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EFFECTS OF PHYSICAL AND CHEMICAL CONDITIONS ON THE *IN VITRO* OXIDATION OF TEA LEAF CATECHINS

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

Abstract—The use of a model fermentation system containing purified green tea shoot catechins and partially purified polyphenol oxidase (EC 1.14.18.1) has provided important data on theaflavin and thearubigin formation. Low oxygen concentration during fermentation, as a result of inadequate aeration or high enzyme concentration, and enhanced by high temperatures, inhibits theaflavin and promotes thearubigin production. Analysis of catechin oxidation under low oxygen tension suggests that the quinones of epicatechin and epicatechin gallate, by virtue of their low redox potentials, are acting as electron carriers in the preferential oxidation of epigallocatechin, epigallocatechin gallate and theaflavin intermediates. Temperature changes between 20° and 30° had little effect on theaflavin values, whereas thearubigin production increased dramatically. Thearubigin–theaflavin ratios over this temperature range increased in oxygen from 1.5:1 to 2.5:1. The pH optima for theaflavin and thearubigin formation were 5 and 6, respectively. The optimization of this factor, together with oxygen concentration, temperature and enzyme concentration, for theaflavin formation, resulted in theaflavin levels 115% higher than those obtained from similar model fermentations occurring under the conditions associated with black tea manufacture in Malawi (pH 5.6; 30×10^{-8} kat of polyphenol oxidase; 27°; 20% oxygen).

INTRODUCTION

Much of the chemical research into tea to the present time has been concerned with identification of the polphenolic components of black tea. Model system *in vitro* studies [1–4] have, in many cases, used simple catechin pairs to synthesize these components and gain further understanding of their chemistry. Few workers have used the model system to assess the reaction conditions under which particular components, beneficial to black tea quality, are best formed.

The importance of the theaflavin in determining tea quality was first demonstrated by Bradfield and Penney [5]. More recently Hilton and Ellis [6] obtained good correlations between theaflavin concentration and the price realized at auction. A knowledge of the conditions of fermentation which produce the highest concentrations of theaflavin is, therefore, of distinct advantage to the tea industry, but the use of macerated whole shoot systems has produced too many fluctuating variables and results have been conflicting.

However, empirical studies have shown that low temperatures are often effective in producing brighter, although perhaps thinner, teas [7]. pH modification, often utilized in instant tea manufacture, has been shown to increase theaflavin content during fermentation [8]. Experimental data from gaseous exchange studies is practically not available due to the difficulties experienced by researchers using macerated shoot systems.

This paper presents data obtained by use of the model system, already described [9], on the effects of physical and chemical conditions on the fermentation products and optimization of these for the production of theaflavin.

RESULTS

The outstanding feature of fermentation occurring under air (Fig. 1A) was the immediate rapid increase in thearubigin. This continued approximately linearly for 30 min and, although the subsequent rate of formation decreased, thearubigin levels continued to increase for a further 30 min. After an initial lag in the production of the theaflavins of *ca* 10 min, levels increased to a maximum at 30–40 min and subsequently decreased. The continual measurement of gaseous exchange throughout fermentation demonstrated that the lag phase in theaflavin coincided with extremely low oxygen concentrations of less than 1% in solution. As oxygen tension began to rise after 10 min, theaflavin formation was also observed. Both oxygen and theaflavin levels continued to increase simultaneously until the oxygen tension of the solution had returned to its initial air value. This was the point in subsequent experiments, where maximum theaflavin yields were being measured, at which the fermentation was terminated (see Experimental). Although catechins were never fully oxidized, subsequent fermentation resulted in theaflavin breakdown and a continued, although slower, increase in thearubigin. It should be pointed out here that, since the extinction coefficients of the thearubigins are unknown and may vary widely, absolute values for thearubigin levels cannot be calculated. It is consid-

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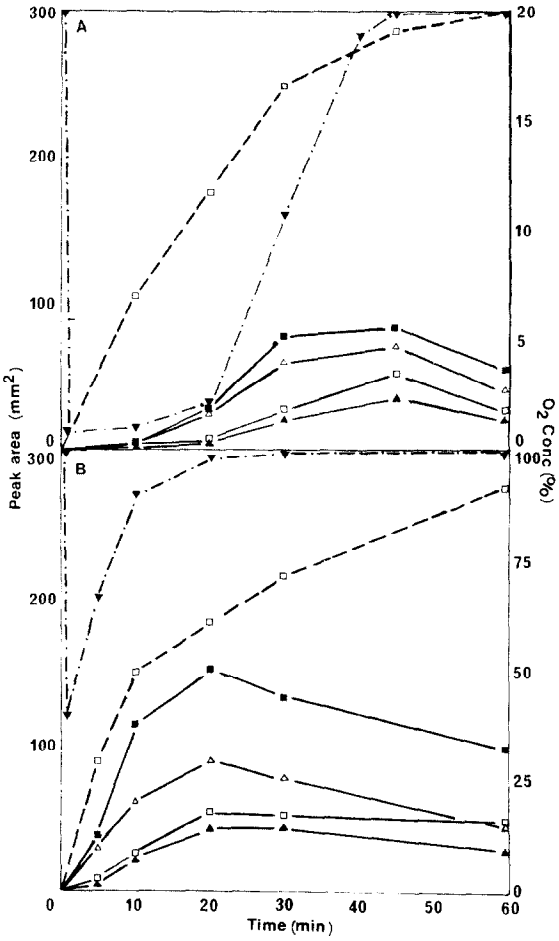

Fig. 1. Time course of a theaflavin and total thearubigin production from model fermentations occurring under air (A) and oxygen (B). Fermentation conditions: standard catechin mixture, pH 5.6, 25°, enzyme activity 30×10^{-8} kat. (□—□) Theaflavin; (▲—▲) theaflavin-3′-monogallate; (△—△) theaflavin-3-monogallate; (■—■) theaflavin-3,3′-digallate; (□- - -□) thearubigin; (▼-·-·▼) % oxygen concentration.

ered valid, however, to compare the total thearubigin levels with those of the theaflavins, due to the large number and variety of compounds present in the thearubigin fraction.

A similar model fermentation occurring under oxygen produced considerably different results (Fig. 1B). The lag phase in theaflavin formation was all but eliminated and after 5 min total theaflavin levels were apparently in excess of those of thearubigin. In addition, the rate of reaction was accelerated producing maximum theaflavin levels and a return of oxygen tension to its initial value after only 20 min. Comparison of oxygen and air fermentations shows that, after the completion of fermentation in both cases, total theaflavin and thearubigin levels were *ca* 50% higher and 40% lower, respectively. Once again, after completion of the enzymic reaction, thearubigin continued to increase as theaflavin levels decreased, suggesting that theaflavin degradation was either the result of a second enzymic reaction or of autoxidation. Since peroxidase has been shown to oxidize theaflavin [10], it was an obvious candidate in this case. In Table 1, fermentations 2–5 were continued for a further 60 min under the conditions shown. The results demonstrate that neither the addition of catalase nor of hydrogen peroxide affects theaflavin breakdown, which is already negligible under nitrogen, when compared with the control. However, degradation of theaflavin under oxygen is considerable, indicating that either polyphenol oxidase, the presence of a second oxidative enzyme, or autoxidation, possibly aided by epicatechin as an electron carrier, is responsible.

The detrimental effects of low oxygen tension on theaflavin formation could be enhanced by increases in enzyme concentration. Figure 2 shows the effects of increasing enzyme concentration on theaflavin and thearubigin production in fermentations under different oxygen regimes. Fermentations carried out under 25% oxygen (Fig. 2A) became oxygen-limited with enzyme concentrations in excess of 15×10^{-8} kat. Up to this level, however, additional enzyme produced increases in theaflavin and thearubigin, although the ratio between the two was low. Further increases in activity to 30×10^{-8} kat caused low oxygen tension and, consequently, thearubigin increased at the expense of theaflavin. With the addition of 45×10^{-8} kat of enzyme, extremely low oxygen conditions ensued and both theaflavin and thearubigin levels decreased.

Increasing the oxygen concentration further (Figs. 2B–D) provided more favourable conditions for theaflavin formation and, hence, fermentations under pure oxygen produced the highest theaflavin–thearubigin ratios. In general, maximum theaflavin levels at any oxygen concentration were obtained by the addition of the highest enzyme activity possible without creating the very low oxygen levels which produce theaflavin inhibition. Consequently, in the model system at an oxygen flow rate of 1 l/hr, a maximum polyphenol oxidase activity of just in excess of 45×10^{-8} kat produced the

Table 1. Effect of various factors on degradation of the theaflavins

Treatment	Peak area ($A_{375\,nm}$)				
	Thearubigin	Theaflavin	Theaflavin-3′-monogallate	Theaflavin-3-monogallate	Theaflavin-3,3′-digallate
Control	250	135	60	180	94
O_2	330	118	59	128	61
N_2	266	135	56	173	90
N_2 + catalase	255	134	54	169	83
$N_2 + H_2O_2$	250	139	57	176	95

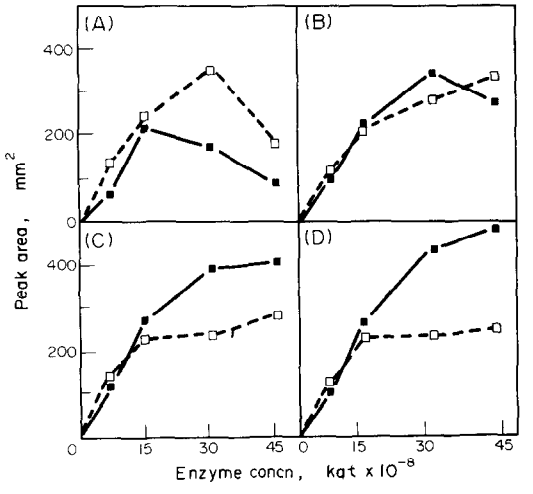

Fig. 2. Effect of increasing enzyme concentration on the formation of total theaflavin (■—■) and total thearubigins (□---□) in model fermentations occurring under various oxygen regimes. (A) 25% O_2; (B) 50% O_2; (C) 75% O_2; (D) 100% O_2. Fermentation conditions: standard catechin mixture, pH 5.6, 25°.

highest theaflavin levels (Fig. 2D). However, it must be stressed that the gas flow was set at the maximum for the reaction chamber dimensions, without creating undue frothing. The gas flow which will maintain more efficient oxygen dissolution within a larger volume of reaction mixture may be considerably greater and, hence, gas mixtures of lower oxygen concentration, but higher flow rates, may produce similar conditions to those occurring with pure oxygen in this system.

Figure 3 shows the time course of theaflavin and thearubigin production under extremely low oxygen concentrations, facilitated by the addition of high enzyme levels (45×10^{-8} kat). Once again, thearubigin formation was observed from the commencement of fermentation, whereas that of theaflavin was not apparent until after 15–20 min, at a point coincidental with increases in oxygen tension. Total theaflavin levels under these conditions were considerably lower than those obtained from a similar fermentation containing *ca* 33% of the enzyme activity (Fig. 1A). This indicates that under low oxygen tension the catechins are in some way diverted to thearubigin formation and are not available for theaflavin production when oxygen is no longer limiting.

The effects of low and high oxygen tensions on catechin oxidation are shown in Figs. 4(A) and 4(B). So that the effect could be seen more clearly, particularly changes occurring in the simple catechins, equal concentrations (55 mM) of all the catechins were used for this experiment. However, similar changes occurred in a standard catechin mixture and, consequently, comparisons between catechin oxidation and theaflavin and thearubigin formation, in the text that follows, are made using the data in Figs. 1 and 4. During the period of low oxygen tension (> 2%) and

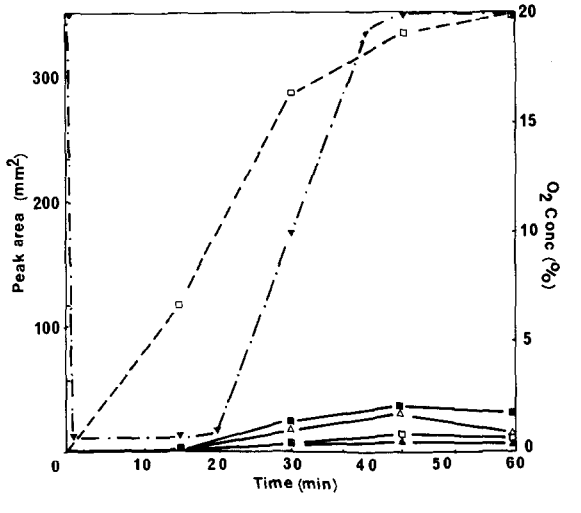

Fig. 3. Time course of theaflavin and total thearubigin production in a model fermentation occurring under low oxygen tension. Fermentation conditions: standard catechin mixture, air, pH 5.6, 25°, enzyme activity 45×10^{-8} kat. (□—□) Theaflavin; (▲—▲) theaflavin-3'-monogallate; (△—△) theaflavin-3-monogallate; (■—■) theaflavin-3,3'-digallate; (□---□) thearubigin; (▼.-.▼) % oxygen concentration.

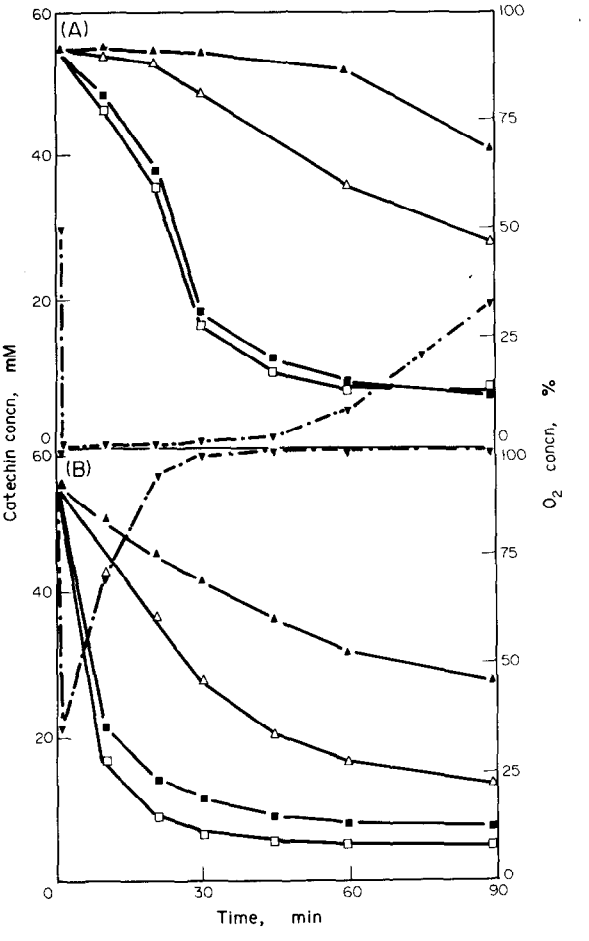

Fig. 4. Time course of catechin consumption in model fermentations occurring in air (A) and oxygen (B). Fermentation conditions: equal individual catechin concentrations (55 mM), pH 5.6, 25°, enzyme activity, 45×10^{-8} kat. (▲—▲) Epicatechin; (△—△) epicatechin gallate; (□—□) epigallocatechin; (■—■) epigallocatechin gallate; (▼·—·▼) % oxygen concentration.

concomitant theaflavin inhibition, no net consumption of the simple catechins was observed. The relatively rapid oxidation of the gallocatechins, therefore, resulted only in the production of thearubigin [9].

After 20 min, oxygen tension and simple catechin oxidation increased simultaneously and theaflavin formation was observed. The more rapid oxidation of epicatechin gallate relative to epicatechin was reflected by the higher concentrations of theaflavin monogallate and theaflavin digallate after fermentation. The presence of oxygen (26%) during fermentation resulted in a more even oxidation of all the catechins from the onset of fermentation and as seen in Fig. 1(B) produced a rapid almost linear increase in the theaflavins.

Figure 5 shows the effects of temperature on total theaflavin and thearubigin levels during both oxygen and air fermentations. Fermentations carried out in oxygen exhibited theaflavin and thearubigin maxima at 30°, whereas in air the theaflavin maximum was at 20°. This latter difference was attributed to unfavourable low reaction medium oxygen tensions for theaflavin formation as a result of increased enzyme activity at higher temperatures. In both air and oxygen fermentations thearubigin formation was clearly more affected by temperature than was theaflavin. A 10° temperature change either side of the theaflavin maximum producted little difference in theaflavin levels, whereas similar changes in temperature at the thearubigin peak either more than halved or doubled the levels. It must be borne in mind, that the fermentations were terminated at the point of maximum theaflavin levels and not when all the catechins had been consumed. Consequently, if the reaction had been allowed to continue to completion with the reduced rates caused by low temperature, considerably higher levels of thearubigins would have been achieved.

The effects of pH modification on total theaflavin and thearubigin and individual theaflavin levels, during air and oxygen fermentations, are shown in Fig. 6. The pH of each reaction mixture was set by dissolving the individual

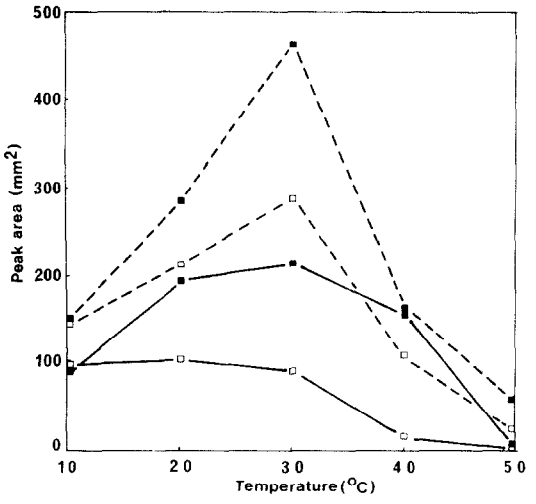

Fig. 5. Effect of temperature on the formation of total theaflavin (———) and total thearubigin (– – – –) in fermentations occurring under air (□) and oxgen (■). Fermentation conditions: standard catechin mixture, pH 5.6, enzyme activity 30×10^{-8} kat.

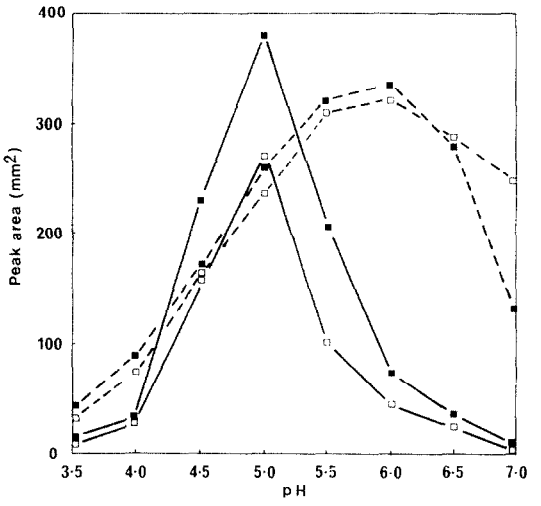

Fig. 6. Effect of pH on the formation of total theaflavin (———) and total thearubigin (– – – –) in fermentations occurring under air (□) and oxygen (■). Fermentation conditions: standard catechin mixture, 20°, enzyme activity 30×10^{-8} kat.

catechins in 0.1 M citrate or citrate–phosphate buffer at the required pH. The formation of theaflavin and of thearubigin can be seen to have separate and distinct pH optima. Theaflavin levels increased above pH 4.0, formed a sharp peak at pH 5.0 and decreased to low levels again at pH 6.0. In contrast, thearubigin formation was less affected by pH and increased at a slower rate from pH 3.5 to form a broad optimum, particularly in air, between pH 5.5 and 6.5.

Time course experiments, carried out at the extreme pHs for theaflavin formation, pH 5 and 6, directly compare the oxidation of the catechins with product formation. At pH 5.0 (Fig. 7B), oxidation of the simple catechins was similar to that occurring in previous fermentations at pH 5.6, whereas oxidation of the gallocatechins was decreased. This resulted in a more favourable ratio of simple catechin quinones–gallocatechin quinones for theaflavin production over a longer fermentation period (Fig. 7A). Breakdown of theaflavin, due to oxidation, was greater over this extended fermentation period and, therefore, maximum theaflavin did not coincide with the point at which substrates were exhausted.

At pH 6.0, although the oxidation rate of the simple catechins was relatively unaffected (Fig. 8B), the gallocatechins were oxidized at a considerably faster rate than at pH 5.0. Thearubigin levels (Fig. 8A), therefore, increased rapidly to a point in time which coincided with the almost total exhaustion of the gallocatechins. As a result of the rapid conversion of the latter to thearubigins, theaflavin production was low.

DISCUSSION

The formation of black tea polyphenols during fermentation may be considered to be the combined result of two reactions. The initial enzymic reaction, catalysed by polyphenol oxidase, is responsible for the oxidation of the catechins to their respective *o*-quinones [11, 12]. In a subsequent reaction, the latter condense to form the

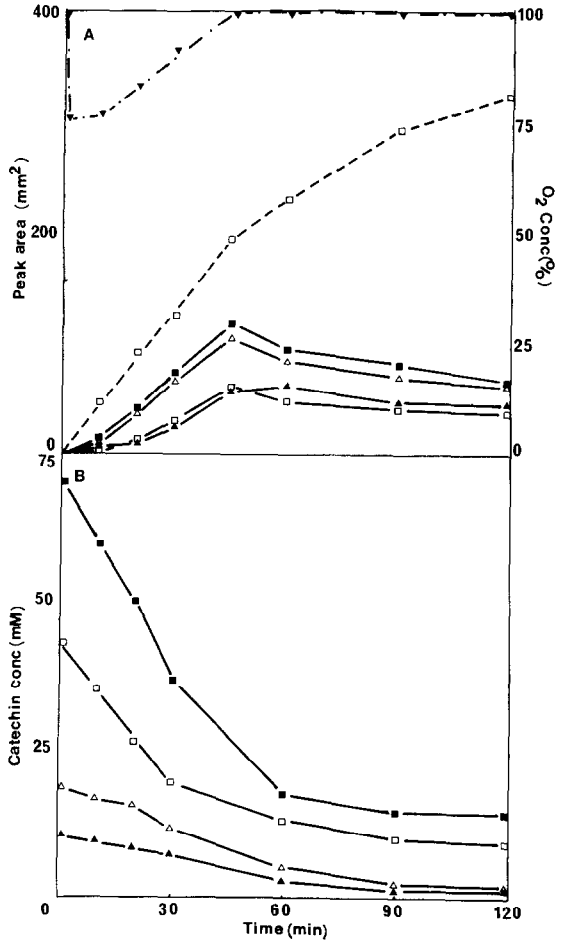

Fig. 7. Model fermentation time courses showing theaflavin and total thearubigin formation (A) and catechin consumption (B) at pH 5.0. Fermentation conditions: standard catechin mixture, pH 5.0, 100% oxygen, 20°, enzyme activity 30×10^{-8} kat. (A) (□—□) Theaflavin; (▲—▲) theaflavin-3'-monogallate; (△—△) theaflavin-3-monogallate; (■—■) theaflavin-3,3'-digallate; (□- - -□) total thearubigin; (▼-·-▼) % oxygen concentration. (B) (▲—▲) Epicatechin; (□—□) epigallocatechin; (△—△) epicatechin gallatc; (■—■) epigallocatechin gallate.

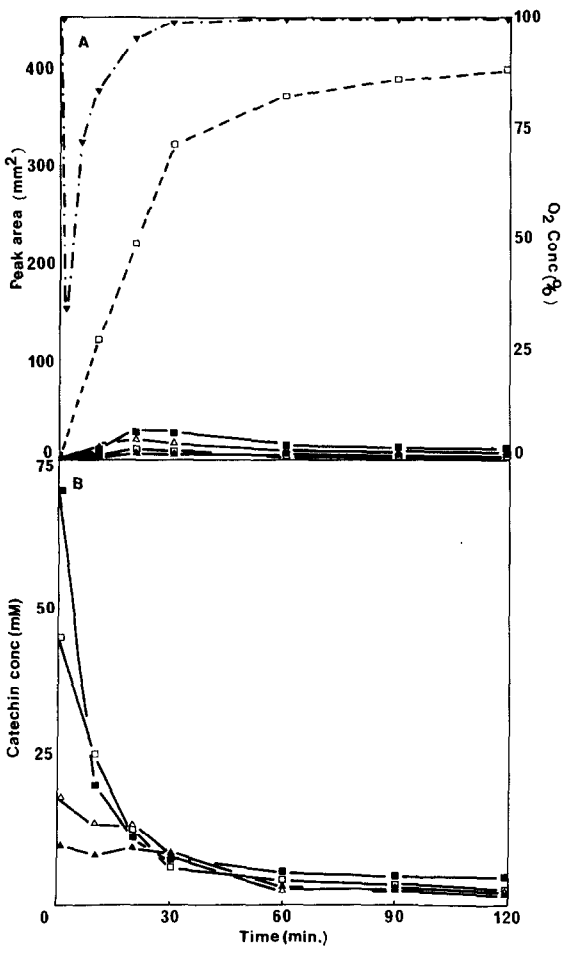

Fig. 8. Model fermentation time courses showing theaflavin and total thearubigin formation (A) and catechin consumption (B) at pH 6.0. Fermentation conditions: standard catechin mixture, pH 6.0, 100% oxygen, 20°, enzyme activity 30×10^{-8} kat. (A) (□—□) Theaflavin; (▲—▲) theaflavin-3'-monogallate; (△—△) theaflavin-3-monogallate; (■—■) theaflavin-3,3'-digallate; (□- - -□) total thearubigin; (▼- -▼) oxygen concentration. (B) (▲—▲) Epicatechin; (□—□) epigallocatechin; (△—△) epicatechin gallate; (■—■) epigallocatechin gallate.

theaflavins and, in addition, numerous chemically undefined reactions occur to produce a series of pigmented components grouped under the general name of thearubigins [13]. The formation of 1 mol of theaflavin requires the oxidation and condensation of 1 mol of a gallocatechin (epigallocatechin gallate or epigallocatechin) with 1 mol of a simple catechin (epicatechin or epicatechin gallate) [14] and, therefore, for the production of maximum theaflavins from any catechin mixture, equal concentrations of these two types of catechin are required. In practice this ideal is never realized since the ratio of simple catechins to gallocatechins, occurring naturally in green tea shoots, is low and is neither compensated for during oxidation by markedly different K_m values of the catechins [15] nor by the presence of substrate specific isoenzymes of polyphenol oxidase. Nevertheless, allowing for these factors, theaflavin levels, obtained after fermentation of green tea shoots or from a model system run under similar conditions, are considerably lower than those theoretically possible. A major reason for this has been attributed to the preferential oxidation of the gallocatechins, as a result of their lower redox values, by the simple catechins during redox equilibration [16]. The results presented in this study confirm this finding. Moreover, they show that the theaflavin and thearubigin levels can be manipulated by increasing the availability and perhaps the stability of the simple catechin quinones under various physical and chemical conditions.

Low oxygen tension during fermentation has been demonstrated to result in an almost total inhibition of theaflavin formation (Figs. 1A and 3). Since no appreciable net consumption of simple catechins occurs during this period (Fig. 4A), and yet it appears from this study that at the concentrations used there is little

difference in the specificity of polyphenol oxidase in its oxidation of catechins, it seems that the simple catechin quinones formed act as electron carriers is subsequent coupled oxidations, such as that of the gallocatechins. In a previous paper [9], it was shown that the gallocatechin quinones are able to react together to form thearubigin components. Consequently, the substantial increases in these products observed on the original HPLC traces (not shown) contribute considerably to the thearubigins formed at this stage of fermentation.

The regulatory mechanism of oxygen tension on theaflavin and thearubigin formation is far from clear, although its effect, as shown in Fig. 4(A), ultimately appears to be through limiting the availability of the simple catechin quinones. There are two possible areas during the formation of theaflavins where oxygen could facilitate such an action. If one considers the scheme in Fig. 9, using theaflavin production from epicatechin and epigallocatechin for example, it becomes apparent that the availability of epicatechin quinones, for either theaflavin or thearubigin formation, is dependent upon the relative rates of enzymic catechin oxidation (r_1) and redox equilibration between the catechins (r_3), where r_1 must be greater than r_3. Epigallocatechin oxidation is not important to the scheme, since its rate of oxidation (r_2), as a result of polyphenol oxidase activity and redox equilibration, produces a relatively high level of free quinones. Low oxygen concentration during fermentation could, therefore, inhibit theaflavin formation by either modifying enzyme activity and reducing r_1 or by accelerating the rate of redox equilibration. Since it is difficult to speculate on how low levels of molecular oxygen could affect a reaction involving electron transfer, such as that of redox equilibration, it seems necessary to suggest that low oxygen concentrations reduce the rates of formation and, therefore, the steady state concentrations of the epicatechin and epigallocatechin quinones. The magnitude of this effect is dependent upon the rate constants r_1 and r_2, but will be significant as a result of the relatively high K_m for oxygen of polyphenol oxidase [15]. The reduction in available epicatechin quinones is further magnified by the effect of the redox equilibration reaction. The results presented in Fig. 4(A) confirm that under extremely low oxygen concentrations, epicatechin and epicatechin gallate consumption was zero, epigallocatechin and epigallocatechin gallate were consumed at a slower rate than in the presence of oxygen (Fig. 4B) and theaflavin production was totally inhibited (Fig. 1A). Additional evidence that low oxygen conditions during fermentation reduce the rate of enzymic catechin oxidation is demonstrated by the similarity between low fermentations, occurring under low O_2 tension (Fig. 1A), and low enzyme activity, respectively [17].

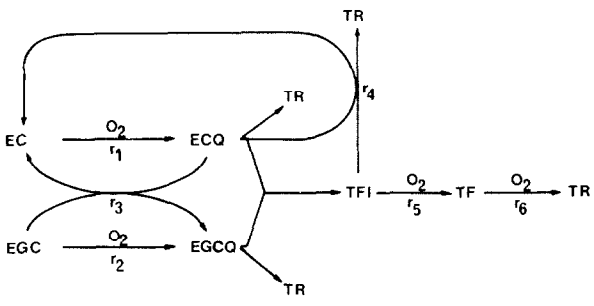

Fig. 9. Scheme showing the formation of theaflavin and thearubigin from the catechin and the possible role played by the simple catechins in coupled oxidations. EC, Epicatechin; EGC, epigallocatechin; ECQ, epicatechin quinones; EGCQ, epigallocatechin quinone; TFI, theaflavin intermediate; TF, theaflavin; TR, thearubigin.

A second mechanism, by which theaflavin levels might be affected during fermentation, again involves the simple catechins as electron carriers, but in the coupled oxidation of theaflavin intermediates to thearubigins (Fig. 9). Although this reaction will occur, in competition with theaflavin production, at higher oxygen concentration, the effect will most likely be enhanced when oxygen tension is low and benztropolone ring formation, also requiring molecular oxygen [18, 19], is inhibited. The reaction can only take place, however, when the steady state concentration of epicatechin quinone is in excess of that utilized in the preferential oxidation of epigallocatechin and, therefore, will not occur under the conditions shown in Fig. 4(A). High gaseous phase oxygen concentration, together with enzyme activities, high enough to effect conditions of low aqueous phase oxygen, should produce theaflavin intermediates which cannot undergo benztropolone ring formation in the absence of molecular oxygen but move to thearubigins by the alternative pathway.

The effect of enzyme activity on theaflavin production may be explained in terms of competition between the rate of simple catechin oxidation and that of the redox equilibration with the gallocatechins. As enzyme concentration is increased, the steady state concentration of the simple catechin quinones increases above the rate at which they are consumed during redox equilibration. The latter remains unaffected and, hence, simple catechins are available for condensation to theaflavin (Fig. 2). Eventually a point is reached, which is dependent upon the gas phase oxygen concentration, where the low oxygen concentration of the reaction medium reduces enzyme activity. Subsequent increases in enzyme level further reduce oxygen tension and, therefore, the steady state concentration of simple catechin quinones and, hence, of theaflavin production becomes totally inhibited. On two occasions during this study, when enzyme levels in the reaction mixture were very high (Fig. 2A), or when enzyme activity was enhanced by temperature (Fig. 3), thearubigin formation was also observed to decrease. Since the breakdown of theaflavin has been shown to be facilitated by molecular oxygen (Table 1) it is speculated that when the oxygen status of the reaction mixture is near to anaerobic, oxidative degradation of the theaflavin is also inhibited.

The effect of temperature on the products formed during fermentation (Fig. 5) is also due, in part, to varying the enzyme activity and the supply of oxygen. The lower solubility of oxygen in the aqueous phase and the considerable increases in enzyme activity with higher temperatures can reduce oxygen concentration to levels detrimental for theaflavin formation. Furthermore, the rates of redox equilibration are also increased with temperature and the combined result of all three effects is reflected in a considerably lower concentration of simple catechin quinones and theaflavin intermediates present for theaflavin formation.

Low temperature fermentation may, therefore, be considered advantageous to tea quality [7], not so much through direct increases in theaflavin, which remained

constant between 20° and 30°, but through its effect on thearubigin levels. The large changes in the latter with temperature, without any significant change in theaflavin levels, suggest that the beneficial increase in catechin oxidation for theaflavin formation is offset by an increase in the rates of coupled oxidation. If this is so, then methods of increasing the production of simple catechin quinones, whilst maintaining low temperatures, should increase theaflavin levels. This concept is of considerable practical significance to the tea industry since the breeding of tea bushes with high enzyme levels, specifically for low temperature fermentation, could be of advantage.

The large increases in theaflavin levels as a result of lowering the pH from 6 to 5 is shown to be due to a selective reduction in gallocatechin oxidation (Figs. 7 and 8). Once again the effect is facilitated through a consequent increase in the availability of simple catechin quinones for reaction with those of the gallocatechins. This result is consistent with the findings of Gregory and Bendall [15], who showed the pH optima for the activity of purified tea polyphenol oxidase with 4-methylcatechol and pyrogallol to be 5.0 and 5.7, respectively. The structure and behaviour of these compounds can be likened to those of the simple and gallocatechins, respectively.

The results presented here, and in a previous paper [9], provide important information on the formation of the thearubigin fraction. Much of the thearubigin produced during the early stages of fermentation is due to the preferential oxidation of epigallocatechin gallate and epigallocatechin and is particularly prevalent as the pH is increased from 5.0 to 6.0. Once formed, these products are stable and do not change significantly during continued fermentation. The presence of similar components in black tea infusions [9] is consistent with these findings and further demonstrates their stability, under the high firing temperatures, during the drying of black tea.

Occurring simultaneously with the formation of thearubigin from the gallocatechins is a second competitive reaction also requiring the electron carrying capacity of the simple catechin quinone to form, as discussed earlier, thearubigin from theaflavin intermediates. The importance of the thearubigin, formed by this route, to the eventual black tea product appears to be dependent upon the oxygen status of the reaction mixture. This may be considerable when aqueous phase oxygen tension is low but both gaseous phase oxygen and enzyme levels are high.

The simple catechins, epicatechin and epicatechin gallate, are also able to form pigmented products, other than theaflavins, when oxidized individually or together. During the early stages of fermentation their utilization in the competing reactions already mentioned, as well as in theaflavin formation, may result in little being available for reaction to thearubigin. However, as fermentation proceeds and gallocatechin concentration is reduced or exhausted, thearubigin resulting from simple catechins [9] becomes more significant.

Oxidative degradation of the theaflavins becomes apparent during fermentation after the gallocatechins have been utilized. Since the largest decreases in theaflavin occur in the mono- and digalloylated species, it is possible that these are the source of gallic acid [19] which increases throughout fermentation. The role of peroxidase [10] in theaflavin degradation has been eliminated, since this is not accelerated by addition of further peroxidase or hydrogen peroxide, nor is it reduced by the addition of catalase. However, the degradation increases under oxygen, decreases under nitrogen and is, therefore, either auto-oxidative or the result of a second oxidative enzyme. Although theaflavin breakdown is not observed initially, due to its high rate of formation, it is probable that degradation occurs from the commencement of fermentation. Since both theaflavin formation and breakdown are stimulated by molecular oxygen, it follows that fermentations should be carried out as quickly as possible and, hence, high enzyme activities, yet not so high as to produce detrimentally low oxygen levels, are necessary for maximum theaflavin yield (Fig. 2D).

The validity of the *in vitro* system in predicting the chemistry of a more complex macerated whole shoot system has already been demonstrated through the quantitative similarities between the time courses of fermentation, the products formed and the levels of enzyme deactivation during fermentation in the two systems. In addition, some of the effects of changing fermentation conditions presented in this paper have also been observed empirically during black tea manufacture. Reducing pH as a method of increasing theaflavin levels, during slurry fermentation for instant tea, has already been patented [8]. The beneficial effects of low temperature during black tea manufacture have also been reported [7].

The results of the model system demonstrate the large increases in theaflavin levels theoretically possible in a homogenous reaction mixture in which conditions are optimized and maintained throughout fermentation. Similar theaflavin increases may not be obtained from macerated tea shoots until the problems of the physical barriers imposed by the tissues [9] are overcome by improved manufacturing techniques.

EXPERIMENTAL

Model system fermentations, product analysis and preparative methods used in this study were carried out according to the methods of ref. [9]. Polyphenol oxidase activity was measured by the method of ref. [15] and is defined in kats. 1 kat is the amount of enzyme required to catalyse 1 mol substrate/sec.

Catechin analysis by HPLC. Based on the method of ref. [20]. Samples (1–5 μl) from Me_2CO extracted green leaves or the model system treated as previously described [9] were injected directly onto a HPLC column (20 × 0.4 cm), packed as described in ref. [9] with Hypersill 5 μm particle ODS. The column was pre-equilibrated with a mobile phase, HCO–NMe_2–MeOH–HOAc–H_2O (18:1.0:0.5:81) at 1 ml/min, run at a flow rate of 2 ml/min, and the eluate was monitored at 280 nm, 0.05 a.u.f.s. The first sample of a series was repeated until a reproducible trace was obtained.

Termination of model fermentations. The points comprising all the figures in this paper were the mean results of individual fermentations carried out in duplicate rather than the analysis of aliquots removed from one reaction mixture. In all expts, other than those involving time courses, the reactions were terminated at maximum theaflavin concn, which was determined by assessment of the oxygen tension throughout fermentation. Initiation of the reaction, by the addition of polyphenol oxidase, results in oxygen consumption during which theaflavin and thearubigin fractions are produced. The subsequent increase and return of oxygen tension to a steady state, close to the starting oxygen concn, reflects the point at which either all the catechins are consumed or, alternatively, just the gallocatechins after preferen-

tial oxidation. In the latter case, the oxygen consumption due to the oxidation of the remaining simple catechins is very low and, therefore, is easily recognizable. In both circumstances theaflavin levels are at their maximum and further fermentation results only in theaflavin degradation.

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REFERENCES

1. Roberts, E. A. H. and Myers, M. (1959) *J. Sci. Food Agric.* **10**, 167.
2. Nakagawa, M. and Torii, H. (1965) *Agric. Biol. Chem.* **29**, 278.
3. Hilton, P. J. (1972) *Phytochemistry* **11**, 1243.
4. Sanderson, G. W., Berkowitz, J. E., Co. H. and Graham, H. N. (1972) *J. Food Sci.* **37**, 399.
5. Bradfield, E. A. and Penney, M. (1944) *J. Soc. Chem. Ind., London* **43**, 306.
6. Hilton, P. J. and Ellis, R. T. (1972) *J. Sci. Food Agric.* **23**, 227.
7. Cloughley, J. B. (1980) *J. Sci. Food Agric.* **31**, 911.
8. Nestle Patent Specification (1966) 1034670.
9. Robertson, A. and Bendall, D. S. (1983) *Phytochemistry* **22**, 883.
10. Stagg, G. W. (1974) *J. Sci. Food Agric.* **25**, 1015.
11. Roberts, E. A. H. (1940) *Biochem. J.* **34**, 500.
12. Roberts, E. A. H. (1952) *J. Sci. Food Agric.* **5**, 193.
13. Roberts, E. A. H. (1958) *J. Sci. Food Agric.* **9**, 212.
14. Brown, A. G., Fulshaw, C. P., Haslam, E., Holmes, A. and Ollis, W. D. (1966) *Tetrahedron Letters* **11**, 1193.
15. Gregory, R. P. F. and Bendall, D. S. (1966) *Biochem. J.* **101**, 569.
16. Roberts, E. A. H. and Myers, M. (1960) *J. Sci. Food Agric.* **11**, 158.
17. Robertson, A. (1983) *Phytochemistry* **22**, 897.
18. Horner, L. Von and Durkheimer, W. (1959) *Z. Naturforsch.* **14**, 741.
19. Roberts, E. A. H. and Wood, D. J. (1951) *Biochem. J.* **49**, 414.
20. Hoeffler, A. C. and Coggan, P. (1976) *J. Chromatogr.* **129**, 460.