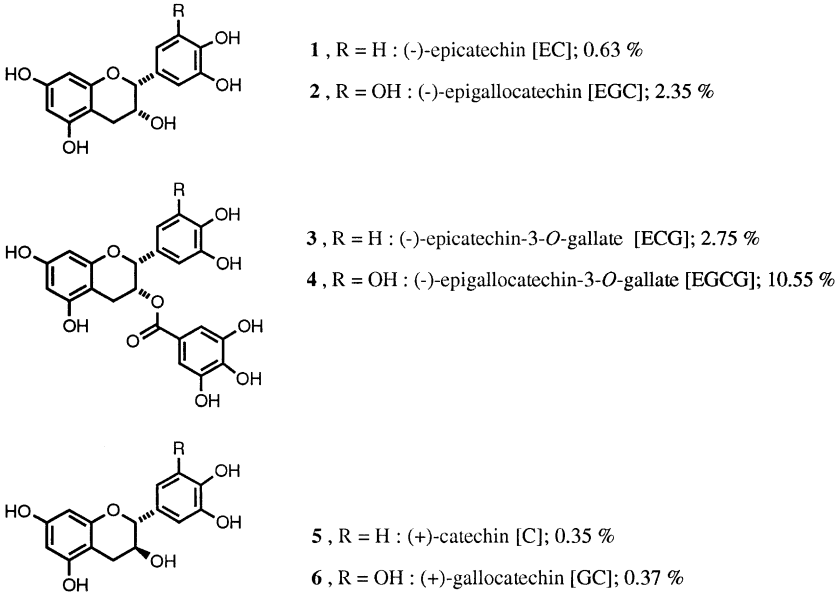


Fig. 1. Principal phenolic flavan-3-ols of a typical Sri-Lankan tea flush, (Lunder, 1988);% composition of dry weight of leaf.

transport of a fruit or vegetable crop, may cause serious problems for the producer. On the other hand the formation of the black tea polyphenols—e.g. theaflavins and thearubigins- occurs when the tissue is deliberately damaged as part of a technical process.

It is generally assumed, although far from proven, that the thearubigins are complex heterogeneous polymers. Because of the problems they have presented to conventional methods of structural analysis over the last forty years they are now often categorised alongside similar oxidatively derived intractable polymers such as melanins and labelled with the soubriquet "too difficult". The object of this review is to re-focus attention on the thearubigins by examining the meagre evidence already garnered concerning their structure and their generation in the technologically driven enzymic browning process, to use informed speculation as a guide and to suggest future avenues of research into these complex, poorly defined, but important children of nature. Evidence is taken from extant published work and key reviews (Roberts, 1962; Sanderson, 1972; Lunder, 1988; Willson and Clifford, 1992; Harbowy and Balentine, 1997; Balentine et al., 1997; Schubert and Spiro, 1997; Davies et al., 1999).

2. Substrates and enzymes

Although absolute concentrations vary, dependent upon factors such as variety and growing conditions (in Japan for example shading the bushes, over a period of two to three weeks is employed to cause the level of phenolic flavan-3-ols to drop), phenolic flavan-3-ols *usually* constitute some 10–25% of the dry matter of the leaf. Despite the varied origins of commercial tea plants, the reported *relative* chemical compositions of the polyphenolic profile in green tea shoots nevertheless show remarkable similarities. Typically the average content of the major phenolic flavan-3-ols (**1--6**) in a Sri-Lankan tea is shown in Fig. 1, (Lunder, 1988). In addition to these metabolites miscellaneous phenolic depsides and other polyphenols, such as flavonols and their glycosides, also occur (~4%) as well as caffeine (~3%).

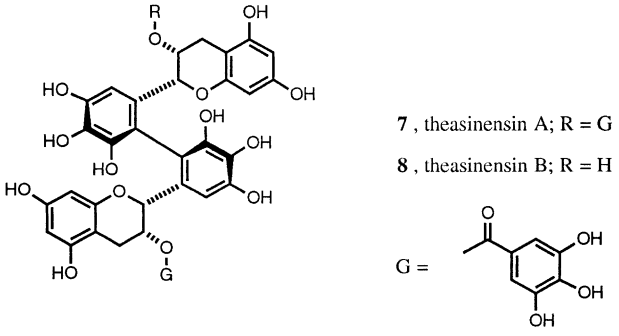


Fig. 2. Minor polyphenolic components of green tea leaf: theasinensins A and B, (Nonaka et al., 1983).

The occurrence of this wealth of phenolic flavan-3-ols in tea leaf might seem to imply the co-occurrence of related proanthocyanidins and their gallate esters. However, as has been noted in earlier work (Haslam, 1989), in plants which metabolise substantial quantities of the gallate esters of flavan-3-ols there is usually found a corresponding diminution in the amounts of the related proanthocyanidins. In studies of green leaf and a commercial oolong tea (shiraore) Nishioka's group (Nonaka et al., 1983; Hashimoto et al., 1989a, b) isolated numerous dimeric proanthocyanidins, free and as gallate esters, (total <0·1%). They also, perhaps more significantly, noted two novel dimeric flavan-3-ol gallate esters, theasinensins A and B (**7** and **8**;~0.05 and 0.013%, respectively, Fig. 2). The latter compounds represent a new class of dimeric flavan-3-ols in which the two flavan units are linked by a C–C bond between the two 'B' rings, forming a biphenyl grouping. The chirality of the twisted biphenyl was shown by the Japanese workers to be *S*.

Since many of the sensory characteristics of manufactured teas derive from the oxidative transformations ('fermentation') of the green leaf phenolics a very great deal of attention has been devoted to this topic. Pre-eminent amongst the enzymes involved in these changes is tea-leaf polyphenoloxidase [PPO; EC 1.14.18.1; monophenol mono-oxygenase (tyrosinase) or EC 1.10.3.2; *o*-diphenol: O_2 oxidoreductase]. The enzyme is a metallo-protein thought to contain a binuclear copper active site, utilises molecular oxygen as electron acceptor and has good functionality in the pH range 4.6–5.6. According to Gregory and Bendall (1966) the tea enzyme was distinguished from the (ubiquitous) tyrosinases by its inability to oxidise monohydric phenols, either by hydroxylation or dehydrogenation. On the other hand the enzyme possessed low activities towards both quinol and *p*-phenylene-diamine, which are characteristically oxidised by laccases . Although gallic acid, caffeic acid and ethyl gallate were oxidised slowly by the enzyme, the preferred substrates of the tea enzyme were *o*-dihydric phenols, such as the tea flavan-3-ols **1–6** (Fig. 1). Since the enzyme could not be referred to definitively as either a tyrosinase or a laccase Gregory and Bendall therefore favoured the nomenclature *polyphenol oxidase*. Mayer and Harel (1979), Mayer (1987) have written two substantive reviews concerning this type of enzyme and the ambiguities and inconsistencies which characterise them.

Peroxidase [POD; EC 1.11.1.7], which catalyses the reductive decomposition of hydrogen peroxide to water, and organic peroxides to the corresponding alcohol, is found in fresh green tea leaf but its role in the oxidation of the polyphenolic substrates is less clearly defined. In plants, peroxidases are involved in IAA metabolism and lignification. They have also been shown to act as efficient reagents to bring about the oxidative coupling of

phenolic substrates, such as gallate esters and the flavan-3-ols which occur in green tea, in the laboratory (Weinges et al., 1969; Mayer et al., 1984). One limit to the possible role of POD is that catalase (EC 1.11.1.16) is also present in tea leaf and this enzyme acts to rapidly remove peroxides. Dix et al. (1981) in a series of model studies suggested that POD is responsible for the oxidative breakdown of theaflavins and the generation of thearubigins as well. Thus purified theaflavins were stable in the presence of tea PPO, but readily oxidised by horseradish POD. In vitro fermentation studies using both PPO and POD also showed the coupled nature of the action of these enzymes (Finger, 1994).

In a detailed study Davies et al. (1999) attempted to determine the relative importance of these enzymes in tea fermentation. They showed that PPO, POD and catalase were all present in fresh tea tissues with activities appropriate for the oxidation of the phenolic flavan-3-ol substrates, but that the ratios of the three enzymes could vary widely. Their studies pointed strongly to the fact that PPO was of prime importance so far as the oxidation of the flavan-3-ol substrates was concerned and this supported the idea that the enzyme had a rate limiting role in the formation of the theaflavins, and presumably other benztropolones. Inhibition of PPO during fermentation, by the action of tropolone or the removal of oxygen, thus led to near complete inhibition of flavan-3-ol oxidation and theaflavin accumulation. However, their results did not entirely rule out the possibility that POD made other contributions to the overall tea fermentation processes. The possibility thus remains that POD may contribute to pathways leading to the thearubigins.

3. Pathways of oxidation

Although various mechanisms (free radical, phenoxonium ion) are theoretically possible to describe polyphenol oxidation, for purposes of illustration the primary step in tea fermentation is probably best envisaged as the overall conversion of the *ortho*-dihydroxy

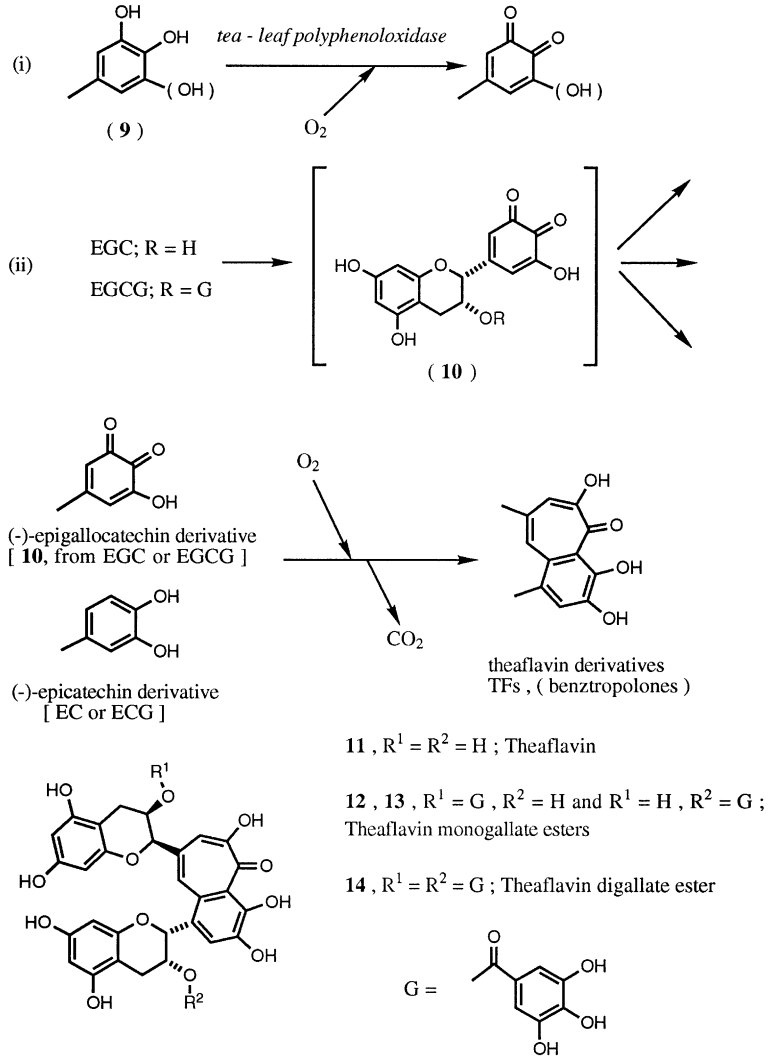


Fig. 3. Oxidation of phenolic flavan-3-ols: route of formation of the principal theaflavins in black teas (Collier et al., 1973).

('catechin') and *ortho*-trihydroxy ('gallocatechin')-phenyl 'B' rings of the substrate polyphenolic flavan-3-ols (**9**) by tea polyphenoloxidase to give the corresponding highly reactive orthoquinone derivatives (Fig. 3, i). The reaction pathways which ensue are illustrated, in the following discussion, using the quinones (**10**) derived from (−)-epigallocatechin (EGC, **2**), and its 3-*O*-gallate (EGCG, **4**). The justifications for this choice of paradigm are twofold. Firstly these derivatives (EGC **2** and EGCG **4**) are quantitatively of greatest significance amongst the green tea leaf polyphenols (Fig. 1) and secondly the 3′,4′,5′-trihydroxy-substituted rings possess the lowest redox potential and are the most readily oxidised (Jovanovic et al., 1994, 1995, 1996). At present three principal divergent pathways [Figs. 3 (ii) and 5], which derive from the reactions of these quinones (**10**), can be defined.

3.1. Enzymic browning

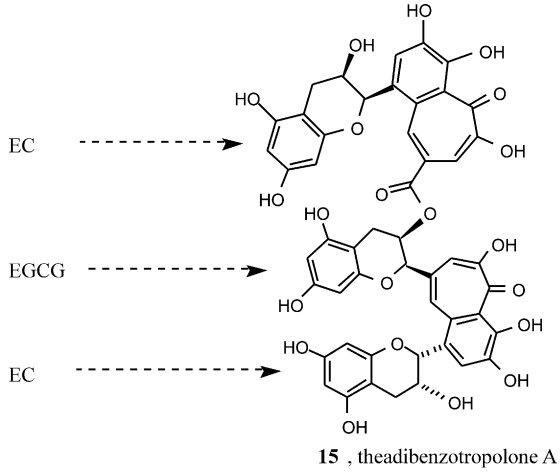
Mayer (1987) cryptically described plant polyphenoloxidases as "enzymes in search of a function". The conspicuous enzymic browning which is generated by PPO in a multitude of plant-pest interactions has led to the view that PPO's primary role is in plant defence. According to this theory, sequestration of PPO within the thylakoid prevents its interaction with phenolics until the cell is disrupted by herbivores, pathogens, senescence, or other injury. The highly reactive electrophilic quinones then cross-link a variety of cellular constituents such as proteins via nucleophilic-SH (cysteine) and-NH_2 (lysine) groups in a process known as 'quinone tanning' (Haslam, 1998). The formation of these adducts—usually coloured brown to black—often present a serious problem in the post-harvest physiology and processing of foodstuffs. About 20% of the original flavonoid content of green tea leaf is thus insolubilised during fermentation (Pierpoint, 1985), and remains with the brown tea leaf residues after infusion. Presumably both the colour and the irreversible binding of phenolics arise from quinone tanning of the tea leaf protein by quinones such as (**10**). Lunder (1988) noted, in support of this thesis, that although proteins constitute 15–23% of the dry weight of black tea leaf, they make up less than 2% of the hot-water solids.

3.2. Theaflavins and other benztropolones

One of the key reactions which these *ortho*-quinones (**10**) undergo is again under purely chemical control and gives the series of discrete yellow-orange pigments, the theaflavins (TFs) and their congeners (Davies et al., 1999). The major theaflavins (**11**–**14**) found in black teas number four (Fig. 3); their total concentration in black teas does not normally exceed 2% and can often be as low as 0.3%. Although their precise fate is not known, prolonged oxidative fermentation progressively decreases the theaflavin concentration in the final black tea (Hashimoto et al., 1992).

The structure of theaflavin (**11**) was determined simultaneously by Takino and Ollis and their colleagues (Takino et al., 1965; Ollis et al., 1966). In addition Takino also neatly demonstrated that oxidation (tea polyphenol oxidase or $K_3FeC_6N_6$) of the appropriate pair of phenolic flavan-3-ol substrates gave the predicted theaflavin derivative according to the mechanism of benztropolone formation put forward earlier by Horner (Horner et al., 1961, 1964); thus oxidation of a mixture of (−)-epicatechin (**1**) and (−)-epigallocatechin (**2**) gave theaflavin (**11**); similarly (−)-epicatechin-3-*O*-gallate (**3**) and (−)-epigallocatechin-3-*O*-gallate (**4**) gave theaflavin digallate (**14**). Consideration of this mechanism for the oxidative formation of benztropolones from catechol and pyrogallol derivatives suggested that black teas should also contain other phenolic benztropolone derivatives, albeit in much smaller amounts, in addition to the theaflavins. Several such compounds, derived from alternative oxidative transformations of phenolic flavan-3-ols (EC **1**, EGC **2**, C **5**, GC **6**) and their gallate esters (**3**, **4**) and gallic acid have now been isolated (Collier et al., 1973; Nonaka et al., 1983; Davies et al., 1999).

The most recent addition to this long list of benztropolone pigments found in black teas is theadibenzotropolone A (**15**; Sang et al., 2002), a compound in which the two pyrogallol type rings in EGCG (**4**; ring B and the galloyl ester group) have each been oxidatively coupled to the catechol type ring B of a molecule of EC (**1**). The new pigment was also formed in model studies of the oxidation of EC (**1**) and EGCG (**4**) with horseradish peroxidase and hydrogen peroxide.



15, theadibenzotropolone A

The mechanism of theaflavin formation (Fig. 3) may be formulated as indicated or as one between the corresponding two quinonoid species (Haslam, 1998). Whichever route is favoured the reaction requires at

least one further oxidation step. Elaboration of the benztropolone group also requires the loss of one carbon atom as carbon dioxide, thus confirming the observations of Roberts (1962) and Sanderson (1972) of the output of carbon dioxide during the early rapid fermentation stage. Finally it should be noted that (except in the case of gallic acid which can undergo decarboxylation during the reaction), the mechanism of formation of theaflavins and analogous benztropolones requires that the complimentary phenolic component which reacts with (**10**) has an unsubstituted double bond (Fig. 3)—i.e. a (−)-epicatechin (**1**) or (+)-catechin (**5**) derivative. It follows directly from this observation that, with high concentrations of (**10**), it is the concentration of these latter polyphenols which determines the maximum possible quantity of theaflavins (TFs) which can be formed in the fermentation. Using the values in Fig. 1 and even assuming quantitative conversion this indicates just under 30% of the quinone (**10**) could be diverted to TFs. Taking into consideration the amount irreversibly bound by quinone tanning, this leaves a minimum of ~50% of (**10**), or its precursors EGC / EGCG (**2**, **4**), unaccounted for.

3.3. Theasinensin formation

It now seems highly likely that, as well as unreacted precursors, this 50% is composed in part of theasinensins (e.g. **7**, **8**; Fig. 2) formed by oxidative coupling of (**10**) with EGC (**2**) or EGCG (**4**), (Nonaka et al., 1983; Hashimoto et al., 1988). Roberts and Myers (1959) showed that among the oxidation products obtained from the polyphenoloxidase promoted oxidation of EGC **2** and EGCG **4** were three compounds, named A, B and C. These could also be identified on paper chromatograms of black tea phenolics. They suggested these compounds were bisflavanols, formed by oxidative coupling, and later work (Vuataz and Brandenberger, 1961; Ferretti et al., 1968) confirmed these suggestions. Direct comparison is not possible now but there can be little doubt that these substances are identical with the theasinensins isolated by Nishioka's group (Nonaka et al., 1983; Hashimoto et al., 1988) including A (**7**) and B (**8**) (Fig. 2). As Roberts correctly implied they are formed by direct oxidative coupling (Fig. 4). In a recent important HPLC study of the analysis of the changes of tea leaf polyphenols during fermentation Hashimoto et al. (1992) showed that the polyphenolic flavan-3-ols EGC **2** and EGCG **4** are more rapidly transformed by endogenous polyphenoloxidase to the theasinensins **7** and **8** (Fig. 2) than to the theaflavins. Theasinensins **7** and **8** were maximal after 2 h. then steadily declined whilst theaflavins reached comparable maximal concentrations only after 4 h before similarly decreasing. Using crude enzyme preparations Hashimoto and his colleagues also prepared, from (−)-epigallocatechin-3-*O*-gallate **3**, in addition to theasinensins A (**7**) and D (the atropisomer of A), the unusual fermentation product oolongtheanin (**16**; Hashimoto et al., 1988). This structure is a significant pointer as to one group of possible reactions which take place in the formation of thearubigins.

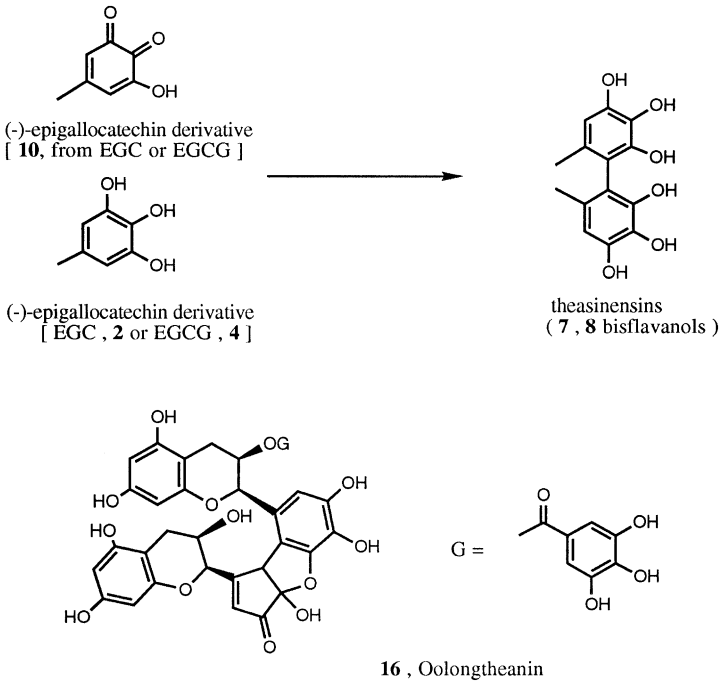


Fig. 4. Theasinensin (bisflavanol) formation by oxidative coupling: structure of oolongtheanin.

4. Thearubigins

"Despite the fact that the thearubigins are the most abundantly occurring of the polyphenolic oxidation products in black tea they are the substances whose chemistry is at present least understood."
E.A.H. Roberts, 1962

"Plus ça change, plus c'est la même chose."
Alphonse Karr, 1849

4.1. Nomenclature and isolation

Roberts (1962) christened this ill-defined group of substances thearubigins (TRs). A recent review (Harbowy and Balentine, 1997) described this nomenclature as a historical artefact and suggested that it should be discarded. However, in the absence of any recommended and suitable alternative this description is retained here. At best thearubigins may be taken as a term to embrace a gallimaufry of indeterminate structures, from the 'monomeric' to the 'polymeric', derived by enzymatic oxidation of the flavan-3-ols in fresh green tea leaf. They are water soluble, acidic, and some, though not neccessarily all, are rust-brown in colour and all display similar ill-defined chromatographic behaviour. Nevertheless it is indisputably the case that since Roberts' pioneering studies (Roberts et al., 1959; Roberts, 1962) little progress has been made towards an understanding of the chemical nature of the thearubigins.

Thearubigins are very soluble in water and Roberts et al. (1959) achieved a partial fractionation—SI, SIa, SII—by solvent (butan-1-ol) extraction and selective precipitation. Partial extraction of both SI and SIa from aqueous solution could also be achieved using ethyl acetate and they suggested that there was probably little difference between these two fractions. The thearubigins were characterised by two dimensional paper chromatography using [butan-1-ol, acetic acid, water] and 2% aqueous acetic acid as irrigants. They were immobile in the latter phase and moved as streaks in the former; SII from the origin as short and pennant-like, whilst SI had an R_F of 0.1–0.8.

The advent of HPLC heralded a new era in the analysis and separation of complex mixtures of phenolic metabolites. Whilst its application exceeded all expectations in the separation and quantification of theaflavins, other benztropolones, theasinensins and minor phenolics in teas, the belief that it would lead to similar major advances in the analysis of thearubigins has, in the event, not been realised (Harbowy and Balentine, 1997). Clifford and Powell (1996) thus succinctly noted their notorious propensity when subjected to HPLC to elute as an 'unresolved Gaussian hump', an observation also well documented by others (Bailey et al., 1991; Powell et al., 1992, Nursten, 1997). Nevertheless an alternative classification of black tea pigments, based upon HPLC fractionation, has been put forward (Nursten, 1997; Bailey and Nursten, 1994). They also introduced the sub-classification of theafulvin. This divides the pigments into three groups. Although it is not crystal clear how this classification of the pigments relates to that of Roberts, a priori, the group III theafulvins (Bailey et al., 1992), appear to resemble most closely the thearubigins described by Roberts.

4.2. Chemistry

Evidence relating to the chemical structure(s) of the thearubigins is sparse. Elemental analysis reveals only vestiges of nitrogen which has often been attributed to traces of caffeine in the samples (Roberts, 1962; Brown et al., 1969a, b). This therefore effectively rules out the possibility that the thearubigins (TRs) contain significant amounts of protein to which quinonoid species, such as (**10**), are covalently attached (see Section 3.1). X-ray analysis of the theafulvin fraction (Bailey et al., 1992) indicated the presence of potassium and lesser quantities of magnesium, manganese, aluminium, phosphorus and sulphur.

Ebullioscopic estimation of the number average molecular weight of various thearubigin fractions gave values of the order of 700, equating to a 'dimeric' flavan-3-ol structure (Roberts, 1962; Roberts et al., 1959). Using size exclusion chromatography values up to around 2,000 were indicated by Clifford and Powell (1996), although other measurements (Millin and Rustidge, 1967) have indicated values up to 40,000, suggesting mammoth 'polymeric' species. Davies et al. (1999) cryptically recorded that a reliable molecular weight could not be measured.

In the 1980s Nishioka's group reported the presence of small quantities of proanthocyanidins and their gallate esters in tea leaf (Nonaka et al., 1983; Hashimoto et al., 1988). In the light of these observations the earlier studies of Ollis and Nursten (Brown et al., 1969a, b; Catell and Nursten, 1976) which demonstrated that thearubigin fractions, broadly correlating with those of Roberts' SI class, exhibited characteristics of the proanthocyanidin group (Haslam, 1998) of natural products, are now more readily comprehended. These thearubigins were thus degraded by mineral acid to give mixtures of cyanidin and delphinidin, with the former predominating (~3:1). Ollis and his group therefore suggested (Brown et al., 1969a, b) that thearubigins are polymeric proanthocyanidins, adding the rider that "*the results so far obtained refer only to the thearubigins extracted from aqueous solution by organic solvents*". Of concern to the authors however was the fact that acid degradation yielded a ratio of cyanidin to delphinidin, the inverse of that of the presumed flavan-3-ol precursors (EC:EGC is ~1:3) in the leaf. However there is,

to date, no evidence that structures of the proanthocyanidin type can be generated from phenolic flavan-3-ols by chemical or enzymatic means. It is therefore suggested that the residual structural fragments of the proanthocyanidin type in these thearubigins derive from the very small amounts of these compounds present originally in the green leaf (Nonaka et al., 1983; Hashimoto et al., 1989a, b). They may be incorporated, as such, into more elaborate structures or simply entrained unchanged by association with the thearubigin fractions (c.f. caffeine). Since the 3′,4′,5′-trihydroxy-substituted 'B' rings possess the lowest redox potential and are the most readily oxidised (see Section 3 above) it is also reasonable to suppose that preferential oxidative transformation of the prodelphinidins occurs during tea fermentations and that the residual thearubigins are correspondingly enhanced in their procyanidin (3′,4′-dihydroxy-substituted 'B' rings) content.

Examination of proton and carbon-13 NMR spectra of thearubigins has proved to be uniformly uninformative. Not unexpectedly the spectra invariably display line broadening and poor signal-to-noise ratios, attributed variously to the heterogeneous, polymeric nature of the thearubigins and to the presence of paramagnetic metals. However, Bailey et al. (1992) suggested, on the basis of the ^{13}C spectra, that theafulvins contained flavan-3-ol species linked via their 'B' rings (C-2 to C-2′). Davies et al. (1999) similarly reported NMR signals which could be attributed to the presence of flavan-3-ol molecules, benztropolone rings and C-2 to C-2′ linkages.

In Roberts' early work on thearubigins (Roberts, 1962; Roberts et al. 1959) the two salient characteristics, besides their **colour**, which emerged were their marked **acidity** and their high **water solubility**, such that in two phase systems [water/ethyl acetate or methyl isobutyl ketone] they partition preferentially to the aqueous one. Thus dissolution of both SI and SII fractions in water gave acidic solutions (pH 3.2). In the case of SII it was not extracted from such solutions by ethyl acetate, but butan-1-ol effected complete extraction. The aqueous solution of SIa was similarly acidic (pH 3·2) and partial re-extraction was achieved using ethyl acetate. Both fractions dissolved freely in aqueous sodium bicarbonate solution and neither ethyl acetate nor butan-1-ol extracted any material from these solutions. Roberts argued (Roberts et al., 1959) that since the pH of a tea infusion is ~5.0 it followed that these acidic substances were therefore present as partially neutralised species . Thus whatever their solubility in organic solvents only that amount in excess of the neutralised portion would be extracted. He also noted that partial neutralisation of other organic acids in tea infusions, e.g. gallic and chlorogenic acids, similarly accounted for their incomplete extraction by ethyl acetate. It seems an inescapable conclusion from these observations that the thearubigins must contain several water solubilising groups and that these are very probably carboxyl functionalities, or their equivalent.

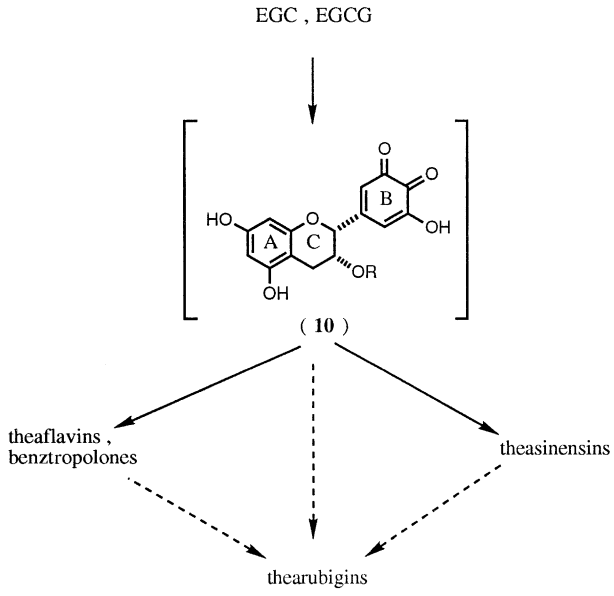
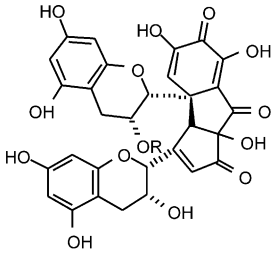


Fig. 5. Possible pathways to thearubigins.

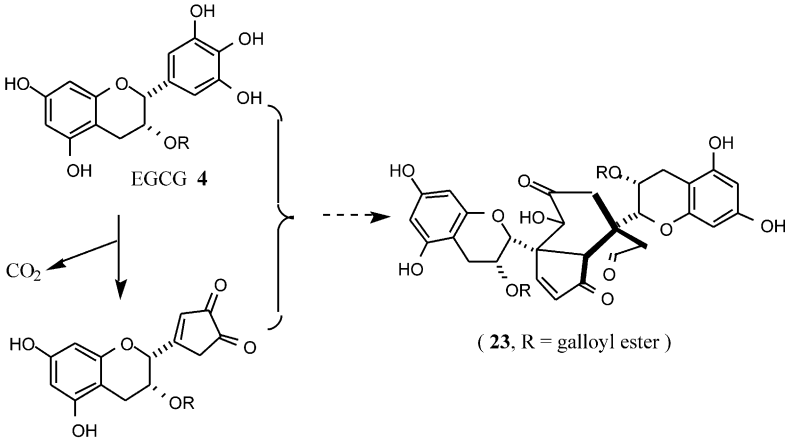
4.3. Thearubigins—structural speculations

Although model tea fermentations (Sanderson, 1972) show that substances with thearubigin-like properties are formed by enzymic oxidation of any one of the tea leaf phenolic flavan-3-ols **1–6** (Fig. 1) it seems highly likely that, as Roberts suggested, the principal substrates are EGC (**2**) and EGCG (**4**), and that the pathways to thearubigins derive largely from the quinone (**10**), theaflavins (**11–14**) and other benztropolones, and from the theasinensins (**7**, **8**) (Fig. 5). Although the theaflavins are stable towards PPO it is thought that they can be oxidised chemically in coupled reactions with other substrates such as EC (**1**) (Davies et al., 1999). At this stage, and in the absence of reliable data, it is probably best to set to one side the question of molecular size, bearing in mind that there are excellent precedents to support the idea that oligomerisation of the precursors, by oxidative coupling, (Haslam, 1998) could occur during fermentation.

One explanation of the marked acidity and water solubility of the thearubigins is that these features derive simultaneously by oxidatively induced ring opening of one or more aromatic rings in the phenolic substrates, generating one or more carboxyl groups in the process. Roberts (1962) first mooted this idea. Chebulinic and chebulagic acids dominated the early investigations of the ellagitannins (Schmidt and Mayer, 1956; Haslam and Cai, 1994). Both contain chebulic acid (**17**) initially presumed but now proven (Tanaka et al., 1996) to be derived by oxidative ring fission of a phenolic ring in its



21, theacitrin A ; R = galloyl ester



(**22**, R = galloyl ester)

(**23**, R = galloyl ester)

putative hexahydroxydiphenoyl ester biogenetic precursor (**18**) [Fig. 6(i)]. An alternative pathway of ring opening of the parent hexahydroxydiphenoyl ester leads, with concomitant loss of carbon dioxide, to a further distinctive ellagitannin metabolite brevifolin carboxylic acid (**19**) [Fig. 6(i)]. In this context it should be noted that oolongtheanin (**16**), from oolong tea, represents a theasinensin in which one of the pyrogallol rings has been oxidatively modified in an entirely analogous manner [Fig. 6(ii)]. Finally, Mayer et al. (1984) reported the isolation of two carboxylic acid derivatives from the mild oxidation of methyl gallate (**20** [Fig. 6(iii)]. In all these instances an entirely satisfactory chemical rationale in which alternative modes of oxidative fission of a pyrogallol ring take place in the precursor substrate can be put forward (Haslam, 1998).

Reference may also be made to further examples which substantiate the idea of oxidative ring fission described above. Thus the 'dimeric' theacitrins (e.g. **21**) are minor yellow pigments which have been isolated from black teas (Davis et al., 1997) . Their formation may be rationalised by oxidative coupling of two phenolic flavan-3-ol precursors (via their B-rings; C-1′ to C-2′), followed by oxidative ring opening of one B-ring phenolic nucleus and further ring closure. More recently Tanaka et al. (2002) in model experiments designed to clarify the oxidative mechanisms involved in the formation of black teas using a green tea homogenate isolated a series of novel 'dimeric' adducts which further illustrate this same idea of oxidative ring fission. Most significantly the product (**23**) is thought to be formed by a cycloaddition reaction involving the species (**22**) in which ring B of one molecule of EGCG (**4**) has undergone oxidative ring contraction accompanied by decarboxylation, (cf. Fig. 6, brevifolin carboxylic acid **19**). However, it should be stressed that none of these particular products has not yet been found in black teas.

There is thus ample circumstantial evidence to support the view that the oxidative fermentation of tea leaf polyphenols could lead, via oxidative ring fission, to carboxylic acids which subsequently contribute to the envelope of substances found in teas and referred to as thearubigins. Unless these substances contain unchanged benztropolone nuclei then they may well be colourless; in this context the reported pale-buff colour of some thearubigin fractions should not therefore be considered unusual.

Nevertheless much of the importance attached to the thearubigins in technological innovations, such as instant and cold-brew teas, derives from the amounts present, their water solubility and their rich brown colour. The browning which ensues when plant tissues are damaged may be explicable by invoking either quinone tanning, the Maillard reaction or caramelisation. A great many of the brown pigments found in nature are, however, melanins, whose structure and synthesis remain as much a mystery as that of the thearubigins. In the classic Raper–Mason scheme of melanogenesis (Mason, 1948; Haslam, 1998) the formation of *eu*mela-

nin from L-tyrosine proceeds via L-dopaquinone and the cyclic indolequinone (**24**). Subsequent reactions occur spontaneously to give the polymeric *eu*melanin (Fig. 7). Highly conjugated structural fragments such as (**25**) are believed to form part of an extended polymer. As a consequence most of the incident light which falls on the pigmented tissue is adsorbed and tissues containing it thus appear brown/black in colour.

When pyrogallol is dissolved in sodium hydroxide solution oxygen is quantitatively abstracted from the surrounding atmosphere. During the exothermic autooxidation which takes place the solution rapidly becomes dark brown. The parallel with melanogenesis is clear, particularly if it is assumed that the hydroxyquinone (**26**), or its equivalent, is an intermediate. But what is the structure of the species formed from pyrogallol in solution? The simplest proposal (Fig. 8) is that it is an oligomeric structure (**27**) of the type elaborated by self-condensation of the hydroxyquinone (**26**), [a tetramer is shown but this is merely illustrative].

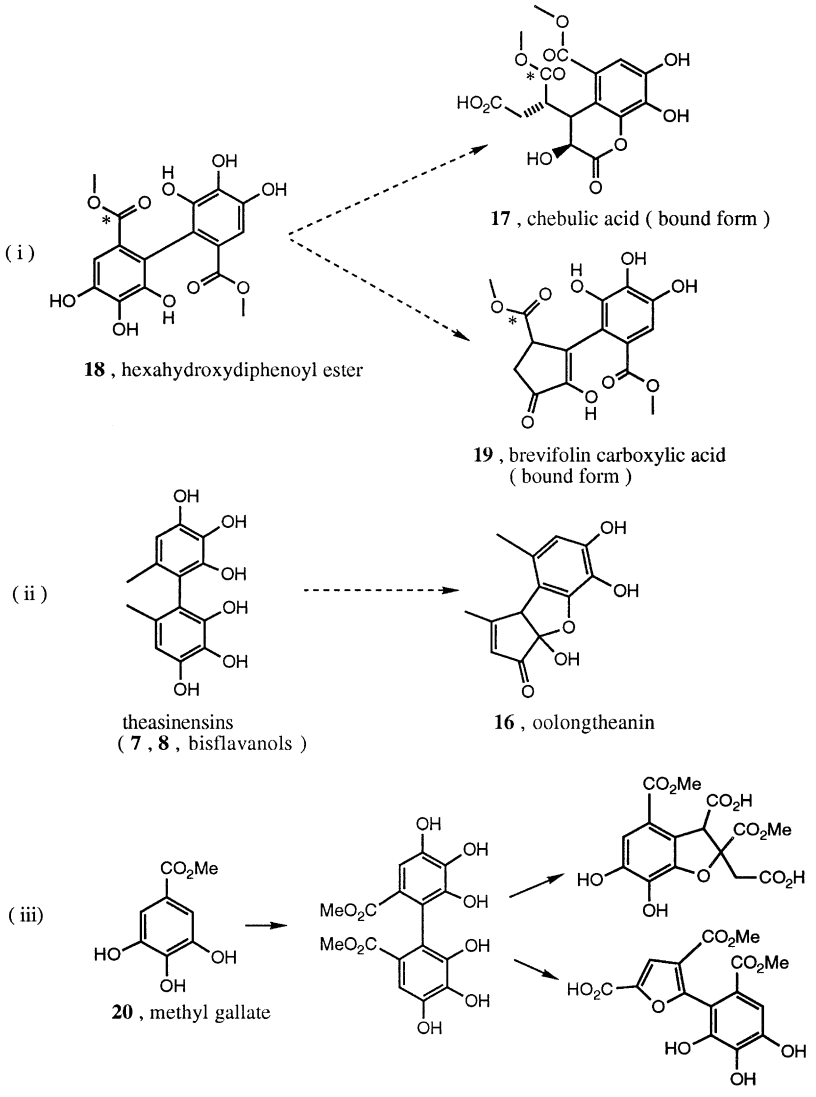


Fig. 6. Oxidative ring opening of phenolic nuclei—in vivo and in vitro.

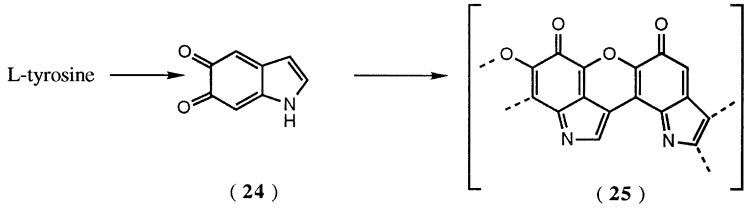


Fig. 7. Melanin formation, (Mason, 1948; Haslam, 1998).

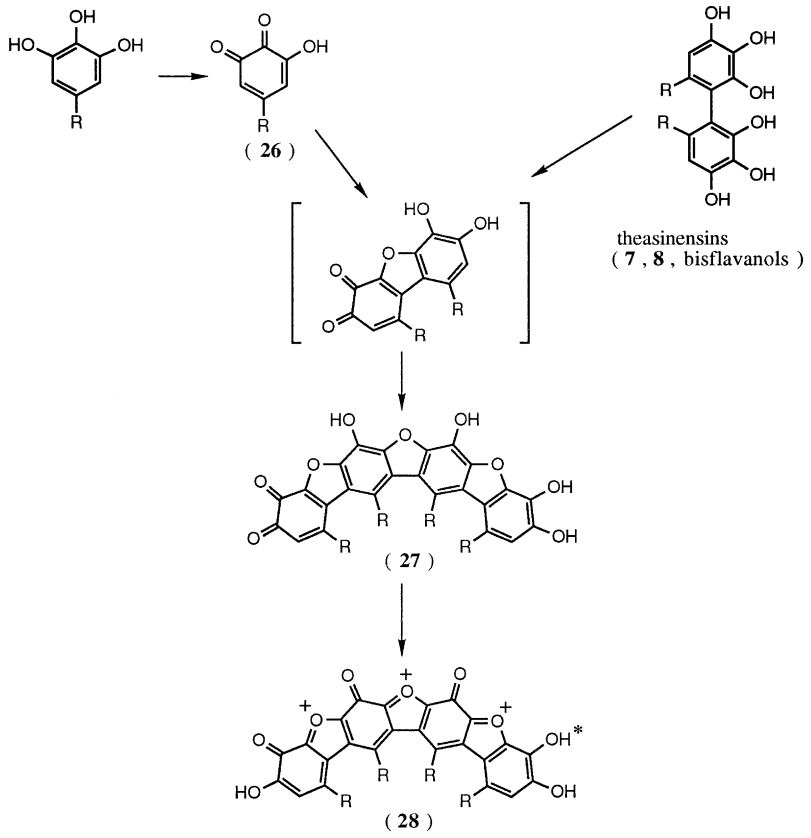


Fig. 8. Suggested structures for the brown pigments developed in alkaline solutions of pyrogallol (R=H); structural proposals for the pigmented thearubigins (R = rings A and C of the flavan-3-ols EGC **2** and EGCG **4**).

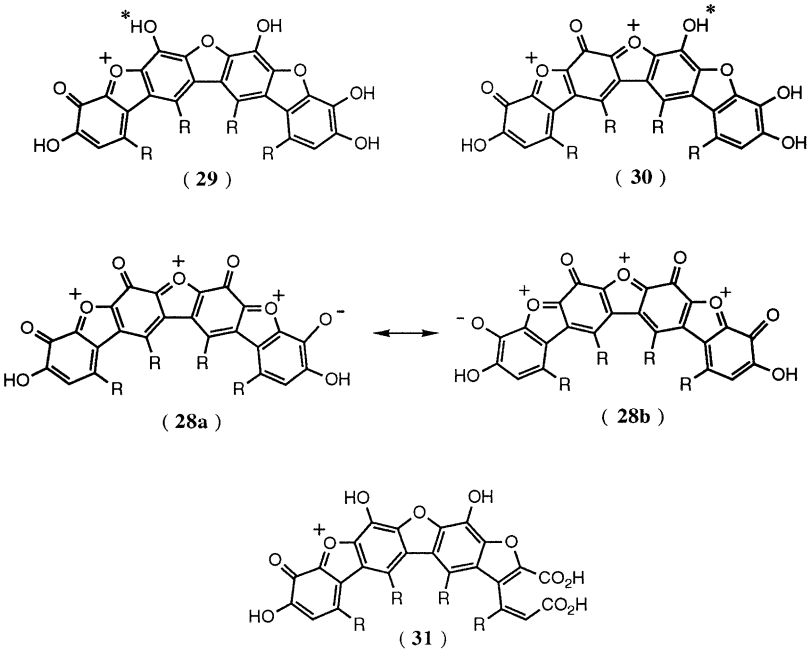


Fig. 9. Rationalisations of the colour and acidity of the thearubigins.

Further intramolecular oxidation might also occur with the *ultimate* formation of highly conjugated cationic structures such as (**28**), which by analogy with melanins, could well be brown in colour.

This mechanistic proposal, it is suggested, can now be extended and used as a basis for a model for the genesis of the rust brown pigmented thearubigins developed in tea fermentations, in which the principal precursors, EGC (**2**) and EGCG (**4**), are treated simply as derivatives of pyrogallol and the group R is taken to designate the rings A and C of the phenolic flavan precursors (Figs. 5 and 8). The following predictions can be made from a scrutiny of the model:

(a) Thearubigins isolated from a typical tea fermentation would comprise a series of very closely related structural types, which range from dimeric and trimeric to tetrameric structures, and possibly greater, and would thus have number average molecular weights in the range ~700–2000. Interestingly in this model, structures such as **27** and **28** would simply represent products of the oxidative dimerisation of the theasinensins (e.g. **7**, **8**), already identified as key intermediates in the initial stages of tea fermentation, (Section 3.3, Fig. 8).

(b) The extensively conjugated aproximately planar structure formed by oxidative oligomerisation of the pyrogallol 'B' rings of the flavan-3-ol precursors, as shown in Fig. 8, would provide the chromophoric group from which the thearubigins derive their rust brown colour. In principle, six other structures exist between **27** and **28** which differ in oxidation states and electron/proton distribution. Structures **29** and **30** are examples of such intermediates.

(c) The acidic properties of the thearubigins would derive from the ionisation of phenolic protons in the chromophore, e.g. $-H^*$ in structures **28**, **29**, **30** and in which delocalisation and stabilisation of the resultant anionic charge would be readily achieved (e.g. **28a**, **28b**). Indeed this extensive delocalisation of charge made possible throughout the chromophore in the ionised species suggests that the rust brown colour of thearubigins derives primarily from such entities (Fig. 9). Conversely, if such ionisation were suppressed then it would be predicted that the depth of colour would be diminished. This effect is precisely that which is observed when lemon (citric acid) is added to tea infusions.

(d) Acidity in the thearubigins could also originate additionally, as described above, by oxidative ring fission of phenolic nuclei in the chromophore, e.g. **31** (Fig. 9).

(e) The extended planar structures suggested for the thearubigins would similarly be expected to provide good templates to complex with caffeine and other phenolic intermediates such as the theaflavins (TFs; **11**–**14**) and hence participate in the important processes of tea creaming.

It should be stressed that these proposals do not, at this point, have the support of specific experimental data. They have evolved as a result of a consideration of the available published information of the chemistry which underlies tea fermentation. As such they merely emphasise the importance of the aphorism that 'theory guides, but experiment decides'. It is for future work to prove or disprove them. One question however remains. During tea fermentations significant quantities of gallic acid are released (Roberts, 1962). But how does this occur? A question surely now best left for another day.

Acknowledgements

The author thanks Alan Davies, Phil Evans, Jim Busby and their colleagues (Unilever Colworth Research) for their introduction to the arcane mysteries of tea and for very many incisive and stimulating discussions concerning the chemical changes which accompany tea leaf fermentation.

References

Bailey, R.G., Nursten, H.E., 1994. Analytical Aspects of the Thearubigins. Soc.Chem.Ind. Lecture Paper, number 26, 10 pp.

Bailey, R.G., Nursten, H.E., McDowell, I., 1991. Comparative Study of the Reversed-Phase High-Performance Liquid Chromatography of Black Tea Liquors with Special Reference to Thearubigins. J. Chromatog. 542, 115–128.

Bailey, R.G., Nursten, H.E., McDowell, I., 1992. The isolation of a polymeric thearubigin fraction from black tea. J. Sci. Food Agric. 59, 365–375.

Balentine, D.A., Wiseman, S.A., Bouwens, L.C.M., 1997. The chemistry of tea flavonoids. Crit. Rev. Food Sci. Nutr. 37, 693–704.

Brown, A.G., Eyton, W.B., Holmes, A., Ollis, W.D., 1969a. Identification of thearubigins as polymeric proanthocyanidins. Nature 221, 742–744.

Brown, A.G., Eyton, W.B., Holmes, A., Ollis, W.D., 1969b. The identification of thearubigins as polymeric proanthocyanidins. Phytochemistry 8, 2333–2340.

Catell, D.J., Nursten, H.E., 1976. Fractionation and chemistry of ethyl acetate-soluble thearubigins from black tea. Phytochemistry 15, 1967–1970.

Clifford, M.N., Powell, C., 1996. A partial resolution of ''black tea tannins'' by HPLC. Polyphenol Communications 96, 217–218.

Collier, P.D., Bryce, T., Mallows, R., Thomas, P.E., Frost, D.J., Korver, O., Wilkins, C.K., 1973. The theaflavins of black tea. Tetrahedron 29, 125–142.

Davies, A.P., Goodsall, C., Cai, Y., Davis, A.L., Lewis, J.R., Wilkins, J., Wan, X., Clifford, M.N., Powell, C., Parry, A., Thiru, A., Safford, R., Nursten, H.E., 1999. Black tea dimeric and oligomeric pigments-structures and formation. In: Gross, C.G., Hemingway, R.W., Yoshida, T. (Eds.), Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology. Kluwer Academic/Plenum Press, New York, pp. 697–724.

Davis, A.L., Davies, A.P., Cai, Y., Lewis, J.R., Wilkins, J., Pudney, P., Powell, C., Clifford, M.N., 1997. A polyphenolic pigment from black tea. Phytochemistry 46, 1397–1402.

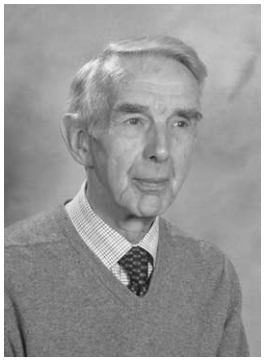
Dix, M.A., Fairley, C.J., Millin, D.J., Swaine, D.E., 1981. Fermentation of tea in aqueous suspension. influence of tea peroxidase. J. Sci. Food Agric. 32, 920–932.

Ferretti, A., Flanagan, V.P., Bondarovich, H.A., Gianturco, M.A., 1968. The chemistry of tea. structures of compounds A and B of Roberts and some model compounds. J. Agr. Food Chem. 16, 756–761.

Finger, A., 1994. In vitro studies on the effect of polyphenoloxidase on the formation of polyphenolic black tea constituents. J. Sci. Food Agric. 66, 293–305.

Gregory, R.P.F., Bendall, D.S., 1966. The purification and some

properties of the polyphenol oxidase from tea (*Camellia sinensis* L.). Biochem.J. 101, 569–581.
Harbowy, M.E., Balentine, D.A., 1997. Tea chemistry. Crit. Rev. Plant Sci. 16, 415–480.
Hashimoto, F., Nonaka, G-I., Nishioka, I., 1988. Tannins and related compounds. LXIX. Isolation and structure determination of B-B′-linked bisflavanoids, theasinensins D-G and oolongtheanin from oolong tea. Chem. Pharm. Bull. 36, 1676–1684.
Hashimoto, F., Nonaka, G-I., Nishioka, I., 1989a. Tannins and related compounds. LXXVII. Novel chalcan-flavan dimers, assamicains A, B and C, and a new flavan-3-ol and proanthocyanidins from the fresh leaves of *Camellia sinensis* L. var. *assamica* Kitamura. Chem. Pharm. Bull. 37, 77–85.
Hashimoto, F., Nonaka, G-I., Nishioka, I., 1989b. Tannins and related compounds. XC. 8-C-ascorbyl-(−)-epigallocatechin-3-*O*-gallate and novel dimeric flavan-3-ols, oolonghomobisflavans A and B, from oolong tea. Chem. Pharm. Bull. 37, 3255–3263.
Hashimoto, F., Nonaka, G-I., Nishioka, I., 1992. Tannins and related compounds. CXIV. Structures of novel fermentation products, theogallinin, theaflavonin and desgalloyl theaflavonin from black tea, and changes of tea leaf polyphenols during fermentation. Chem. Pharm. Bull. 40, 1383–1389.
Haslam, E., 1989. Plant Polyphenols: Vegetable Tannins Revisited. Cambridge University Press, Cambridge. p. 35..
Haslam, E., 1998. Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action. Cambridge University Press, Cambridge.
Haslam, E., Cai, Y., 1994. Plant polyphenols (vegetable tannins): gallic acid metabolism. Nat. Prod. Rep. 11, 41–66.
Horner, L., Durckheimer, W., Weber, K-H., 1961. Zur Kenntnis der o-Chinone, XIX. Hydrolysestudien an 2-Substitutieren 1,3 Dicarbonylverbindungen als Beitrag zum Mechanismus der Purpurogallinbildung. Chem. Ber. 94, 2881–2887.
Horner, L., Durckheimer, W., Weber, K-H., Dolling, K., 1964. Zur Kenntnis der o-Chinone, XXIV. Synthese, Struktur und Eigenschaften von 1′,2′-Dihydroxy-6,7-Benztropolone. Chem. Ber. 97, 312–324.
Jovanovic, S.V., Steenken, S., Tosic, M., Marjanovic, B., Simic, M.G., 1994. Flavanoids as Anti-oxidants. J. Am. Chem. Soc. 116, 4846–4851.
Jovanovic, S.V., Steenken, S., Hara, Y., Simic, M.G., 1995. Anti-oxidant potential of gallocatechins. a pulse radiolysis and laser photolysis study. J. Am. Chem. Soc. 117, 9881–9888.
Jovanovic, S.V., Steenken, S., Hara, Y., Simic, M.G., 1996. Reduction potential of flavonoid and model phenoxy radicals. which ring in flavonoids is responsible for anti-oxidant activity? J. Chem. Soc. (Perkin Trans. II) 2497–2504.
Lunder, T.V., 1988. Tea. Nestec Ltd. Technical Assistance, Nestlé Research Centre, Vevey, pp. 42.
Mason, H.S., 1948. The chemistry of melanin—mechanism of oxidation of DOPA by tyrosinase. J. Biol. Chem. 172, 83–92.
Mayer, A.M., Harel, E., 1979. Polyphenol oxidases in plants. Phytochemistry 18, 193–215.
Mayer, A.M., 1987. Polyphenol oxidases in plants—recent progress. Phytochemistry 26, 11–20.
Mayer, W., Hoffman, E.H., Lösch, N., Wolf, H., Wolter, B., Schilling, G., 1984. Dehydierungsreactionen mit Gallussäureestern. Liebig's Annalen 929–938.
Millin, D.J., Rustige, D.W., 1967. Tea Manufacture. Process Biochem. 2, 9–13.
Nonaka, G-I., Kawahara, O., Nishioka, I., 1983. Tannins and related compounds XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. Chem. Pharm. Bull. 31, 3906–3914.
Nursten, H.E., 1997. Chemistry of tea infusions. In: Schubert, R., Spiro, M. (Eds.), Chemical and Biological Properties of Tea Infusions. U & M Verlag mbh, Frankfurt/Main, pp. 22–38.
Ollis, W.D., Brown, A.G., Haslam, E., Falshaw, C.P., Holmes, A., 1966. The constitution of theaflavin. Tetrahedron Lett. 1193–1204.
Pierpoint, W.S., 1985. Phenolics in food and feedstuffs: the pleasures and perils of vegetarianism. In: van Sumere, C.F., Lea, P.J. (Eds.), The Biochemistry of Plant Phenolics, Annual Proceedings of the Phytochemical Society of Europe, Vol. 25, pp. 427–451.
Powell, C., Clifford, M.N., Opie, S.C., Ford, M.A., Robertson, A., Gibson, C.L., 1992. Tea cream formation. the contribution of black tea phenolic pigments determined by HPLC. J. Sci. Food Agric. 63, 77–86.
Roberts, E.A.H., 1962. Economic importance of flavonoid substances: tea fermentation. In: Geissman, T.A. (Ed.), The Chemistry of Flavonoid Compounds. Pergamon Press, Oxford, pp. 468–512.
Roberts, E.A.H., Cartwright, R.A., Oldschool, M., 1959. The phenolic substances of manufactured tea. I. Fractionation and paper chromatography of water-soluble substances. J. Sci. Food Agric. 8, 72–80.
Roberts, E.A.H., Myers, M., 1959. The phenolic substances of manufactured tea. IV. Enzymic oxidations of individual substrates. J. Sci. Food Agric. 10, 167–179.
Sanderson, G.W., 1972. The chemistry of tea and tea manufacturing. Rec. Adv. Phytochemistry 5, 247–316.
Sang, S., Tian, S., Meng, X., Stark, R.E., Rosen, R.T., Yang, C.S., Ho, C.T., 2002. Theadibenzotropolone a, a new type pigment from enzymatic oxidation of (−)-epicatechin and (−)-epigallocatechin gallate and characterised from black tea using LC/MS/MS. Tetrahedron Letters 43, 7129–7133.
Schmidt, O.Th., Mayer, W., 1956. Naturliche Gerbstoffe. Angew. Chem. 68, 103–115.
Schubert, R., Spiro, M., 1997. Chemical and biological properties of tea infusions. U & M Verlag mbh, Frankfurt/Main. pp. 110..
Takino, Y., Ferretti, A., Flanagan, V., Giancurto, M., Vogel, M., 1965. Structure of Theaflavin, a Polyphenol of Black Tea. Tetrahedron Lett. 4019–4025.
Tanaka, T., Kouno, I., Nonaka, G-I., 1996. Glutathione-mediated conversion of the ellagitannin geraniin to chebulagic acid. Chem. Pharm. Bull. 44, 34–40.
Tanaka, T., Mine, C., Kuono, I., 2002. Structures of two new products of green tea polyphenols generated by model tea fermentation. Tetrahedron 58, 8851–8856.
Vuataz, L., Brandenberger, H., 1961. Plant phenols. III. Separation of fermented and black tea polyphenols by cellulose chromatography. J. Chromatogr. 5, 17–31.
Weinges, K., Ebert, W., Huthwelker, D., Mattauch, H., Perner, J., 1969. Oxidative Kuplung von Phenolen II. Konstitution und Bildungsmechanismus des Dehydro-dicatechins A. Liebig's Annalen 726, 114–124.
Willson, K.C., Clifford, M.N., 1992. Tea: Cultivation to Consumption. Chapman & Hall, London. pp. 739.



Edwin Haslam was educated at the University of Sheffield (1949–1955) and held a Sir William Ramsay Fellowship in the University of Cambridge (1955–1958) before returning to the Deparment of Chemistry in Sheffield. He served for a period as Head of Department, held the first Hugh Kelly fellowship in Rhodes University, S.A. (1975) and was visiting Professor in the University of the South Pacific (1984). He has published widely on plant phenolics, the shikimate pathway and secondary metabolism and is the author of six books. Awards include the PSE prize (1977), the Procter and Wolstenholme Memorial lectureships of the Society of Leather Trades Chemists (1987 and 1996), the Groupe Polyphenols Medal (1998), and the third North American Tannin award (1998). He was created Professor Emeritus in 1995.