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EFFECTS OF CATECHIN CONCENTRATION ON THE FORMATION OF BLACK TEA POLYPHENOLS DURING *IN VITRO* OXIDATION

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Key Word Index—*Camellia sinensis*; Theaceae; model fermentation system; HPLC; catechins; theaflavins; thearubigins.

Abstract—An *in vitro* model fermentation system, containing purified catechins and partially purified polyphenol oxidase (EC 1.14.81.1) from green tea shoots, has been used to determine the effect of catechin mixtures of different concentration and proportions on the formation of theaflavin and thearubigin. Increases in total catechin concentration, 25% above that typical in green tea shoots of Malawi-grown bushes, inhibited polyphenol oxidase activity and, consequently, depressed theaflavin levels. Individual or combined concentrations of epicatechin gallate and epigallocatechin gallate in excess of 110 mM were shown to be responsible for enzyme inhibition, whereas epicatechin and epigallocatechin had no effect. Fermentation of a catechin mixture, containing the four major catechins, epicatechin, epigallocatechin, epigallocatechin gallate and epicatechin gallate, at equal individual concentrations (55 mM), produced, after 30 min, total theaflavin levels 68% higher and thearubigin levels only 25% higher than those from a standard catechin mixture fermented under similar conditions. Continued fermentation of this mixture produced no further theaflavin, but the thearubigin fraction increased significantly, due to subsequent oxidation of the excess of simple catechins. A new catechin mixture was, therefore, calculated to give a similar level of theaflavin to that of the previous mixture without leaving an excess of unoxidized simple catechins. The catechin proportions and concentrations of the latter mixture agree well with those of the green shoots of quality Kenyan teas or similar quality Malawi teas grown during the dry cold season. The results indicate that a high ratio of simple to gallocatechins will facilitate a high theaflavin–thearubigin ratio in black tea.

INTRODUCTION

In a previous paper [1], experiments using an *in vitro* model fermentation system demonstrated that theaflavin levels, from a fermenting mixture of catechins, can be considerably increased by changing the reaction conditions from those occurring in whole tea shoots during factory fermentation. Throughout these experiments the catechin mixture was maintained constant and was based on the average green leaf composition of 12 Malawi clonal teas measured on several different occasions throughout the year. However, there is evidence to suggest that changes in the concentrations and proportions of the catechins during growth are also important in producing changes in the theaflavin levels and, consequently, the quality of black tea [2, 3].

The shoot used in black tea manufacture is, on average, composed of a bud and three leaves. The distribution of the catechins within the shoot varies with the age of the tissue [4, 5] and it is generally agreed that the concentrations of epigallocatechin gallate, epicatechin gallate and epicatechin decrease as the leaf ages, whereas epigallocatechin levels increase. Tea making quality has been attributed to a high ratio of epogallocatechin–epigallocatechin gallate but, although this ratio is higher in the older leaves, it is the second leaf which generally produces the highest quality tea. High quality in black teas has also been correlated with high total catechin content and high epicatechin [2].

Total and individual catechin levels also change with the growth rate of the bush [6]. Tea shoots under conditions of slow growth as a result of low temperature, low light or drought, generally produce the highest quality teas. In addition, theaflavin levels have also been shown to increase and decrease at the troughs and peaks, respectively, of tea shoot growth and decrease in response to the increased growth rate with nitrate application [7].

In this study an attempt has been made, using the model fermentation system previously described [8] together with precisely controlled reaction conditions, to elucidate the anomalies which exist at present between the results of the above-mentioned workers. Results are reported in this paper which define the exact role of the catechins in producing high theaflavin values during fermentation.

RESULTS

The 'standard catechin mixture', used with the model system experiments previously described [1, 8], was based on the average annual catechin composition of green tea shoots in Malawi, adjusted to compensate for the water deficit, incurred during the withering process, prior to manufacture. These catechin concentrations were taken to be the 100% levels in subsequent experiments, where catechin proportions and concentrations were varied.

Figure 1 shows the effect of increasing total catechin

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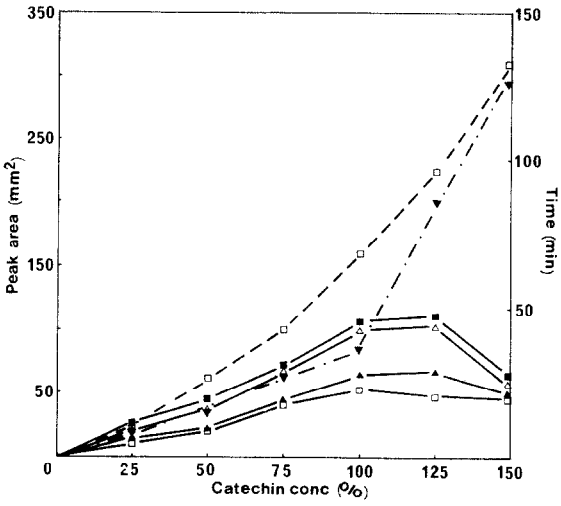

Fig. 1. Effect of increasing total catechin concentration, whilst maintaining the individual catechin ratio, on the formation of the theaflavins and total thearubigin (100% = standard catechin mixture). Fermentation time was the time taken to reach maximum theaflavin levels [1]. (□–□) Theaflavin; (△–△) theaflavin-3'-monogallate; (▲–▲) theaflavin-3-monogallate; (■–■) theaflavin-3,3'-digallate; (□ - - - □) thearubigin; (▼···▼) fermentation time.

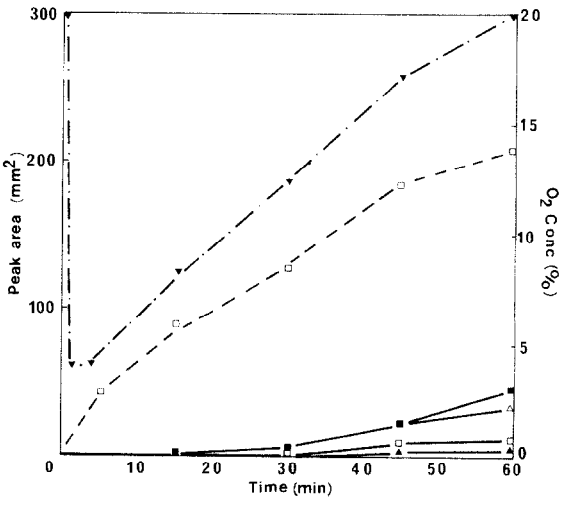

Fig. 2. Time course for the fermentation of theaflavin and thearubigin from a standard catechin mixture containing low enzyme levels (6.7×10^{-8} kat). (□–□) Theaflavin; (△–△) theaflavin-3'-monogallate; (▲–▲) theaflavin-3-monogallate; (■–■) theaflavin-3.3'-digallate; (□ ····· □) thearubigin; (▼···▼) percentage oxygen concentration.

concentration on the formation of theaflavin and thearubigin, whilst maintaining the same ratios between the catechins. Both increased linearly with catechin concentration until the 100% level was reached. Further increases resulted in a considerable decrease in the rate at which fermentation proceeded, suggesting enzyme inhibition by one or more of the catechins, and a concomitant increase in thearubigin levels at the expense of theaflavin. Figure 2 shows a similar effect of low polyphenol oxidase activity on the fermentation of a standard catechin mixture. Here, as in the case of high catechin enzyme inhibition produced in Fig. 1 the rate of fermentation is reduced and theaflavin levels are particularly low. Although there are no data on catechin consumption available to complement this time course, it is speculated that, during the first 15–20 min of fermentation, the simple catechin quinones are utilized predominantly in gallocatechin oxidation [1]. Only when the levels of gallocatechin have diminished substantially, are simple catechin quinones available for theaflavin production, which is seen to rise between 20 and 30 min.

In an attempt to demonstrate which of the catechins was responsible for enzyme inhibition, each was increased from 0 to 200%, whilst those remaining were maintained constant. Each fermentation was terminated when theaflavin levels were maximal [1]. The percentage change in total theaflavin with increase in each catechin is shown in Fig. 3. Theaflavin increases, effected by epigallocatechin or epicatechin, were linear and enzyme activity was, consequently, unaffected by these two catechins at the 200% level. However, epigallocatechin gallate and epicatechin gallate were responsible for decreases in total theaflavin formation above 125% (93.75 mM) and 175% (33.23 mM), respectively, indicating that substrate inhibition of polyphenol oxidase was limited to the catechin gallates. Since epigallocatechin gallate and epicatechin

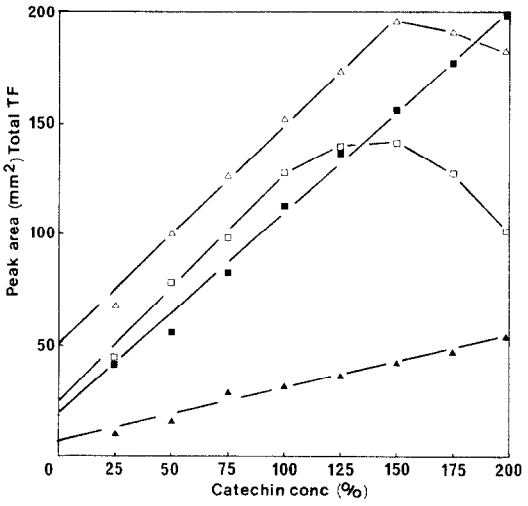

Fig. 3. Effect on total theaflavin of increasing the concentration of each individual catechin of a standard catechin mixture whilst maintaining the concentrations of the remaining catechins constant (100% = concentration of each catechin in the standard catechin mixture). (▲–▲) Epicatechin; (■–■) epigallocatechin; (□–□) epigallocatechin gallate; (△–△) epicatechin gallate.

gallate were maintained constant at standard catechin mixture levels, whilst one or other was increased, the combined concentrations of the catechin gallates at their points of inhibition were 112 and 108 mM, respectively. These two figures are consistent with, and are further substantiated by, the combined catechin gallate concentration of 105 mM at which inhibition of theaflavin formation occurred in Fig. 1. Substrate inhibition by epigallocatechin gallate has been reported by Gregory and

Bendall [9], although the concentration at which inhibition was observed in their system was many times lower than that shown in Figs. 1 and 3. It would appear, therefore, that the enzyme kinetics, using a concentrated catechin mixture and an impure polyphenol oxidase preparation, vary significantly from those obtained with pure enzyme and single substrate. Figure 4 shows the effect of increasing the four major catechins in equal concentration on the levels of theaflavins and thearubigin after fermentation. A mixture, taken as 100%, containing the catechins at individual concentrations of 37.5 mM, i.e. equal in total catechin concentration to that of a standard catechin mixture (150 mM), produced theaflavin levels 20% higher and thearubigin levels 17% lower than the standard catechin mixture. Furthermore, the individual concentrations of the catechins present in this ratio could be increased to 55 mM (total catechin concentration of 220 mM) before substrate inhibition of polyphenol oxidase occurred. The combined concentration of the catechin gallates at this point was, therefore, 110 mM, a value which is in good agreement with those calculated from Figs. 1 and 3. At this concentration, total theaflavin levels were *ca* 68% higher and thearubigin levels only 25% higher than those from the standard catechin mixture.

Time courses for the production of theaflavin and thearubigin (Fig. 5) and oxidation of the catechins (Fig. 6), from a model fermentation containing equal individual catechin concentrations of 55 mM, show improved theaflavin formation, although simple catechin consumption was still considerably slower than that of the gallocatechins. Maximum theaflavin levels occurred after 30 min and coincided with a point at which no further oxidation of the gallocatechins was observed. Since no further theaflavin formation was possible, subsequent fermentation resulted in the oxidation of the remaining simple catechins and of the theaflavins to form additional

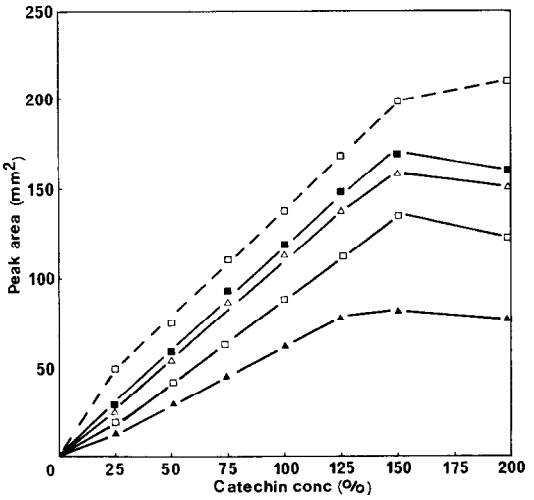

Fig. 4. Effect of increasing the total catechin concentration, of a mixture consisting of equal catechin concentrations, on the formation of theaflavins and total thearubigin (100% = total catechin concentration equal to the 150 mM of the standard catechin mixture). (□–□) Theaflavin; (△–△) theaflavin-3′-monogallate; (▲–▲) theaflavin-3-monogallate; (■–■) theaflavin-3,3′-digallate; (□ – – – □) total thearubigin.

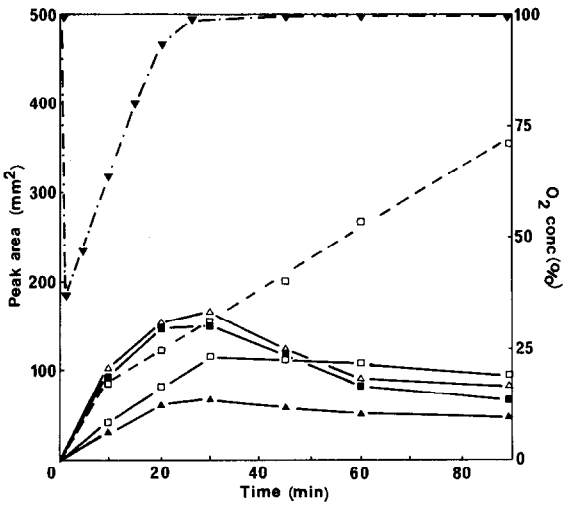

Fig. 5. Time course for the fermentation of theaflavins and total thearubigin from a catechin mixture containing equal individual catechin concentrations (55 mM). (□–□) Theaflavin; (△–△) theaflavin-3′-monogallate; (▲–▲) theaflavin-3-monogallate; (■–■) theaflavin-3,3′-digallate; (□ – – – □) total thearubigin; (▼···▼) percentage oxygen concentration.

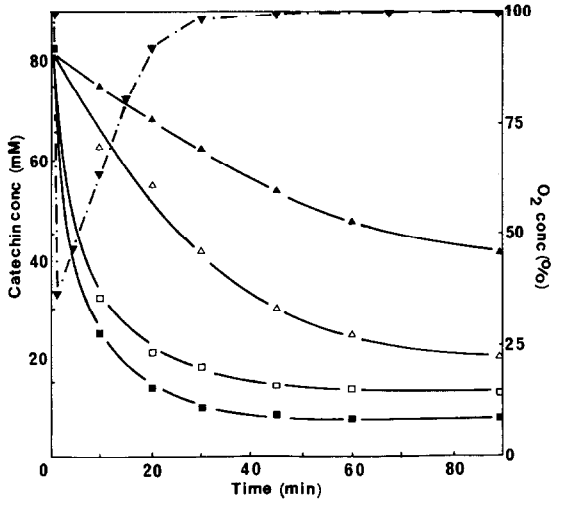

Fig. 6. Fermentation time course showing the disappearance of catechins from a mixture of equal individual catechin concentration (55 mM). (▲–▲) Epicatechin; (■–■) epigallocatechin; (△–△) epicatechin gallate; (□–□) epigallocatechin gallate; (▼···▼) percentage oxygen concentration.

thearubigin [1]. It was apparent, therefore, that the concentrations of the simple catechins were in excess of those actually required. Remaining simple catechins, in addition to forming thearubigins, might also act as electron carriers in the oxidation of the theaflavin already formed.

The point in Fig. 6 at which no further oxidation of the gallocatechins occurred was used to calculate new catechin ratios which, after fermentation, would produce similar theaflavin levels but total exhaustion of all the

catechins. The catechins oxidized in the first 20 min were calculated to be: epigallocatechin, 52 mM; epigallocatechin gallate, 41 mM; epicatechin gallate, 28 mM; and epicatechin, 12 mM. However, the total catechin gallate concentration of this mixture could be increased 58% without polyphenol oxidase inhibition and, hence, each catechin was increased by that percentage to give the following proportions: epigallocatechin, 82 mM; epigallocatechin gallate, 65 mM; epicatechin gallate, 44 mM; and epicatechin, 19 mM.

The time courses for the products formed during catechin consumption in this mixture are shown in Figs. 7 and 8, respectively. Maximum theaflavin levels were observed after 30 min and, although the ratios of the individual theaflavins were different, total theaflavin levels were similar to those in Fig. 5. Catechin levels decreased at rates similar to those in Fig. 6 to converge at a point corresponding to maximum theaflavin formation. Little remained of unoxidized catechins at this point and subsequent fermentation effected only a modest increase in the level of thearubigin.

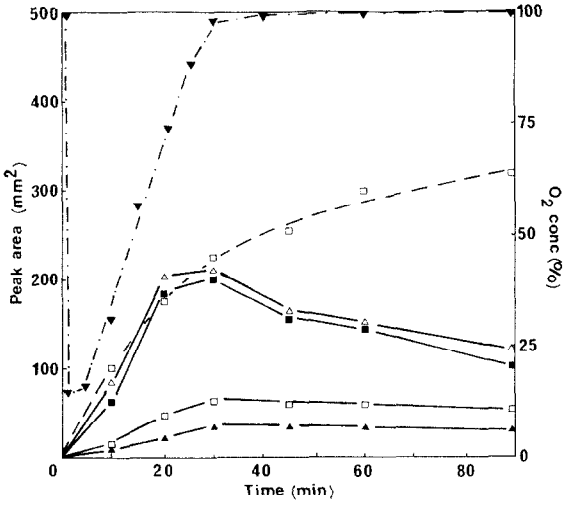

Fig. 7. Time course for the fermentation of theaflavins and total thearubigin from the calculated optimum catechin mixture. (□–□) Theaflavin; (△–△) theaflavin-3'-monogallate; (▲–▲) theaflavin-3-monogallate; (■–■) theaflavin-3,3'-digallate; (□ – – – □) thearubigin; (▼···▼) percentage oxygen concentration.

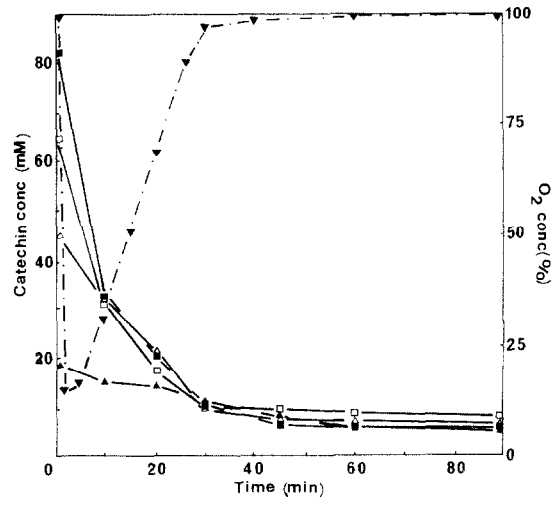

Fig. 8. Time course of catechin consumption during fermentation of the calculated optimum catechin mixture. (■–■) Epigallocatechin; (□–□) epigallocatechin gallate; (△–△) epicatechin gallate; (▲–▲) epicatechin; (▼···▼) percentage oxygen concentration.

A balance sheet quantifying the levels of epicatechin and epicatechin gallate present in the theaflavin and thearubigin fractions, 20 min after the commencement of the two model fermentations shown in Figs. 5 and 7, is given in Table 1. The theaflavin levels were calculated using the original HPLC peaks and the extinction coefficients reported by Collier *et al.* [10] and adjusted to compensate for anticipated theaflavin breakdown due to further oxidation [1]. This was determined by measuring the rate of disappearance of those theaflavins, containing epicatechin and epicatechin gallate as components, after the point at which the gallocatechins had been depleted and no further theaflavin could be formed. It was assumed that the rate of theaflavin breakdown over the first 20 min of fermentation was similar.

Figure 9 is a compilation of HPLC data to demonstrate changes in total thearubigin during 90 min fermentation of the optimum catechin mixture shown in Fig. 7. The inclusion of 'baselines' indicates the formation of two distinct thearubigin fractions over this time course. The partially resolved peaks above the 'baseline' are the result of oxidation and reaction of the gallocatechins [8].

Table 1. Recovery of the simple catechins in the theaflavin and thearubigin fraction after fermentation of optimized (A) and equimolar (B) catechin mixtures

Fermentation mixture	Catechin	Uptake (μmol)	Recovery from theaflavin (μmol)	Anticipated breakdown (%)	Corrected recovery from theaflavin (μmol)	Catechins in thearubigins (μmol)
A	Epicatechin	5.0	3.0	10	3.3	1.7
	Epicatechin gallate	28.0	10.0	33	14.0	14.0
B	Epicatechin	9.5	4.4	5	5.0	4.5
	Epicatechin gallate	22.0	7.7	50	11.5	10.6

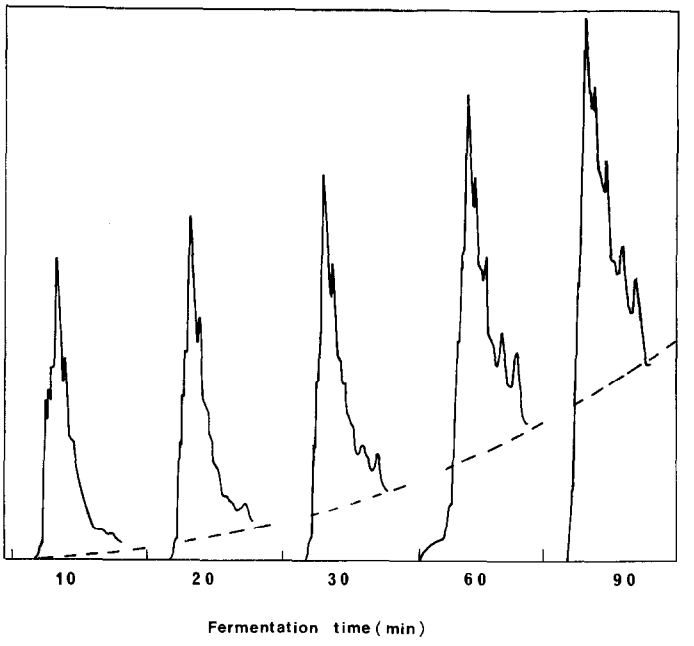

Fig. 9. Changes in the thearubigin fraction as shown by HPLC during fermentation. Inclusion of a baseline (----) shows the formation of unresolved thearubigin below that formed from the gallocatechins.

Consumption of the latter and maximum theaflavin levels occurred after 30 min (Figs. 7 and 8) and this is confirmed by the fact that little increase in the total area of this fraction occurred during the subsequent 60 min of fermentation. The second thearubigin fraction appears on HPLC as a mass of unresolved material which merely increases the 'baseline' below the resolved thearubigin. Simple catechins, when oxidized enzymically and reacted with themselves, produce a similar type of fraction (data not shown). Thearubigin formed in this way, in addition to the substantial amounts formed by the further oxidation of theaflavin intermediates [1] and theaflavin itself, will all probably contribute to this unresolved fraction.

Table 2 shows the changes which occur in the catechin composition of fast and slow growing tea bushes and compares them with the optimum composition calculated using the model fermentation system. During the slow growing season, epigallocatechin gallate levels decrease, whilst the levels of the other catechins increase [6]. The general effect is to increase the ratio of simple catechins to gallocatechins to one similar to that of the optimum. Assuming non-specificity of polyphenol oxidase, a more equal oxidation will occur of the simple catechins and the gallocatechins and a higher ratio of the corresponding quinones will be present for theaflavin formation. Fast growing tea shoots contain a low simple catechins to gallocatechins ratio although there is little difference in the total catechin concentrations.

Table 2. Effect of growth rate on the catechin ratio in Malawi tea clone SFS 204 and comparison of these with the optimum catechin concentration for theaflavin formation as assessed by the model system

	Catechin concentration (mM)		
Catechins	Slow growing May–Nov.	Fast growing Dec.–Apr.	Optimum
Epigallocatechin gallate	75	100	65
Epigallocatechin	60	30	82
Epicatechin gallate	35	20	44
Epicatechin	22	8	19

DISCUSSION

The formation of theaflavin requires the oxidation and condensation of a simple catechin and a gallocatechin [11]. Ideally, for the production of maximum theaflavin levels in a given mixture, equal concentrations of the two types of catechin quinones are required. Improved ratios of the two types of quinones can be obtained, therefore, by fermentation of catechin mixtures under conditions which will increase simple catechin oxidation, decrease gallocatechin oxidation or increase the stability of the resultant quinones from both. Results have recently been presented [1] which demonstrate that high oxygen tensions, low pH and low temperature during fermentation are effective in producing such conditions. In this paper, using these optimum conditions for theaflavin formation, already defined, model system experiments have shown that theaflavin levels can be increased still further by changing the catechin composition normally found in Malawi green leaf.

Epigallocatechin gallate concentrations of the growing tea shoots are particularly high [4, 5] and this, in addition to the epicatechin gallate concentrations also present, produces a total catechin gallate concentration close to that at which substrate inhibition of polyphenol oxidase was observed (Figs. 1 and 3). Further increases in one or

both of the catechin gallates consequently reduced enzyme activity and depressed theaflavin levels.

The effect of reduced enzyme activity in producing poor theaflavin levels is also shown in Fig. 2 in which a model fermentation containing low enzyme concentration was carried out. The phenomenon is probably the net result of two reactions. Simple catechin oxidation is reduced to a rate closer to that of redox equilibration [1] so that substantially lower simple catechin quinone levels are available for theaflavin formation. In addition, since the fermentation rate is reduced, further oxidation of the theaflavin which has been synthesized will also be greater.

The natural limits on catechin levels, imposed by the catechin gallates, indicate that little improvement in theaflavin formation can be obtained by increasing total catechin concentration in the existing ratio. By moving from catechin ratios associated with green tea shoots to one using equal individual concentrations, but of similar total concentration to that in the green tea shoots, a 25% increase in total theaflavin was achieved. In addition, as a result of the lower catechin gallate levels of this new ratio, the total catechin concentration was increased considerably, without inhibiting polyphenol oxidase activity, to give theaflavin and thearubigin levels 68% and only 25% above, respectively, those from a standard catechin mixture. Total simultaneous consumption of the catechins was still not achieved and even though enzymic oxidation of each catechin was perhaps equal, subsequent redox equilibration favoured gallocatechin quinone formation [1, 12]. Such high concentrations of the simple catechins were, therefore, unnecessary and a new ratio was calculated which gave similar total theaflavin levels at the point at which total oxidation of the catechins had occurred (Fig. 8). Further fermentation, therefore, resulted in relatively little thearubigin production from unoxidized simple catechins.

The optimum catechin ratio quoted in this paper is only so, however, provided that fermentations are carried out under conditions which are not limiting for theaflavin production. Should oxygen become limiting, or the pH increase from 5.0 to 6.0, preferential coupled oxidations leading to thearubigin formation will occur [8]. Furthermore, the stated concentrations were calculated for 70% withered leaf and, therefore, similar concentrations present in green leaf, prior to plucking, will be supraoptimal after wither. Substrate inhibition of polyphenol oxidase may well occur in this case.

The various components of the thearubigin fraction, and their sources, have been discussed in previous papers [1, 8]. There seems little doubt from this study using a model fermentation system and combined HPLC analysis that enzymically oxidized catechins can react to form both theaflavin and thearubigin components. Although the experiments were designed primarily to demonstrate theaflavin changes during fermentation, competition for common precursors and efficiency of the HPLC system has also yielded information on the thearubigin fraction.

The term thearubigin was originally designated [13] to cover a group of low MW pigments found in black tea, which partitioned into the aqueous phase of an ethyl acetate–water mixture [14]. However, their heterogeneity became apparent subsequently when they were chromatographed on paper [15] and classified into two groups, SI and SII, according to their mobility. The SII components were almost stationary and have since been reported to consist of large MW non-dialysable material which could be separated into at least three more pigmented bands when chromatographed on Sephadex LH-20 [16]. The original term which described thearubigins as a group of similar dimeric compounds [17] has, therefore, become outdated and has perhaps restricted the development of a much broader concept, which includes those compounds more polar than the theaflavins and consisting of both low- and high-MW polymeric polyphenolic material, formed by very different routes and of possibly very different structures. In addition to the purely phenolic structures, macromolecular phenolic complexes containing proteins, nucleic acids and polysaccharides are also considered to be present after fermentation [18]. Taking this into account it is perhaps remarkable that the thearubigin fraction obtained by HPLC from a black tea infusion is so similar to that from a model system containing only six purified catechins [8]. This fact demonstrates, particularly well, the importance of the high green leaf catechin concentration to the eventual black tea product. However, the only thearubigin components to be resolved by HPLC in this study were those from the gallocatechins and so the mass of material lying underneath these peaks (Fig. 9) might well be different in the real and model systems.

Thearubigins, obtained from a model system containing only 4–6 major catechins, are formed by four separate routes, which will now be discussed. Although the reaction mixture of macerated green tea shoots is considerably more complex, there is no contradictory evidence to suggest that similar reactions do not occur during black tea manufacture. The levels of each of the four fractions formed is dependent upon the reaction conditions [1], the respective concentrations of the catechins and polyphenol oxidase, and the point in time at which the fermentation is terminated. During the early stages of fermentation, regardless of how favourable the conditions are for theaflavin formation, redox equilibration between the simple catechins and gallocatechins produces an excess of gallocatechin quinones which react to form thearubigin components [8]. The amount of thearubigin formed by this reaction is dependent upon oxygen tension which, if particularly low, will facilitate preferential oxidation of accumulated theaflavin intermediates, a second major source of thearubigins [8]. Table 1 shows that the relative amounts of simple catechins found in thearubigins after the fermentation of two catechin mixtures, under conditions optimized for theaflavin production, can be as high as 50%. Although, in the absence of gallocatechins and theaflavin intermediates, the simple catechins will react together to form the third source of thearubigins, it is anticipated that little of the 50% found in thearubigin during a normal fermentation will be derived from this reaction. In high oxygen fermentations, or after the exhaustion of gallocatechins, the contribution of simple catechins to the thearubigin fraction might be more significant. The fourth source of thearubigins and perhaps responsible for the larger MW components as well as the production of gallic acid during fermentation, lies in the oxidation of theaflavin [1]. Although the results of a previous paper suggest that this process is auto-oxidative, requiring molecular oxygen, the role of a second oxidative enzyme cannot be ruled out.

Due to the complexity of the thearubigins and to the lack of suitable separation techniques, no direct data on their structure or on their contribution to black tea quality, is available. Although high levels of theaflavin are

considered to be necessary for quality teas [6], there is little doubt that a tea containing low thearubigin is considered 'thin' or bodiless. Since the formation of theaflavin and thearubigin is competitive, an increase in the former by the methods outlined in this and previous papers [1, 8] will necessarily reduce the levels of the latter. The need for further information on the thearubigins in relation to tea quality is, therefore, apparent but structural data is a pre-requisite.

The results presented here and in a previous paper [1] demonstrate that the production of theaflavin, during fermentation, is far from being the predominant process. Under natural growing temperatures, taking into account the physiological pH of the leaf cell, the relatively low concentration of oxygen in air and the poor diffusion of oxygen into the aqueous phase, the conditions are near optimal for thearubigin formation. In addition, the concentrations of three of the major catechins, epigallocatechin, epicatechin gallate and epicatechin, in green tea shoots are suboptimal for theaflavin formation and the fourth, epigallocatechin gallate, is supraoptimal to the point of inhibiting the whole process. The growing conditions in Malawi do not encourage better catechin ratios [6]. Catechin compositions, approaching the optimum presented here, are best obtained under slow-growing conditions and the higher quality Malawi teas are, therefore, produced during the cold dry months between May and November when yields are low. Table 2 shows the changes in the catechin composition of fast- and slow-growing tea bushes of the clone SFS 204 and it can be seen that, during the fast growing season, already suboptimal levels of epigallocatechin, epicatechin gallate and epicatechin decrease further, while epigallocatechin gallate increases considerably. The catechin composition of the slow-growing tea is considerably closer to that of the optimum.

These results essentially explain the success of the Kenyan tea industry where, due to their climatic conditions and altitude, tea grows slowly throughout the year so that total annual yields are comparable to those of Malawi, but quality is considerably higher. Further improvement of black tea quality must also be due to the naturally lower fermentation temperature, which as demonstrated [1] will increase theaflavin–thearubigin ratios.

Attempts to understand tea quality in terms of the catechin composition of the green leaf have, in the past, been confusing. Possibly much of this confusion has been the result of relating liquor characteristics to the composition of leaf which has been fermented under conditions that vary considerably and are far from optimum. High theaflavin levels have been corelated with a high epigallocatechin–epigallocatechin gallate ratio [6] and, although this is often the case, anomalies have arisen which cannot be explained by this theory. One such anomaly is the decrease in epigallocatechin gallate levels and increase in those of epigallocatechin as the leaf ages, and yet the highest quality liquors are attributed to the younger leaves [2]. The simultaneous reduction of epicatechin gallate and often that of epicatechin also with leaf age has, however, been overlooked and in the context of the results presented here it becomes apparent that the relevant change is not in the ratios of the individual gallocatechins to one another, but in the ratio of total simple catechins to gallocatechins.

EXPERIMENTAL

Model fermentation system expts were carried out as described previously [8] under pure O_2, at 20°, and pH 5.0 and the reactions were initiated by the addition of 30×10^{-8} kat of polyphenol oxidase. 1 kat is defined as the amount of enzyme to convert 1 mol of substrate per sec. These conditions were optimum for theaflavin formation [1]. The purification procedures for both polyphenol oxidase and the catechins were described by Robertson and Bendall [8]. Epicatechin was also purchased from Sigma. HPLC analysis of theaflavin and thearubigin has also been described by Robertson and Bendall [8]. Analysis of catechins by HPLC was described by Robertson [1].

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