Studies on the 'Fermentation' of Ceylon Tea

6. THE NATURE OF THE TEA-OXIDASE SYSTEM

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Recent investigations on tea fermentation, both in Ceylon and Assam, have led to distinct differences of opinion regarding the nature of the oxidase system that is responsible for fermentation. After modifying his earlier views on this question at least twice, Roberts [1939; 1940] has now identified the tea-oxidizing enzyme with cytochrome oxidase. In previous studies from these laboratories [Lamb & Sreerangachar, 1940], not only have objections to the cytochrome theory of tea fermentation been presented, but also definite evidence for the view that tea oxidase is a polyphenol oxidase with an established specificity for *o*-dihydric phenols.

Despite this, the considerable body of indirect evidence that Roberts adduced in favour of his theory made it appear for the moment a reasonable assumption, but further work has shown that alternative explanations could be found for most of his material observations. Perhaps the most serious objection to the cytochrome theory of tea fermentation is that so far there is no direct evidence for the presence in tea leaf of cytochrome and cytochrome oxidase, which seems quite essential for the acceptance of the theory. The present investigation was, therefore, undertaken with a view to obtaining some definite information which would serve either to establish or confute the presence of these components in tea.

Spectroscopically, cytochrome is easily detected in its reduced state by its characteristic absorption spectrum. Since chlorophyll may interfere in the observations on the leaf it may be extracted by solvents such as acetone. Any possible inactivation of the oxidase in the process does not affect either the content or the character of cytochrome. Following this procedure, Yakushiji [1935] has reported seeing the α -bands of cytochrome *b* and *c* in acetone-extracted spinach leaves. Choosing the basal portion of the stem, probably with a view to obviating the chlorophyll interference, Roberts [1941], with Miss K. Bhagvat, claims to have demonstrated the presence of cytochrome in the tea plant. They appear to have observed an absorption band corresponding to the '*b*' band of cytochrome in the green region of the spectrum. It is not clear whether it is the component *b* or the spectral band β that they have observed, and it is noteworthy that other bands of either cytochrome *b* or *c* (it cannot

be component *a* as its β band is unknown) are not observed.

Spectroscopy can also be used in detecting the presence of cytochrome oxidase. On oxidation by the enzyme the sharp absorption bands of reduced cytochrome give place to the diffuse bands of oxidized cytochrome which are hardly perceptible in the micro-spectrophotometer. Disappearance of reduced cytochrome bands would therefore indicate the action of the cytochrome oxidase.

Before proceeding to describe the experimental evidence in the present investigation, a brief reference may be made to those points of dissension between workers here and in Assam not previously discussed by us.

Methods of investigation. In earlier work we have relied mostly on the titrimetric methods in the estimation of the tea-oxidase activity, and we have recognized the limitations of the method. Roberts [1941] has criticized this method on the ground that the iodine titre is affected by condensation of *o*-quinones produced, but has admitted that these errors are likely to be very small.

However, an alternative method of estimating enzyme activity, using ascorbic acid as substrate as described in Part 4 of this series, has yielded identical results as regards the optimum pH and temperature of the enzyme reaction. Therefore condensation errors in the iodine titration method could only be very small, and in a comparative experiment the conclusions are practically unaffected by these errors. It is interesting to note in this connexion that Roberts [1941] has himself obtained results of optimum pH (5.4) and temperature (29°) which are very close to those found by us. Such correspondence detracts very considerably from the relevance of his criticisms of the accuracy of our method. From the point of view of our present contention about the specific nature of the oxidase, the quantitative accuracy of the method is relatively less important. It is sufficient that the direct oxidase activity of the enzyme on polyphenols has been demonstrated beyond doubt.

The study of simpler component reactions affords an easy approach to the understanding of the most complicated biological processes *in vivo*. Preparation of purified enzyme and a study of its reaction *in vitro* on pure chemical substances is therefore a fruitful line of investigation [e.g. Hussein & Cruess, 1940], and we have always followed this course in our study of the mechanism of tea fermentation and the substrate specificity of the enzyme.

Substrate specificity. The importance of this property of the enzyme cannot be over-emphasized. Roberts's summing up [1941] of evidence on this point unfortunately lacks clarity. We admit that substrates like *p*-phenylene-

diamine and ascorbic acid, when added to fermented tea-leaf suspensions, are doubtless oxidized. But is it due to the existence of a cytochrome system? Is it not possible that the very reactive *o*-quinone reacts with these added substrates while it is itself being reduced to polyphenol? The enzymic nature of stimulation of O_2 uptake would thus be maintained and tea polyphenol would act as a carrier in the reaction. Thus the oxidations of ascorbic acid and *p*-phenylene-diamine would be only secondary reactions, and the fact that polyphenol-free enzyme preparations fail to oxidize directly these substances as well as quinol lends further support to this view.

Our enzyme preparations display a clear-cut specificity for *o*-dihydric phenols, and such a marked specificity is incompatible with the presence of cytochrome + cytochrome oxidase. However crude our enzyme preparation may be, it must be regarded as relatively pure when compared with tea-leaf mince and, unlike the latter, its reaction on substrates is free from the interference of tea polyphenols. To that extent, our results on substrate specificity of the oxidase system are more reliable than those of Assam workers.

Effect of organic solvents. Acetone inhibition seems to be characteristic of cytochrome oxidase, and so Roberts has inferred that our acetone-prepared enzyme would have undergone a substantial loss in activity. Nevertheless, we have obtained strong activity of this acetone-prepared enzyme on catechol and other *o*-dihydric phenols. The assumption that tea leaf originally contained cytochrome oxidase and that it was considerably inactivated during acetone treatment would be consistent with the failure of the enzyme to oxidize quinol and *p*-phenylene-diamine. But how are we to explain the strong activity on catechol unless, of course, it is granted that the leaf originally contained polyphenol oxidase also?

It is likely that besides cytochrome oxidase other plant oxidases are also inhibited to a different degree by acetone. For instance, Srinivasan [1936] has shown that ascorbic acid oxidase is inhibited by acetone. Although we have found the greater part of the original enzyme activity in our preparations, it is possible that they may not have retained the same polyphenol-oxidizing activity as in fresh leaf.

Oxygen tension. Further indirect evidence for the cytochrome theory consists in Roberts's observation that the initial rate of O_2 uptake by fermenting tea leaf is markedly accelerated by an increase in the O_2 tension. But it is doubtful whether this is an established property of cytochrome oxidase. Green [1940] states that 'the rate of oxidation of the ferrocytochrome by molecular oxygen in presence of the enzyme is practically independent of the partial pressure of O_2 '. In fact, since the respiration of yeast and other micro-organisms is found to be independent of O_2 tension, this is taken as evidence for the functioning of cytochrome oxidase in aerobic systems.

Evidence for the cytochrome theory has also been inadequate on other points. For instance, the failure to depress the rate of O_2 uptake when catechol is added to fermenting tea leaf cannot be considered as proof for the carrier nature of the tea oxidase, as is shown in Part 5 of this series. The existence of both soluble and insoluble enzymes in tea makes any evidence based on solubility inconclusive. If the enzyme is considered too insoluble to be polyphenol oxidase, it is also too soluble to be cytochrome oxidase.

EXPERIMENTAL

Spectroscopic investigations

Presence of cytochrome in tea. Employing a micro-spectrophotometer we made observations on the finely ground tissues in presence of both sodium succinate and $Na_2S_2O_4$. Contrary to Roberts's results, examination of the basal portion of the stem in tea plant did not give any positive indications for the presence of cytochrome. Similarly, other tissues of the plant, when separately collected and tested, failed to display any characteristic absorption bands which could be identified with those of cytochrome.

No cytochrome could be detected in the acetone-extracted tea leaf from which the possible interfering substances like chlorophyll and tannin were removed by solvent extraction. Attempts to prepare cytochrome *c* from leaf by the method of Keilin & Hartree [1937] yielded negative results. Both Ceylon and Indian tea leaves were used in this investigation.

From certain considerations it seemed reasonable to expect the presence of cytochrome *c* in our enzyme preparations. First, Yakushiji's [1935] findings made the presence of cytochrome probable in the acetone-extracted tea leaf, from which our oxidase preparations were obtained. Secondly, the ammonium sulphate saturation employed in the preparation of cytochrome *c* from ox heart [Theorell, 1936] was also the method used for the purification of the soluble enzyme, as will be described later. Furthermore, since Roberts attributed the oxidizing activity of the enzyme preparations to the cytochrome system, it was reasonable to expect the presence of cytochrome *c* in highly active enzyme concentrates. But it was found that none of these preparations, when spectroscopically examined, displayed any specific absorption bands. Figs. 1 and 2 give the absorption spectra of a highly active tea oxidase solution. The results in the visible region of light (Fig. 2) were obtained in a Gaertner's polarizing spectrophotometer, while for a study of absorption in the ultra-violet region (Fig. 1) a Hilger's instrument was employed. 10 mm. cells were used in both cases.

It will be seen that there is no selective absorption either in the visible region or in the ultra-violet region studied, and therefore the preparation does not contain cytochrome. The component cytochrome a_3 , which Keilin & Hartree [1939] identify with cytochrome oxidase, has a characteristic absorption (430 and 590 $m\mu$), and most of the other haematin have a strong absorption in the extreme violet between 400 and 420 $m\mu$ [Dixon, Hill & Keilin, 1931]. But the spectrum of the tea-oxidase solution cannot be related to any of these.

Detection of cytochrome oxidase. The detection of cytochrome oxidase was based on its *in vitro* reaction on cytochrome *c*, which could be followed in the spectrophotometer. In view of the possible complications that would be introduced, the fresh-leaf mince or the finely ground tissue could not be used for this investigation. On the other hand, the acetone-prepared enzyme was considered a suitable material; for, if the observed activity of the preparation was attributed to the cytochrome system, it should be possible to demonstrate cytochrome oxidase in it.

Fig. 1. Ultra-violet absorption spectrum of tea oxidase.

Preparation of tea oxidase. In order to minimize the inactivating effect of the solvent on the enzyme, the acetone extraction of the oxidase was carried out at about -20° in a bath of ice and salt under the following conditions, which are a refinement of those previously used by us. The leaf material was derived from young tea plants in the garden of the Indian Institute of Science, Bangalore. About 10 g. of fresh leaf were finely ground up, soon after plucking, with 30 g. of ice crystals and some iron-free sand and then introduced into a beaker which was kept immersed in a bath of ice and salt at -20° . Acetone (300 ml.), previously cooled to the same low temperature, was then added with vigorous stirring. The acetone extract was sucked off by means of an inverted thistle funnel, packed at the mouth with glass-wool, and immersed in the beaker. Renewal of cold acetone and repetition of the extraction three or four times rendered the preparation almost completely free of polyphenol, as tested by FeCl_3 and also by self-digestion of the preparation in the Warburg manometer. The material was quickly dried *in vacuo* and then extracted with 100 ml. phosphate buffer at pH 7.0, under cold conditions. After filtration the extract, which was

highly active towards catechol, was brought to pH 5.0 with acetic acid and mixed with sufficient ammonium sulphate to saturate to the extent of 1/3. From the time of plucking up to this stage, when the enzyme may be expected to be more stable, the time taken was only 4–5 hr., and since the operations were, as far as possible, done in the cold, the inactivation of the enzyme must have been minimal during the preparation. The fluid was left overnight in the ice-box.

The slight precipitate that appeared was then filtered off, and to the filtrate more ammonium

Fig. 2. Visible region absorption spectrum of tea oxidase.

sulphate was added to bring it to half-saturation. There was a further precipitate which was also filtered off, and the filtrate was then saturated with ammonium sulphate, when again there was a precipitate. The three precipitates obtained were dispersed in water and dialyzed in the cold against running distilled water. The dialyzed suspensions, obtained by one-third or one-half saturation, contained for the most part inert matter with comparatively little enzyme, and they were therefore discarded. The precipitate at full saturation gave, on dialysis, a pale yellow solution constituting a very active preparation of oxidase. This could be preserved in the refrigerator for a considerable period without appreciable loss of activity.

The residue, after extraction of soluble enzyme from the acetone-prepared leaf powder, was further ground into a fine suspension and also preserved in the ice-chest.

Experiments with reduced cytochrome *c* and cytochrome oxidase from heart muscle. Cytochrome *c* was prepared by the method of Keilin & Hartree [1937], and it was found that goat's heart proved a better source than ox heart. The dialyzed cytochrome solution was preserved in the ice-chest. Reduction

of cytochrome was carried out by passing H_2 in presence of either freshly prepared palladinized charcoal [Hartung, 1928] or of platinized asbestos (E. de Haen's preparation). An active preparation of cytochrome oxidase was also obtained from fresh goat heart muscle by the method of Keilin & Hartree [1938].

The heart-muscle oxidase readily catalyzed the oxidation of reduced cytochrome, with consequent disappearance of the latter's characteristic absorption bands in the microspectrophotometer. But the tea-oxidase solution prepared as above, when added to the reduced cytochrome substrate, did not have

It will be seen from Figs. 4 and 5 that the absorption characteristics of reduced cytochrome remained unaffected in presence of tea-oxidase preparations, while heart oxidase (Fig. 3) brought about a complete change. In the case of heart enzyme and tea-insoluble enzyme the reaction mixtures had to be filtered before taking measurements in the spectrophotometer, and the slight shift in the curve in the latter instance is perhaps due to the absorption of cytochrome *c* on the solids of the tea-residue suspension.

The degree of oxidation of reduced cytochrome *c* is related to the fall in the intensity of its absorption

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 3. Oxidation of cytochrome by heart oxidase. I, absorption spectrum of reduced cytochrome; II, reduced cytochrome + heart-muscle enzyme.

Fig. 4. Action of tea-soluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-soluble enzyme.

Fig. 5. Action of tea-insoluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-insoluble enzyme.

any effect whatever on the absorption bands, which remained practically steady for about 24 hr. It must, therefore, be concluded that the specific cytochrome oxidase was absent from the tea-oxidase preparation. The activity towards catechol, on the other hand, was very marked, as determined by its rapid oxygen uptake ($-Q_{O_2} = 170$).

A suspension of the insoluble enzyme also gave similar spectroscopic results, which points to the same conclusion as in the case of the soluble enzyme.

The course of reaction of these enzyme preparations on reduced cytochrome, as compared with that of the heart oxidase, was further followed in the polarizing spectrophotometer. 0.5 ml. enzyme was allowed to react with 2.5 ml. reduced cytochrome solution (3×10^{-4} M) for 2 hr. at room temperature, and spectroscopic determinations were carried out in 10 mm. cells.

The kinetics of the oxidation can therefore be followed by measuring in the above reactions the extinction coefficients at 550 $m\mu$, the maximum of reduced cytochrome *c*. Fig. 6 represents these data, and it will be seen that whereas the relative concentrations of reduced and oxidized cytochrome remain unaltered in the case of tea oxidase, the heart oxidase action results in a rapid fall in the reduced cytochrome concentration.

These results show that, judged on its specificity of reaction, tea oxidase cannot be identified with cytochrome oxidase.

Roberts [1941] has reported observing the reduced cytochrome bands when cytochrome *c* and succinate were added to the finely ground tea tissue and that these bands disappeared on vigorous shaking. In this reaction there was no need for any extraneous addition of cytochrome *c*, since he had

already observed cytochrome in the tissue by its 'b' band. Further, he has remarked elsewhere in his paper [Roberts, 1941] that when cytochrome c of ox heart is added to tea-tissue suspension it would combine with tea tannin and would thus be 'inactivated', thereby meaning perhaps an irreversible chemical change in cytochrome. If that is so, the disappearance of the reduced cytochrome bands may not be due to the presence of cytochrome oxidase. In our experiments we have found that

Fig. 6. Oxidation of cytochrome. I, tea oxidase; II, heart oxidase.

when reduced cytochrome is added to tea-tissue suspensions there is a rapid colour change from original red to a dirty yellow. The spectrum of the filtered liquid does not correspond to that of oxidized cytochrome and it cannot be reduced again either by Pd and H_2 or by $Na_2S_2O_4$. The change suffered by reduced cytochrome is not, therefore, a simple reversible oxidation, and under the circumstances the presence of cytochrome oxidase in the tissue becomes doubtful.

Manometric investigations

The oxygen uptake measurements were made in Warburg manometers with all necessary controls. All reactions were carried out at pH 7.0, total volume 2.5 ml., and temperature 30° . The first point established was the marked activity of the enzyme solution on catechol and its inactivity on reduced cytochrome c as substrate (Table 1).

Reduced cytochrome had not been reacted upon, as was shown by its absorption bands when tested at the end of the reaction.

In the *in vitro* reconstruction of the well-known respiratory system of cytochrome c, cytochrome oxidase, succinate and its dehydrogenase, it was desired to find out if the enzymes in the system could be replaced by tea-oxidase preparation. Results of oxygen uptake by the substrate, reduced

Table 1. Oxygen uptake of tea oxidase with catechol and cytochrome

Oxygen uptake when 0.5 ml. of diluted enzyme (original solution $\times 4$) was allowed to react on 1 ml. of catechol solution (M/20) and 1 ml. of reduced cytochrome solution, respectively, in presence of 1.0 ml. of phosphate buffer at pH 7.0.

Time period (min.)	5	10	20	30	45	75	90	120	150
Catechol substrate (μ l. O_2)	5	12	22	35	54	91	112	145	184
Reduced cytochrome substrate (μ l. O_2)	3	1	4	1	0	0	0	0	4

cytochrome + succinate, in presence of tea-soluble and insoluble enzymes on the one hand and the heart enzyme on the other are given in Table 2.

Table 2. Oxygen uptake of different enzyme systems on cytochrome c + succinate

In the case of the heart enzyme the cytochrome concentration was 1.2×10^{-5} M; in the case of tea enzymes 6.0×10^{-5} M. 0.5 ml. enzyme and 0.5 ml. 1% succinate were used in all cases.

Time period (min.)	5	20	60	90	120	150
Tea-soluble enzyme (μ l. O_2)	0	3	3	3	6	—
Tea-insoluble enzyme (μ l. O_2)	0	4	8	6	3	—
Heart enzyme (μ l. O_2)	4	—	46	59	90	110

Failure of tea enzyme to stimulate oxygen uptake in the above reactions may be due to the absence of either cytochrome oxidase or succinic dehydrogenase or both in the tea-oxidase preparations. If this mixture of reduced cytochrome c + succinate + tea oxidase is supplemented by a further factor, namely, succinic dehydrogenase free from cytochrome oxidase, oxygen uptake will then proceed, provided that tea oxidase contains cytochrome oxidase. But succinic dehydrogenase and cytochrome oxidase are invariably concomitant in animal tissues, and attempts to separate them by ultracentrifugation, heat inactivation and so on were not successful. However, if tea oxidase contains cytochrome oxidase, its addition to a succinic dehydrogenase preparation with only a weak cytochrome oxidase activity should result in an acceleration of the rate of oxygen uptake of the substrate mixture because of an increase in the cytochrome oxidase concentration. A succinic dehydrogenase preparation obtained from goat heart muscle by the method of Ogston & Green [1935] was found to have only a poor cytochrome oxidase activity, but strong succinic dehydrogenase activity, as tested by the usual methylene-blue technique. Addition of this preparation to the system 'reduced-cytochrome + succinate + tea oxidase' did not increase the rate of O_2 uptake in the reaction to any appre-

ciable extent. For the sake of clarity the comparative oxygen uptake values are summarized in Table 3.

the two places. For instance, in our previous studies we have shown that catechol is oxidized by our enzyme preparations at a higher rate than tea

Table 3. *Oxygen uptake by various reaction systems*

Reaction										Oxygen uptake μl./hr.
Catechol + tea oxidase	...	...	...	...	...	...	...	...	...	198
Reduced cytochrome c + tea oxidase	...	...	...	...	...	...	...	...	...	—
" + succinate + tea oxidase	...	...	...	...	...	...	...	...	...	3
" + " + succinic dehydrogenase preparation	...	...	...	...	...	...	...	...	...	9
" + " + " + tea oxidase	...	...	...	...	...	...	...	...	...	11

The absence of stimulation of oxygen uptake by the tea enzyme in all reactions in which cytochrome c formed the substrate must be interpreted as due to lack of cytochrome oxidase. The results obtained demonstrate unequivocally that tea oxidase cannot be identified with cytochrome oxidase. But, on the other hand, since catechol is readily oxidized by the enzyme; there can be little doubt about its polyphenol oxidase nature.

DISCUSSION

A similarity in a few properties led Roberts to propose his cytochrome theory of tea fermentation. Since then, these similarities have been shown to be spurious, and according to his own findings there are serious and 'very marked differences' between tea oxidase and animal-cytochrome oxidase. But these differences he attributes to a dissimilarity that may exist between plant-cytochrome oxidase and animal-cytochrome oxidase. Unfortunately, very little is known to date about plant-cytochrome systems, and until they are fully investigated it may be premature to base any conclusions on them.

In explaining the failure of ox-cytochrome c to accelerate tea-tannin oxidation, Roberts has gone even further. He has assumed tea-leaf cytochrome to be different from the ox-heart cytochrome in that the latter is inactivated by combination with tea tannin while the former is endowed with some type of immunity from tannin. If this be true it seems to be something specific to tea and perhaps to other tanniferous plants; for Brown & Goddard [1941] have observed that ox-cytochrome c does stimulate the oxidation of *p*-phenylene-diamine by the cytochrome oxidase present in wheat embryos. However, Roberts's postulate, of the presence in tea of a cytochrome immune to tea tannin, is reminiscent of his previous postulate of an immune peroxidase and protective substances, which has not as yet been confirmed.

Certain differences pointed out in the oxidase systems concerned in tea fermentation in Ceylon and Assam appear, on closer examination, to be a consequence of the use of different techniques in

tannin, and this has been confirmed. The results of Assam workers with their material appear to be exactly the reverse. The difference is only apparent, not real, because the conditions were not the same in both cases; the Assam experiments were carried out with minced leaf in which tea-tannin oxidation products had accumulated, but these were eliminated in our reaction mixtures. The tannin-oxidation products do interfere with the rate of subsequent oxidations by the enzyme, as is fully discussed in Part 5 of this series. Such an interference is in fact found to be responsible for the reversal in the rates of oxidation of catechol and tea tannin as observed by Assam workers. The suggestion of a difference in the nature of Assam and Ceylon enzymes receives, therefore, no support from the above consideration. A point of some interest in this connexion is that the present investigation also included the enzyme derived from tea plants raised on Indian seeds and grown under Indian conditions.

The spectroscopic and manometric evidence obtained in the investigation goes to show conclusively that tea oxidase cannot be identified with cytochrome oxidase, which forms the keystone of the entire structure of the cytochrome theory of tea fermentation. Absence of cytochrome further justifies the rejection of the theory. On the other hand, our original finding that the tea-fermentation enzyme was a polyphenol oxidase needs no modification whatever. The mechanism of tea fermentation based on this finding, whilst not so complex as the cytochrome theory, is consistent with the main facts so far observed.

SUMMARY

1. Attempts to demonstrate directly by spectroscopic methods the presence of cytochrome in tea have been unsuccessful.
2. Spectroscopic investigation of highly active tea-oxidase preparations showed that the enzyme could not be identified with cytochrome oxidase. Manometric results have confirmed this conclusion.
3. Some of Roberts's previous results have been reviewed in the light of the later work and certain

differences have been satisfactorily accounted for. From the evidence presented, Roberts's cytochrome theory of tea fermentation cannot be considered valid.

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— Studies on the 'Fermentation' of Ceylon Tea

7. THE PROSTHETIC GROUP OF TEA OXIDASE

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Earlier investigations in this laboratory on the specific nature of the oxidizing enzymes in tea [Lamb & Sreerangachar, 1940; Sreerangachar, 1943] have shown that the enzyme belongs to the family of polyphenol oxidases of which the potato oxidase and the mushroom oxidase are well-known members. The work of Kubowitz [1937; 1938] and of Keilin & Mann [1938] respectively on these enzymes has established that these polyphenol oxidases are Cu-protein compounds, and undergo a cycle of transformation from cupric to cupro form and vice versa, in the course of their catalytic activity. Cu is also found in the prosthetic groups of other oxidative enzymes such as laccase, ascorbic acid oxidase and tyrosinase, but the specific protein carrier in each of these enzymes is considered to be different. The Cu-proteins, unlike the Fe-porphyrin protein enzymes, show no specific absorption bands in the visible region of the spectrum, the absorption, if any, being only diffuse. A spectroscopic examination of tea oxidase has failed to reveal any characteristic absorption bands [Sreerangachar, 1943], and the enzyme is, therefore, analogous in this behaviour to Cu-proteins.

Consequent on the finding that tea oxidase was a polyphenol oxidase with respect to its substrate specificity, further investigations of the material

and chemical nature of this enzyme became desirable, and it was thought that a study of the prosthetic group was a fruitful line to follow in the first instance. In particular, it was intended to see whether, like the other known examples of polyphenol oxidases, tea-polyphenol oxidase was also a Cu-protein compound.

Cu is easily detected in tea-oxidase preparations, but it was found that the crude preparations also contained Mn and Fe. As these two metals are also frequently associated with certain oxidizing enzymes, the possibility of their forming the active group of tea oxidase had to be considered in the early stages. According to Bertrand & Mallèvre [1895], there exists a close connexion between Mn and laccase, and Shaw & Jones [1935] have emphasized the importance of Mn in the study of the oxidative enzyme in tea. That the fresh tea leaf contains appreciable amounts of Mn can be seen by the fact that the ash is generally green because of the presence of manganous salts, which readily change over to the characteristic permanganate colour when wetted with oxidizing solutions. But in spite of this, the acetone-prepared enzyme contains only a fraction of the original amount (200–400 µg./g.). Further, Mn is almost completely absent even in partially purified preparations of enzyme and there-