175. STUDIES ON THE "FERMENTATION" OF CEYLON TEA

1. THE NATURE OF THE ENZYME SYSTEM

2. OXIDIZING ENZYMES

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1. THE NATURE OF THE ENZYME SYSTEM

"FERMENTATION", as understood in the tea industry, is the process during which "black tea" develops its characteristic aroma and colour of liquor. The development of black tea characteristics may be arrested by steaming freshly gathered tea leaf and this device is employed in making green tea. It is at once fairly obvious therefore that fermentation is an enzymic process and this has been confirmed by several workers [Gadd, 1932; Benton, 1938]. Benton [1938] has made an intensive study of secondary effects in fermentation due to micro-organisms. The tea bushes in cultivation at present are not anywhere much more than a selection from a thoroughly heterogeneous collection of accidental hybrids, and the conditions of cultivation are so diverse, ranging from tropical to almost temperate, that we wish to make it clear that we are not attempting to explain anything more than our own observations in Ceylon, so far confined to leaf grown at high elevations (4500 ft.).

However, we cannot pass on to an account of our investigations in Ceylon without some reference to work in other countries which has been principally concerned with three classes of enzymes, namely, oxidases, peroxidases and catalases, and their function in the main reaction which appears to be the oxidation of polyphenols. Thus Nanninga [1901], Aso [1901], Newton [1902] were the earliest to describe the enzyme as oxidase (thease) and Mann [1901], Bernard & Welter [1911], Kursanov [1935], and Oparin [1935] showed the presence of both oxidase and peroxidase. More recently Roberts & Sarma [1938] have realized the interest of investigations on tea fermentation "to the biochemist, not only as an illustration of the growing importance of enzyme chemistry in industry, but also as a detailed study of the chemical changes taking place in damaged plant tissue". These workers studied a peroxidase system in Assam leaf but were unable to detect oxidase, the latter observation, however, having been qualified in a joint letter [Lamb & Roberts, 1939].

Roberts & Sarma [1938] state that "various qualitative studies have been made at the several Tea Research Institutes, but no thorough investigation has been made apart from that of Oparin [1935]". Quantitative studies have actually been in progress in Ceylon since Evans [1927] first investigated the absorption of O_2 by fermenting tea leaf. Evans found that leaf dipped in 1% $HgCl_2$ before crushing fermented quite normally and in this way ruled out micro-organisms; he then proceeded to measure O_2 uptake and found that rapid

absorption of O_2 occurred during the first 3 or 4 hr. of fermentation after which the rate decreased. From the figures obtained Evans was able to plot a smooth "die-away" curve characteristic of enzyme reactions. The rate of O_2 absorption was also unaffected by the presence of hydrogen cyanide, an observation which is of considerable interest in connexion with the role of peroxidase, marked quantities of which enzyme are present in Ceylon leaf. Catalase was also studied by the same worker who found that catalase activity declined rapidly during the fermentation process but he was unable to interpret the significance of his findings. The present investigation in Ceylon started in 1936, brief progress reports having been published [Lamb, 1936; 1937].

Fermentation of tea in Ceylon

In Ceylon, tea is pruned down every 2 to 4 years according to elevation and climatic factors, thus maintaining the bushes in a state of active vegetative growth. The buds with two partially matured leaves ("flush") are harvested at intervals of 7–12 days, and taken to the factory where:

(1) The flush is slowly withered to 50–55 % moisture content by means of conditioned air, or natural breezes. The operation normally takes about 18 hr. and the leaf becomes flaccid.

(2) The withered leaf is then rolled in lots of 200–400 lb. in machines which apply a twisting motion. The effect on the leaf may be likened to the wringing of a damp cloth. During this process some of the juices are worked out of the leaf and form a varnish-like coating on the surface. Fermentation is started by the rolling process which is normally continued in successive stages of 30 min. The leaf is sifted after each stage until 80–90 % of the leaf is twisted (and broken) into a size which will allow passage through standard sieves.

(3) The leaf sifted from the rolling process is spread in layers 2 in. thick, usually on cement slabs, to continue fermentation, which is timed from the commencement of rolling and ranges from $1\frac{1}{2}$ to 4 or more hours.

(4) Subsequent stages involve drying with hot air at 85–95° to a moisture content of 3 %, cleaning, grading etc.

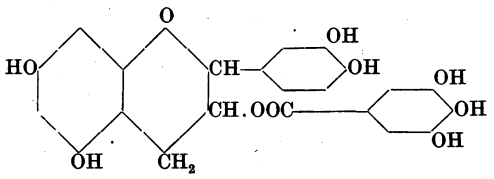
The substrate

Again, it is necessary to emphasize the fact that tea bushes in cultivation at present cannot by any test be considered as a pure line crop. The observations which follow cover only the material gathered from our own estate. Although selection work is in active progress it has not so far been found possible to work with clonal material. It has, however, been practicable to manufacture the leaf from single bushes and these investigations have given ample proof of the heterogeneity of the material in cultivation on our own estate [Lamb, 1939]. The polyphenols which have received the most attention may be roughly divided into three classes: (*a*) tannins, (*b*) catechins, (*c*) simple polyphenols. Tsujimura [1929] considers the principal tannin to be the galloyl ester (I) of a tea catechin (II). Deijs & Dijkman [1936], working with Java tea, produced evidence in support of this structure and succeeded in splitting off gallic acid by means of *Aspergillus niger*. Oshima [1936] succeeded in synthesizing a tannin (III) which he claims to be identical with the natural product of Formosan leaf. In Ceylon we have not succeeded in splitting gallic acid from the amorphous tannin preparations from our leaf. The culture of *A. niger* used for the purpose split gallic acid from commercial white tannin and grew vigorously on our tea tannin

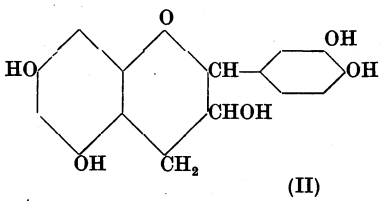
preparation, but without producing any gallic acid. Ceylon leaf, however, contains marked quantities of gallic acid in an apparently free state. It seems quite probable that at least two different tannins may occur in tea, but under our conditions the galloyl ester type does not appear to be present in any marked quantity.

The two principal catechins in Ceylon leaf appear to be *l*-epicatechin (II) also called tea catechin [Tsujimura, 1929] and gallo-catechin (IV) of Oshima. The quantities, however, are small compared with the tannins. Deijs [1939, 1, 2] has recently isolated these catechins from Java leaf.

Of the simple polyphenols, gallic acid, as mentioned above, occurs in marked quantities. Catechol is also probably present in traces:



(I)



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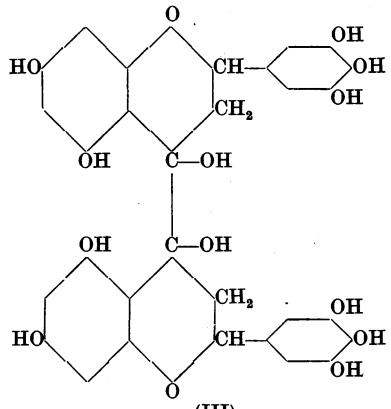

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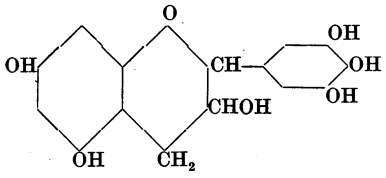

(IV)

Reference must also be made to some of the other constituents of Ceylon tea leaf. In view of the work of Roberts & Sarma [1938] it is necessary to mention that ascorbic acid[1] is present in amounts varying between 0·2 and 0·4 mg. per g. of fresh weight. Sugar in the various parts of the flush ranges from 1·41 to 0·73 % of dry weight, and starch, which does not clear at night from the leaves of some varieties, ranges from 2·96 to 0·82 % of dry weight. Some chlorophyll degradation takes place during fermentation and is of importance in connexion with the formation of the insoluble pigments of made tea as will be shown later. Caffeine, which will precipitate some of the polyphenolic constituents of tea leaf [Shaw, 1935], occurs to the extent of 3·5 % of the dry weight of the average sample of Ceylon tea leaf.

[1] Estimated by means of 2:6-dichlorophenolindophenol and Drumstick ascorbic acid oxidase [Sreerangachar, results prepared for publication].

The enzyme system

On crushing either fresh or withered Ceylon tea leaf the development of a coppery red colour commences in the crushed tissue exposed to the air, and within a period of roughly 4 hr. almost all the initial green colour of the crushed leaf has changed to a coppery red tint. Extraction of different portions of such crushed leaf with water at intervals shows intensification of colour in the aqueous extract from an initial greenish-lemon to a deep reddish brown, whilst the extracted tissue retains an insoluble pigment which is initially pale yellowish green and finally a bright coppery red. The final extract is well illustrated by the liquor from the ordinary domestic brew of tea and the insoluble pigment by the colour of the spent leaves or "grouts".

Addition of H_2O_2 to the crushed leaf causes a very marked change of colour in a short period, consistent with the activation of a peroxidase. The crushed tissue will liberate O_2 from H_2O_2. If fresh or withered leaf be heated above 120° F it remains green after crushing. Fermentation is also inhibited by preventing access to O_2. CO_2 continues to be evolved by crushed withered leaf, but at a greatly reduced rate compared with uncrushed fresh or withered leaf [Evans, 1928].

EXPERIMENTAL

In order to save space, much experimental detail must be condensed but we shall endeavour to give a logical summary of the successive stages of our work and to enlarge only upon the more important points.

(1) *Preparation of enzyme system.* A weighed quantity of fresh or withered leaf is ground with iron-free sand under acetone and rapidly filtered under suction, repeating the operation until the coloured constituents are leached out. The greenish white residue is then dried *in vacuo*. McIlvaine's or Sørensen's phosphate buffer may be employed to extract soluble enzymes from the resultant dry powder, for which purpose one extraction of 30 min. is sufficient. The extract gives a faint ferric chloride test.

(2) *Preparation of substrate.* A weighed quantity of undamaged fresh or withered leaf is immersed in boiling water for 5 min. in order to inactivate enzymes. The material is then drained and the juices expressed and stored in a non-actinic bottle under toluene. This stock solution keeps for at least 14 days. Stock solution may be diluted with McIlvaine's or Sørensen's phosphate buffer as required.

(3) *Quantitative method for measurement of oxidizing activity.* 10 ml. of reaction mixture consisting of substrate solution diluted with buffer, and in some cases enzyme extract, are mixed with 20 ml. $N/10$ I_2 and 12·5 ml. N NaOH. The mixture is then kept in the dark for 15 min. and back-titrated with $N/20$ $Na_2S_2O_3$ after the addition of 25 ml. of 1·5N H_2SO_4, using a starch indicator. The method is essentially that of Shaw [1935] and is reliable for the principal tannin of tea, and may be used to follow oxidative changes taking place during fermentation. Its advantages are speed and definition of end point, together with a degree of simplicity and directness which safeguards against the confusion which may arise from precipitation methods.

The limitations of the method must, however, be borne in mind. It is not in any way specific, even for polyphenols, and does not afford any indication of changes such as condensation. The method can only be regarded as a measure of changes in oxidizable material. However, in the enzymic reactions studied, the changes in titre have been directly proportional to O_2 absorption value as will be shown in § 12.

(4) In vitro *reaction with soluble enzyme.* Both quantitative and qualitative reactions are carried out in a series of boiling tubes through which washed air may be bubbled. The bubbling also serves to keep all reaction mixtures well stirred. Controls for autoxidation are similarly aerated, the enzyme preparation, if in solution, being in each case replaced by an equal volume of buffer solution. For most quantitative experiments the reaction is allowed to proceed for 4 hr., and if it is desired to observe peroxidase activity, 0·25 ml. portions of $N/10$ H_2O_2 solution are added at suitable intervals. A total volume of 25 ml. is normally convenient to work with, a typical reaction mixture for observation of soluble enzyme activity being 10 ml. of enzyme solution, 10 ml. of substrate solution diluted 5 times with buffer and the whole made up to 25 ml. The relative amounts of enzyme and substrate may be arranged so that they are both equivalent to a given amount of fresh leaf.

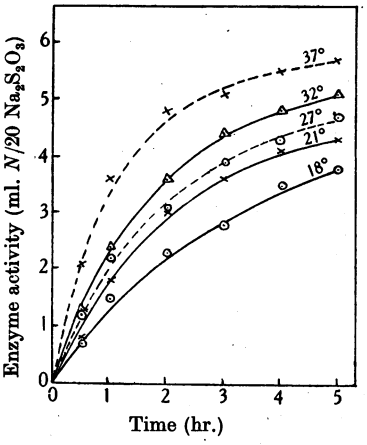

Fig. 1.

Soluble enzyme preparations are sensitive to high concentrations of tannin and are practically inhibited by the undiluted substrate solution. Soluble enzyme activities are therefore observed in diluted substrate. The insoluble enzyme preparations described later are fully active in undiluted substrate. The addition of H_2O_2 causes an increase in enzyme activity measured by titration. In a typical case the titration difference increased from 3·7 to 5·0 ml.

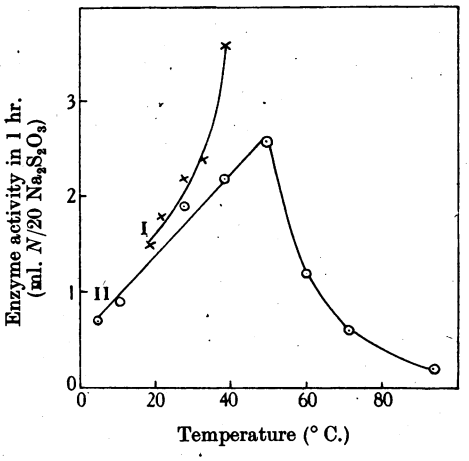

Fig. 2.

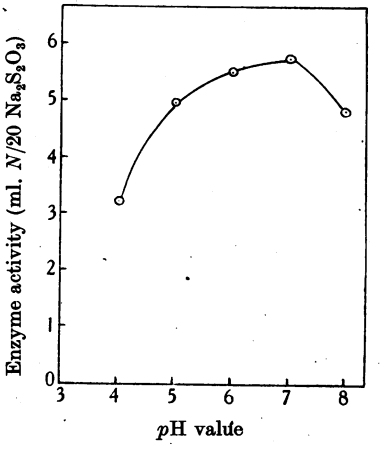

Fig. 3.

(5) *Kinetics, pH and temperature in relation to soluble enzyme.* Fig. 1 shows oxidizing activity of the soluble enzyme plotted against time in hr., over a temperature range of 18–37°. Fig. 2 shows that the optimum temperature for oxidation in the enzyme-substrate system prepared from Ceylon tea leaf is in the region of 49°, and Fig. 3 indicates that the optimum *p*H for the same system

is between 6·0 and 7·0. In all three cases corrections have been made for autoxidation of substrate.

(6) *Purification of soluble enzyme.* The soluble enzyme system is precipitated from buffer solution by 80 % alcohol. Half saturation with ammonium sulphate also brings about precipitation of active material, but in neither case is the full activity of the enzyme system preserved. The juice of fresh leaf may also be treated with acetone or alcohol to precipitate a similar white gelatinous mass which shows marked activity. This precipitate is partly soluble in water.

Ordinary dialysis and ultrafiltration revealed that part of the enzyme system is apparently of low molecular weight since it passes through collodion and parchment membranes, the ultrafiltrate containing active enzyme. Attempts at absorption on alumina C and kaolin have failed, the solution of the enzyme system retaining its full activity. On heating a concentrated solution of the active enzyme system in water there is no indication of any heat coagulation. These last two observations support the suggestion that the activity of the system is due to substances of low molecular weight. In our hands attempts to crystallize active materials from the active solution have so far failed.

A search for accelerators at the time of the dialysis experiments gave some indication that Cu and Mn together have some accelerating effect on the enzyme system. This observation requires further investigation in view of the fact that certain Nyasaland [1937] teas which failed to ferment properly were induced to behave normally by spraying with copper solutions. Keilin & Mann [1938] have described a polyphenol oxidase which consists of a copper-protein complex.

(7) *Mechanism of the oxidation of polyphenols by the soluble enzyme system.* Purified enzyme was prepared by the following method. 100 g. fresh leaf were minced and mixed with 100 ml. water at *p*H 6·5. This mass was then pressed out and the juice precipitated by adding alcohol to a concentration of 80 %. The precipitate was filtered, washed with 80 % alcohol and dispersed in water. This suspension was then filtered and the filtrate made up to 50 ml. The solution was almost water-white when fresh but discolored on keeping for 4–5 days. It gave a very faint reaction with ferric chloride.

Pugh & Raper's work [1927] with tyrosinase suggested to us the investigation of the action of the enzyme system upon catechol. Following their technique we were able to isolate dianilino-*o*-benzoquinone (M.P. 190°; N found 9·78 %; calc. for $C_{18}H_{14}O_2N_2$ 9·66 %).

Subsequently anilinoquinones were prepared from Gambir catechin isolated from B.P. catechu and Tsujimura's tea catechin isolated from Ceylon leaf. The difficulties of crystallization were, however, such that by the time clean crystals were obtained the quantity was insufficient for analysis. White amorphous tea tannin and gallocatechin are also oxidized by the enzyme and in the presence of aniline gave a cumulative precipitate during the reaction.

The progressive formation of precipitates when the reactions are carried out in the presence of aniline is considered to be an indication that quinones are formed, which indication is supported by the observation (*vide* following paper) that the oxidation product of amorphous tea tannin oxidizes ascorbic acid. Quinones formed during these reactions are either unstable or themselves powerful oxidizing agents since complex reactions follow their production usually with intense colour formation.

Within the limits imposed by the difficulties explained above we were able to investigate the specificity of the soluble enzyme system, the results being set out in Table 1.

Table 1

Substance	Nature of substance	Reaction (2–3 hr.)
Monohydric phenols:		
Phenol	—	None
Cresols	*o*- and *p*-Hydroxytoluene	None
Tyrosine	*l*-*p*-Hydroxyphenylalanine	None
Dihydric phenols:		
Catechol	*o*-Dihydroxybenzene	Quinone formation
Guiacol	Ditto with one OH group methylated	None
Diacetyl catechol	Fully acetylated catechol	None
Resorcinol	*m*-Dihydroxybenzene	None
Quinol	*p*-Dihydroxybenzene	None
Orcinol	*m*-Dihydroxytoluene	None
Adrenaline	*o*-Dihydroxybenzene with side chain	Very rapid oxidation
Trihydric phenols:		
Pyrogallol	1:2:3-Trihydroxybenzene	Oxidation
Phloroglucinol	1:3:5-Trihydroxybenzene	None
Hydroxymonocarboxylic acids:		
Protocatechuic acid	3:4-Dihydroxybenzoic acid	Oxidation
Gallic acid	3:4:5-Trihydroxybenzoic acid	Oxidation
Nadi reagent	α-Naphthol and *p*-phenylenediamine	Very slight oxidation

The results set out in Table 1 show that the enzyme system is highly specific in that it oxidizes polyphenols having hydroxyl groups in the *o*-position only. Very careful tests failed to give any indication of oxidation of monophenols and, unlike peroxidase, the system showed no sign of activity with *p*-hydroxyphenol (quinol). The slight but definite oxidation of Nadi reagent may be attributed to the action of traces of the quinones of polyphenols associated with the enzyme, as is more fully explained later (*vide* following paper). The soluble enzyme system oxidizes all the catechin and tannin preparations so far obtained from Ceylon tea leaf and there is little reasonable doubt that the first stage of the oxidation is the production of an *o*-quinone. This statement is consistent with the constitutional formulae depicted in the section above dealing with the substrate.

Since ascorbic acid is eliminated by the method of preparation of the enzyme system used in studying the specificity there cannot be any question of the formation of H_2O_2 by an ascorbic acid–ascorbic acid oxidase system. Furthermore, it appears that peroxidase activation does not take place since peroxidase is not so specific in action. Further investigation of these reactions, along the lines recently followed by Jackson [1939] in his most interesting studies of oxidation by polyphenol oxidase, is desirable.

(8) *The insoluble enzyme system.* Having gathered information about the characteristics of the soluble part of the enzyme system we next directed attention to the residual activity of the preparation after removal of soluble enzyme. Repeated and thorough extractions with several aqueous solvents such as buffer solutions of different *p*H and glycerol did not render the residue inactive. Accordingly, quantitative determinations were made on different samples before and after extraction.

Mention has been made of the heterogeneity of the tea crop cultivated in Ceylon. Careful repetition of all major experiments is thus necessary and the variations encountered are well illustrated in Table 2, where figures obtained from 4 different samples are given. For each determination 0·5 g. of enzyme preparation was taken and the same quantity of stock substrate used in each reaction.

Table 2

System	Activity (ml. $N/20$ $Na_2S_2O_3$)			
	Sample I	Sample II*	Sample III	Sample IV
Unextracted powder	25·25	24·4	52·0	9·0
Extract	18·0	23·2	8·8	12·8
Residue	32·25	41·6	65·6	17·2

* Triplicates agreed to within 0·6 ml.

The stock substrate was not necessarily the same stock; it has to be freshly prepared from time to time. The determinations for any one sample were, however, all carried out with the same stock solution.

Despite the variations in the figures, the same general conclusion may be drawn from the results with each different sample that the residue retains a very high degree of activity which actually appears to be enhanced by extraction of the soluble enzyme. In these experiments the residue was dehydrated with acetone before determination of its activity. A further series of experiments was next carried out to determine the effect of successive extractions with buffer solution. Although the enzyme preparation is relatively tannin-free it actually contains titratable amounts of polyphenols and there was at least a possibility that such substances might act as inhibitors of insoluble enzyme activity. Table 3 shows the result of these extraction experiments. Activities were determined on 10 ml. of a stock substrate solution sufficient in quantity to cover the whole experiment. The wet residue plus filter paper was in each case introduced into 10 ml. buffer solution at *p*H 5·4 before addition to 10 ml. of substrate solution. For each extraction 20 ml. of buffer solution were used and after filtration 10 ml. were titrated in order to determine the amount of oxidizable material extracted.

Table 3

Figures represent ml. $N/20$ $Na_2S_2O_3$.

No. of extractions	Oxidizable material in 10 ml. extract	Activity of 0·25 g. of enzyme preparation
Nil	—	14·2
1	5·6	13·6
2	1st 5·8 2nd 1·8	15·0
3	1st 5·1 2nd 2·1 3rd 0·5	14·0
4	1st 5·6 2nd 2·0 3rd 0·3 4th 0·1	15·6

The figures in Table 3 confirm the fact that a high degree of activity is retained by the residue after successive extractions but do not afford any definite evidence of activation by removal of inhibiting substances.

A separate experiment was carried out at *p*H 5·4 (with different material) in order to study the effect of successive extractions upon the amounts of soluble enzyme removed from the enzyme preparation. The figures are given in Table 4.

If, after the reaction, the residue containing insoluble enzyme is washed free of colour and added to further substrate, it is observed that activity is retained by the residue. This process has been repeated three times and after oxidizing

Table 4

Extract	Activity of extract (expressed in ml. $N/20$ $Na_2S_2O_3$)
1st	12·0
2nd	6·5
3rd	3·0
4th	1·0
Activity of residue	25·5

three successive quantities of substrate, the residue was found still to possess the power of oxidizing further substrate, thus indicating the insolubility of the enzyme system in a buffered solution of its natural substrate. The extractability of the soluble enzyme system from the dry powdered enzyme preparation varies markedly with the *p*H of the buffer solution employed for extraction.

Table 5 displays figures which are a measure of activities resulting from different extractions. Since the optimum *p*H for the oxidizing activity of the soluble enzyme system in tea extract substrate is between 6·0 and 7·0, both *p*H of extraction and *p*H of activity have to be taken into account. The solubility rises markedly between *p*H 5·0 and 8·0, and an extract made at *p*H 8·0 shows very high activity in tea extract substrate at about the optimum *p*H for the system (6·5).

Table 5

	Activity in ml. $N/20$ $Na_2S_2O_3$ per equiv. of 1 g. enzyme powder		
Column I *p*H of extraction and/or reaction	Column II Extraction and reaction *p*H as in column I	Column III Extraction *p*H 7·0 Reaction *p*H as in column I	Column IV Extraction *p*H as in column I Reaction *p*H 6·0–6·5
5·0	31·2	59·4	20·4
6·0	32·4	65·4	30·0
7·0	46·8	68·4	56·4
8·0	61·4	57·6	91·2

The removal of soluble enzyme does not appear to affect the amount of insoluble enzyme activity in the residue of the enzyme preparation. It might be expected that extraction by buffer at *p*H 8·0 would remove most of the activity of the preparation. However, Table 6 shows that the activity of the residue is unaffected by the amount of soluble enzyme removed, since the residue is just as active after successive extractions at *p*H 8·0 as it is after a single extraction at *p*H 5·0.

Table 6

Aliquot no.	Extraction	Activity of insoluble enzyme in tea extract substrate at optimum *p*H 5·4 (*vide* § 10) (ml. $N/20$ $Na_2S_2O_3$)
1	One at *p*H 5·0	6·7
2	One at *p*H 8·0	6·5
3	Four successive extractions at *p*H 8·0	6·2

The relation between the soluble and insoluble parts of the enzyme preparation we have described has caused us much thought and investigation. We intend to refer to only one of many possible explanations, and this arises from an observation that the soluble enzyme activity apparently increases during the process of

withering Ceylon tea leaf. It seemed possible that some process such as cellular lysis might cause the formation of soluble enzyme from insoluble enzyme. Table 7 gives the details of these observations.

Table 7

Preparation	Activity (ml. $N/20$ $Na_2S_2O_3$)
Soluble enzyme from 1 g. of enzyme preparation made from a sample taken from a quantity of well-bulked fresh leaf (moisture content 76%)	25·6
Similar preparation from another sample taken after withering to 63·2% moisture content in 18 hr.	43·2
Another sample withered to 46·4% moisture content in 18 hr.	57·6

The increase of soluble enzyme activity during withering has been confirmed by several experiments and was reported by one of us [Lamb, 1936]. Similar observations in other countries have been reported by Mann [1901] and Jones [1937–8]. Roberts & Sarma [1938], and Deijs [1939, 1, 2] have observed an increase in peroxidase activity. Similar determinations of the activities of insoluble enzyme before and after withering reveal little change; if anything, a slight tendency to increase. The figures in Table 3 show that extraction of soluble enzyme tends to activate the insoluble part of the system. This makes it very difficult to say whether the increase of soluble enzyme during withering is at the expense of the insoluble system.

While the whole question of solubility of the oxidizing enzyme system of Ceylon leaf requires further investigation, we regard it as a problem subsidiary to our main investigations and have pressed on, intending to return to it at a later date. We have, however, investigated the possibility of proteases causing changes in the solubility of these oxidizing enzymes. Papain did not cause the formation of any soluble enzyme from the insoluble enzyme system but, on the other hand, inhibited the oxidizing activity of the preparation.

Roberts & Sarma [1938] have stated that fermentation of finely minced tea leaf suspended in water is inhibited by the addition to the suspension of a liver catalase preparation. They attribute this effect to decomposition of H_2O_2 and consequent effects upon peroxidase activity. No details of the catalase preparation are given, but if it contains any protease there is an alternative explanation of their observations.

(9) *Endo-enzyme.* One of us [Sreerangachar, 1939] has referred to the insoluble enzyme system as endo-enzyme. In the light of subsequent results we have formed the opinion that the term endo-enzyme can only serve to confuse and have accordingly adopted the term "insoluble enzyme" to express the same meaning.

(10) *Optimum pH and temperature of insoluble enzyme activity.* The optimum *p*H and the optimum temperature for the oxidizing activity of the insoluble enzyme in tea extract substrate are widely different from the optima of the soluble enzyme system. Figs. 4 and 5 show these optima to be at *p*H 5·4 and 27°. The latter figure is remarkably low for optimum enzyme activity. The widely different optimum temperatures may explain some of the curious differences in activities recorded in § 8.

(11) *Mechanism of the oxidation of polyphenols by the insoluble enzyme system.* The insoluble enzyme system exhibits the same specificity for *o*-dihydric phenols as the soluble enzyme described in § 7. With *p*-cresol, however, there is a very slight activity more suggestive of impurities than of a definite reaction such as

may be observed with polyphenols containing *o*-hydroxy groupings. Slight oxidation of Nadi reagent was also observed. The anilinoquinone of catechol was again isolated and identified.

Between the soluble and insoluble enzyme systems there is, however, one most important difference. Whereas soluble enzyme tends to be inactivated by

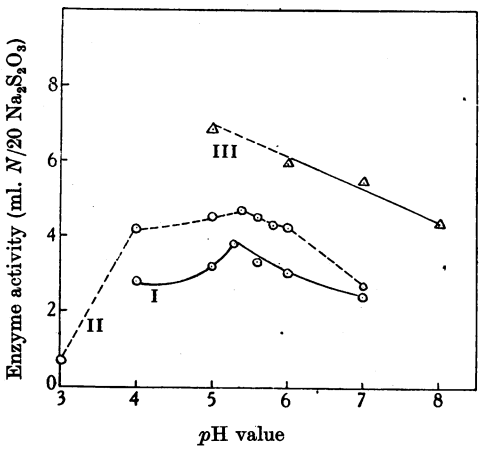

Fig. 4.

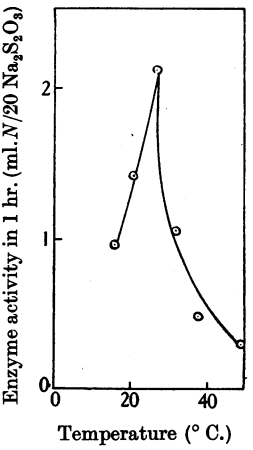

Fig. 5.

high concentrations of substrate the insoluble enzyme remains almost fully active. White amorphous tea tannin preparations moistened with a few drops of buffer are oxidized by the insoluble enzyme when stirred into a paste.

Some evidence of the adsorption of polyphenol by the enzyme preparation was obtained by shaking substrate solution with the powdered enzyme preparation under anaerobic conditions at a low temperature and leaving for 1 hr. On filtering at the end of this period there was a fall in the titre of the substrate solution. Adsorbed tannin may be washed out by buffer solution with some difficulty. Heat-inactivated enzyme preparation has the same property of adsorbing substrate from solution. Glutelin, salt-soluble proteins, starch and pectins were each separately extracted from enzyme preparations without any considerable effect upon the adsorptive power of the residue. In measurement of activity, errors due to the absorptive power of the enzyme powder have been avoided by obtaining initial titration values after the addition of enzyme to the reaction mixture.

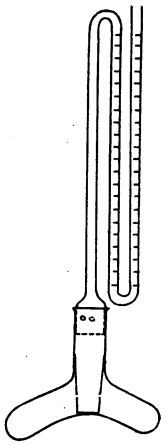

Fig. 6. Respirometer.

(12) *O_2 absorption.* A pair of simple respirometers, illustrated in Fig. 6, were adapted to measure O_2 absorption during reactions between the enzyme systems and various substrates. This particular type of respirometer is very limited in its application but careful work afforded evidence useful in confirming previous findings. It is hoped later considerably to extend investigations in this field with a more sensitive and adaptable apparatus of the Barcroft-Warburg type.

First, we were able to confirm the fact that CO_2 is not evolved as a result of reaction between the soluble or insoluble enzyme system

and either tea extract substrate, amorphous tea tannin or simple polyphenols. Secondly, the apparatus afforded a means of establishing the enzymic nature of the O_2 uptake.

Fig. 7 consists of four curves plotted from data obtained on various occasions when *different* enzyme and substrate preparations have been studied at 27° (thermostat).

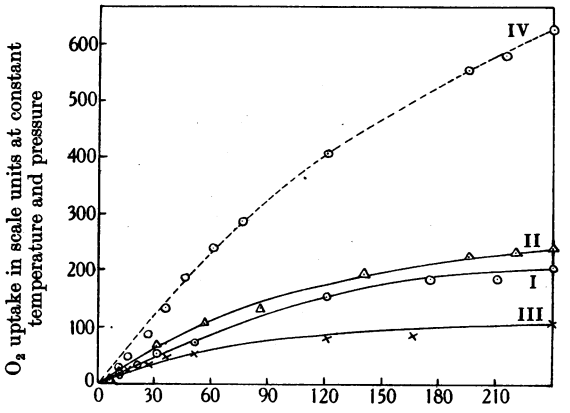

Fig. 7.

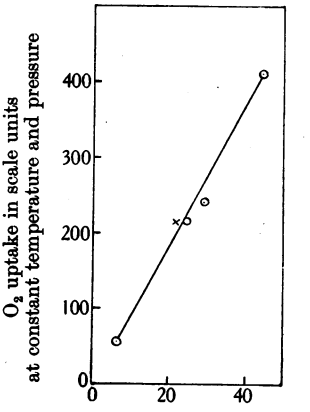

Fig. 8.

Fig. 7. I. 2 ml. concentrated tea extract substrate + 0·3 g. insoluble enzyme powder. II. 3 ml. tea extract substrate + 0·3 g. unextracted enzyme powder. III. 2 ml. amorphous tea tannin solution (1·41 %) + 0·3 g. insoluble enzyme. IV. 3 ml. 1 % catechol solution + 0·3 g. unextracted enzyme powder.

The slow diffusion of O_2 into the wet tissue makes it impossible to extract any information about the relative activities of these various systems although it has been shown that slowness of O_2 uptake is accompanied by slowness of titrimetric changes and that there is a fairly constant relation between the two methods of observing activity. This relation is shown in the third column of Table 8, and in Fig. 8.

Table 8

Substrate	Enzyme	Ratio O_2 absorbed to change in titre
Tea extract	Mixed enzymes*	8·6
,,	,,	8·1
,,	,,	9·1
,,	Insoluble enzyme	8·6
Amorphous theotannin solution	Mixed enzyme	9·6

* I.e. unextracted enzyme powder.

These experiments have not taken any account of the CO_2 evolved by fermenting leaf but show clearly that O_2 absorption and tannin oxidation may proceed independently of CO_2 production in the primary oxidation processes involving polyphenols.

Evans [1928] studied the evolution of CO_2 by fermenting Ceylon tea leaf and gives the following figures (Table 9):

Table 9

System	ml. CO_2 produced by 40 g. leaf in 1 hr.	ml. O_2 absorbed by 40 g. leaf in 1 hr.	Ratio CO_2/O_2
Fresh leaf	13·9	17·4	0·8
Fresh leaf crushed	14·1	17·6	0·8
Leaf withered to 55%	9·8	11·0	0·9
Leaf withered to 55% and crushed	3·4	26·0	0·13

The same worker showed a slight decrease in the value of CO_2 produced by rolled leaf in each consecutive hr. over a period of 7 hr., the amounts being 4·6 ml. in the 1st hr. and 3·8 ml. in the 7th. The uptake of O_2 is very rapid for the 1st hr. and decreases slowly until the ratio CO_2/O_2 again approximates to unity. Evans drew attention to differences in the behaviour of fresh and withered leaf after crushing but was unable to offer any explanation. The increase in the oxidizing activity of the preparation of soluble enzyme which we have studied offers some explanation and the joint observations point to some vital changes in the respiratory processes of withered tea leaf which under Ceylon conditions is in a moribund condition, the withering process being carried beyond the permanent wilting point of the greater part of the leaf. The qualification with respect to the greater part of the leaf is necessary because we have observed surprising differences in the rate of wither below 65% moisture content even between pieces of flush gathered from the same bush [Lamb, 1936]. This finding is confirmed by Leniger & Deijs [1937] in Java.

Roberts [1939] has conducted an extensive manometric investigation of the fermentation of Assam tea leaf and most of his findings with *crushed fresh leaf* must apply to Ceylon leaf. Since, however, it is the practice in Ceylon to wither leaf much harder than in Assam some caution must be exercised in comparing results.

Discussion

Our study of the fermentation process has so far been analytical in its conception. The *in vivo* processes may be considerably more complicated than those in any of the component systems we have studied, but their eventual elucidation should be simplified by studies of the simpler component reactions.

It is of interest, however, to compare titrimetric estimations of activity *in vivo* with titrimetric estimations of activity *in vitro*. 0·1 g. of dry matter of freshly rolled leaf in the average case contains oxidizable substances equivalent to about 27 ml. $N/20$ $Na_2S_2O_3$. After 4 hr. fermentation this falls to the equivalent of about 21 ml. $N/20$ $Na_2S_2O_3$. In a typical *in vitro* experiment, an enzyme plus substrate system, roughly equivalent to 0·1 g. of dry matter of whole leaf, gave titrations of 26 and 20 ml. before and after a 4 hr. reaction period. The oxidizing activity observed in our reactions is therefore of the same order as the activity *in vivo* and there does not appear to be any doubt that our enzyme preparations contain the greater part of the polyphenol-oxidizing enzymes.

Undoubtedly, the enzyme preparation contains an oxidase system, which is independent of ascorbic acid, and there appears to be some doubt as to whether peroxidase activation takes place at all in the system we have studied.

The enzyme systems we have described have some properties in common with the several polyphenol oxidases but at present we will not confuse the

issues by the use of specific names or by conjectures about the parallels with plant respiratory systems which have been described by other workers. There is some evidence that the normal respiratory processes of tea leaf undergo vital changes during the withering process practised in Ceylon, and until such matters are further investigated reactions associated with respiratory changes must be examined with some care before they are considered in conjunction with the fermentation processes of Ceylon tea manufacture. It is conceivable, for instance, that the peroxidase and catalase systems of tea leaf function only in respiration and remain inactive during the fermentation process, except possibly in fragments of under-withered and comparatively undamaged leaf still respiring normally.

SUMMARY

An outline of the production of tea in Ceylon is given, and it is explained how enzyme oxidation of polyphenolic substances in the leaf is thought to be the principal change occurring during manufacture.

Methods are described for the preparation of enzyme practically free from polyphenols and a polyphenolic substrate free from active enzyme. A titrimetric method is employed for the observation of enzyme activity in the prepared substrate which is aerated during the course of the reaction.

The enzyme preparation may be resolved into a buffer-soluble part showing markedly increasing solubility from *p*H 5·0 to 8·0 and an insoluble part unaffected by successive leachings at any *p*H in the same range.

Oxidizing activity of the soluble enzyme in the prepared substrate is optimum at *p*H 6·0–7·0 and at 49°. The corresponding optima for the insoluble enzyme are *p*H 5·4 and 27°, the latter temperature being remarkably low for optimum enzyme activity of plant enzymes.

Both soluble and insoluble enzyme systems show marked specificity in oxidizing polyphenols with *o*-hydroxy groupings only. The primary oxidation products are most probably *o*-quinones but this has only been established by analyses in the case of catechol.

The addition of H_2O_2 to either the soluble or the insoluble enzyme systems causes increased activity but the system becomes less specific in action and will then oxidize *p*-dihydroxybenzene.

Enzymic absorption of O_2 accompanies the reaction between the enzyme preparations and the tea substrate, but CO_2 is not evolved when these enzyme systems oxidize tea substrate, although CO_2 is evolved during the *in vivo* fermentation processes.

The activity of the enzyme system studied is sufficient to account for all the polyphenol oxidation taking place during the fermentation process practised in the manufacture of tea in Ceylon.

2. OXIDIZING ENZYMES

ROBERTS & SARMA [1938] state that the fermentation of tea leaf in Assam is brought about by peroxidase and that oxidase is present only in negligible quantities. Roberts has latterly qualified this statement but Deijs [1939, 1, 2] has still more recently published findings to the effect that oxidase does not occur to any marked extent in Java leaf. Deijs used methods similar to those employed by Roberts and it is possible that, as with Roberts, the enzyme system capable of direct oxidation of tea tannins, which may exist in Java leaf, was also

inhibited by alcohol treatment at high laboratory temperatures, as reported in a previous communication [Lamb & Roberts, 1939].

The enzyme system which we have described readily oxidizes polyphenols without the addition of H_2O_2. In other words the system is an oxidase system. Our enzyme preparations are, however, more active in the presence of added H_2O_2 and the question arises as to whether the oxidase system contains any mechanism which may generate H_2O_2 to activate the peroxidase or whether the polyphenols are oxidized without peroxidase activation.

Experimental

Ascorbic acid oxidation

Roberts's [1939] first views were that H_2O_2 is generated from an ascorbic acid-ascorbic acid oxidase mechanism. Ascorbic acid is present in fresh Ceylon tea leaf in amounts varying from 0·2 to 0·4 mg. per g., but the specific ascorbic acid oxidase does not appear to occur in any of the Ceylon leaf we have examined. In our enzyme preparations, however, ascorbic acid is eliminated by the method of preparation, for leaching with acetone and, in the instance of the soluble enzyme, precipitation of buffer-soluble enzyme by means of alcohol, cannot leave any ascorbic acid associated with the enzyme preparation. In spite of this we have shown conclusively that the enzyme system will oxidize pure polyphenols without the addition of H_2O_2. If ascorbic acid oxidase were present in the enzyme preparation and H_2O_2 could be generated by oxidation of ascorbic acid, the addition of ascorbic acid to a reaction mixture of enzyme and polyphenol would be expected to cause acceleration of reaction. Careful experiments have completely failed to reveal any such acceleration. Roberts [1939] records similar observations. If ascorbic acid oxidase were present in fresh leaf, the mode of preparation of the enzyme powder with acetone would, according to Srinivasan [1936], be expected to preserve 33 % of the ascorbic acid oxidase activity of the powder. When ascorbic acid is added to an enzyme and catechol reaction mixture, the oxidation of the catechol, as shown by the formation of anilinoquinone in the presence of aniline, far from being accelerated is inhibited until the oxidation of ascorbic acid, followed by means of 2:6-dichlorophenol-indophenol, is complete. *o*-Quinones possess the power of oxidizing ascorbic acid and it has been shown that the rate of oxidation of ascorbic acid by our enzyme preparation is related to the production of quinones. Unfortunately none of our enzyme preparations has been completely free from polyphenols; as already mentioned, they all give a weak $FeCl_3$ test. However, the figures in Table 1 show that there is only a limited amount of quinone or potential quinone in the preparation and that the addition of polyphenol markedly accelerates the oxidation of ascorbic acid.

Table 1

Time min.	Control ascorbic acid solution	Enzyme from 0·05 g. powdered preparation (mg. ascorbic acid)			
		Soluble enzyme	Soluble enzyme + catechol	Insoluble enzyme	Insoluble enzyme + catechol
0	0·24	0·24	0·26	0·24	0·25
5	0·24	0·16	0·13	0·21	0·12
10	0·24	0·10	0·06	0·18	0·05
30	0·23	0·04	0·00	0·16	0·00

The acceleration of oxidation by catechol is less marked with the soluble enzyme which, as shown in Part I, Table 3, contains more of the polyphenolic residue after extraction by buffer than the insoluble enzyme preparations. Preparations of white amorphous tannin made from tea leaf have the same accelerating effect as catechol, thus further supporting the evidence for quinone formation from tea tannin.

KCN inhibition

Sensitivity to KCN is a well-known property of peroxidase. Evans [1927] stated that the uptake of O_2 by crushed (Ceylon) tea leaf is unaffected by the presence of HCN whilst Roberts & Sarma [1938] have stated that "small amounts of KCN completely inhibit fermentation" (in Assam). Accurate observation of the effect of addition of KCN to crushed leaf tissue is a difficult matter in practice because the intimacy of mixture is questionable, and because of possible interaction between the cyanide and substances not directly connected with enzyme activity. We have circumvented these difficulties by methods described in Part I and Table 2 gives the results of a detailed study of the inhibitory effect of KCN upon our enzyme preparations.

Table 2

	Concentration of KCN in reaction mixture. Enzyme activities expressed in ml. $N/20$ $Na_2S_2O_3$					
	0	$M \times 10^{-6}$	$M \times 10^{-5}$	$M \times 10^{-4}$	$M \times 10^{-3}$	$M \times 10^{-2}$
Enzyme powder + tea extract substrate	4·3	4·1	4·2	3·9	—	—
Enzyme powder + tea extract substrate + H_2O_2	6·3	5·8	4·6	3·4	—	—
Enzyme powder + purified amorphous tea tannin substrate	4·9	4·6	4·5	3·9	1·6	0
Enzyme powder + purified amorphous tea tannin substrate + H_2O_2	5·7	4·9	4·7	3·5	1·6	0

The conclusions to be drawn are quite clear and are that KCN up to a concentration of $M \times 10^{-4}$ has little effect upon oxidase activity while peroxidase activity stimulated by addition of H_2O_2 is markedly affected at $M \times 10^{-5}$. Oxidase activity is easily discernible at $M \times 10^{-3}$ concentration of KCN at which peroxidase stimulation is completely inhibited. Oxidase activity is inhibited at $M \times 10^{-2}$ concentration of KCN. These conclusions have been confirmed several times and appear to apply also to the soluble enzyme system, the peroxidase activity of which is inhibited at [KCN] $M \times 10^{-4}$ in catechol and gallic acid substrates while oxidase activity is still apparent. The dianilino *o*-quinone of catechol (M.P. 190°) was prepared by means of insoluble enzyme powder in the presence of $M \times 10^{-4}$ KCN.

Reviewing the data we have obtained we cannot avoid the conclusion that our enzyme preparation contains an oxidase system capable of oxidizing polyphenol without peroxidase activation. On searching the literature we have abstracted the following data concerning inhibition by KCN:

(*a*) Peroxidase activity stops at $5M \times 10^{-5}$ [Srinivasan, 1936].

(*b*) Ascorbic acid oxidase activity is very weak at $M \times 10^{-3}$ [Srinivasan, 1936].

(*c*) Cytochrome oxidase activity is inhibited at $1{\cdot}2M \times 10^{-4}$ [Stotz *et al.* 1938].

These data support our findings and cast doubt upon the presence of cytochrome oxidase in our preparations.

Non-fermenting leaf

During the course of plant selection work on tea, with which the Department is associated on account of selection for quality, we have come across a single bush of considerable biochemical interest. The flush from this bush does not ferment, however carefully the leaf is crushed and aerated. The following observations throw some light on the possible normal role of peroxidase:

(1) The tannin concentration is normal.

(2) The ascorbic acid content is normal.

(3) The addition of ascorbic acid and ascorbic acid oxidase does not induce any apparent change, either separately or in the form of lemon juice or apple juice.

(4) The addition of H_2O_2 induces fermentation as observed by colour changes of leaf and infused liquor.

(5) Preparations of enzymes from the leaf made by the methods previously described do not show any oxidase activity with our substrate preparation. Peroxidase action is stimulated by the addition of H_2O_2.

(6) Admixture of an active insoluble enzyme preparation (1·5 g. powder to 25 g. of leaf) induces a normal fermentation. In this case the colour of the infused leaf (tea-taster's "infusion") is more normal than in the case where H_2O_2 is added.

(7) Addition of Cu and Mn salts (*vide* Part I, § 6) does not induce any apparent change.

We admit that care must be exercised in applying conclusions drawn from abnormal specimens to normal cases, but we feel that the data obtained from the non-fermenting leaf support the evidence against the participation of ascorbic acid and ascorbic acid oxidase in normal fermentation. It also affords valuable indication that our enzyme preparation contains the essential part of the polyphenol oxidizing enzyme system of tea leaf.

Catechol oxidation

Oxidase and peroxidase activity in 0·2 % catechol solution in the presence of aniline both yield a dianilinoquinone, M.P. 191°. In the absence of aniline, peroxidase activity produces duller and more brownish products from catechol than oxidase activity, and it has been noted that whereas oxidase action produces very little change in I_2 titre, peroxidase produces a marked change in titre, the suggestion being that peroxidase activity results in the formation of more condensation products than does oxidase activity. This is probably true of other substrates and it appears to us that the addition of H_2O_2 to fermenting tea leaf results in duller and less desirable products from the tea-making point of view than does normal fermentation. Further investigation of this aspect of tea fermentation is desirable and may have important practical bearings.

The possible role of cytochrome

In a joint note [Lamb & Roberts, 1939] reasons were given why our Ceylon enzyme preparation might be expected to contain a cytochrome *c*-cytochrome oxidase system. Further investigations have led us away from the cytochrome theory though Roberts informs us of yet unpublished work in which he finds support for the idea that a cytochrome system exists in the insoluble enzyme preparation.

First, we have attempted to prepare cytochrome *c* by the method of Coolidge [1938]. We were able to follow this method without any difficulty and obtained a preparation similar to that described by the author but, in the absence of any

spectrometric apparatus, we were unable definitely to establish the presence of cytochrome *c*. However, the preparation was found to induce the oxidation of quinol and *p*-phenylenediamine by a washed beef muscle preparation (indophenol oxidase), which affords strong presumptive evidence of the presence of cytochrome *c* in our preparation. The addition of part of this preparation to our insoluble enzyme preparation did not accelerate the oxidation of tea extract substrate nor did it induce the oxidation of quinol or *p*-phenylenediamine.

Secondly, as mentioned in a previous section, our insoluble enzyme preparation retains some activity in a KCN concentration of $M \times 10^{-3}$, whereas Stotz *et al.* [1938] report inhibition of cytochrome oxidase activity at $1{\cdot}2M \times 10^{-4}$ concentration of KCN.

Thirdly, the insoluble enzyme does not oxidize quinol or *p*-phenylenediamine as a cytochrome *c* and cytochrome oxidase system would be expected to do. The insoluble enzyme powder, after several hours in a well aerated 0·1% quinol solution at *p*H 5–5·5, shows no sign of oxidizing activity. 0·1% *p*-phenylenediamine substrate under similar conditions shows some sign of oxidation in that the enzyme powder becomes stained with a blue colour, and after some considerable period the substrate itself becomes faintly blue to an extent slightly more pronounced than may be accounted for by autoxidation. Addition of catechol or white tea tannin preparation greatly accelerates the development of this blue colour. In the absence of added polyphenol the blue colour developed in the substrate is not extracted on shaking with toluene, the toluene layer remaining practically colourless. When catechol is added to the *p*-phenylenediamine substrate the development of blue colour is accelerated and a rose-coloured substance is extracted by toluene. If *p*-phenylenediamine be added to a catechol substrate + enzyme system which has been allowed to react for a time, the blue colour is developed instantaneously and toluene extraction results in a yellow colour in the toluene layer. Pure *p*-quinone rapidly causes the formation of the blue colour in *p*-phenylenediamine solution in a manner very similar to the enzyme plus catechol system. Since, as stated above, the insoluble enzyme powder contains adsorbed polyphenols it would appear that quinones and not direct enzyme activity are responsible for the slight observed oxidation of *p*-phenylenediamine. When α-naphthol is present together with *p*-phenylenediamine (Nadi reagent) similar observations have been recorded, but in this case toluene extraction results in a deep rose-coloured extract. Pure *p*-quinone produces instantaneous development of the blue-coloured substance soluble in toluene when added to Nadi reagent. Quinol in the presence of appreciable quantities of catechol or white amorphous tea tannin also appears to contribute to the development of a colour which cannot be accounted for by the oxidation of these latter polyphenols and there appears to be reason to suspect that quinol is also oxidized by the *o*-quinones of catechol and tea tannin although not so easily as *p*-phenylenediamine.

During the course of these investigations we found that a solution containing pure *p*-quinone and quinol slowly develops a red colour on the addition of H_2O_2, similar to that produced by tea peroxidase activity. The colour is not produced when either H_2O_2 or *p*-quinone alone is added to quinol solution. The possibility of quinone produced by oxidase giving apparent peroxidase activity in the presence of added H_2O_2 requires investigation.

A further characteristic of the system cytochrome oxidase plus cytochrome *c* appears from the literature to be its inhibition in reduced light intensity by CO, and the restoration of this activity in bright light. We have exposed crushed tea leaf, and enzyme preparation moistened with buffer, to an atmosphere of pure

CO for 30 min. under conditions of very low light intensity but have not been able to observe the slightest sign of inhibition in either case when compared with controls. The crushed leaf naturally does not ferment in an atmosphere of CO but fermentation commences immediately after exposure of the leaf to air. We have not observed the effect of mixtures of CO and air since, from the literature at our disposal, we gather that the inhibitory effect of CO upon cytochrome oxidation is permanent in low light intensities. The above investigations present a considerable bulk of evidence against the idea that the insoluble enzyme preparation contains cytochrome oxidase. However, we intend to make every endeavour to have a spectroscopic examination made on our enzyme preparation so that direct evidence for or against cytochrome *c* will be available.

Discussion

The ascorbic acid–ascorbic acid oxidase mechanism advanced by Roberts to explain the activation of peroxidase in tea leaf fails to account for the oxidizing activity of enzyme preparations made by us from Ceylon tea leaf. The oxidation of ascorbic acid added to fermenting tea leaf may be explained by the action of *o*-quinones upon ascorbic acid. Further work in Assam has led Roberts to modify his views and we have had the much appreciated opportunity of reading Roberts's unpublished work which has led him to abandon the theory.

The question therefore arises as to whether peroxidase activation takes place at all during fermentation or whether peroxidase is a respiratory enzyme which does not function during the degradation processes which constitute tea manufacture. We cannot at this stage obtain a complete answer to this question since we have only studied a part of the whole (*in vivo*) fermentation process, but the component which we have studied is sufficiently active to be able to account for all of the polyphenolic oxidation *in vivo*. The KCN-sensitivity of the oxidizing action of this component leaves little room for doubt about its independence of H_2O_2. Added H_2O_2 causes acceleration of oxidizing activity but this activity is rapidly reduced by KCN to the level of oxidizing activity in a system to which H_2O_2 has not been added. Furthermore, the system has been shown to possess the power of forming anilinoquinones in the presence of quantities of KCN which are known to inhibit peroxidase activity.

The specificity of the oxidizing activity with respect to *o*-dihydroxy groupings in polyphenolic substrates, pointed out in Part I of this series, also precludes peroxidase activation because on adding H_2O_2 the increased oxidizing activity is accompanied by loss of specificity, the enzyme systems then being capable of directly oxidizing polyphenols containing *p*-hydroxy groupings. The enzyme system in the presence of catechol or amorphous tea tannin, that is to say, when *o*-quinones are formed, appears to oxidize *p*-phenylenediamine and also quinol but this oxidation is in fact a secondary reaction due to *o*-quinones. Both the soluble and insoluble enzyme systems may, therefore, give a false impression of activity in the case of *p*-phenylenediamine, or Nadi reagent, and quinol, on account of *o*-quinone formation, whereas the enzyme systems free from any marked quantities of *o*-dihydric polyphenols are quite inactive. This appears to be a parallel to the ascorbic acid–ascorbic acid oxidase and the cytochrome c–cytochrome oxidase systems in other plants where a highly specific oxidase, through the medium of a substrate containing specific groupings, catalyses the initial changes of a series of reactions. Since tea leaf contains a much higher amount of polyphenolic substances than is usual in plant tissues, and is indeed cultivated for this reason, it would not be surprising if it contained also enzyme systems of rather specialized functions.

The literature on oxidizing enzymes contains many references to H_2O_2 formation as a result of the oxidation of dihydric phenols to quinones by polyphenolases. Pugh & Raper [1927] discuss this reaction in relation to "direct oxidases" which have a direct bearing on the problem of tea leaf oxidases. Our results do not lend any support to the suggestion that any H_2O_2, formed during the reaction between the enzyme systems we have studied and polyphenolic substrates, activates peroxidase. KCN-sensitivity and the specificity of these enzyme systems afford definite evidence to the contrary.

The conclusion we draw from our investigations is, therefore, that the soluble and insoluble enzyme systems are capable of direct oxidation of *o*-dihydric polyphenols without the activation of peroxidase, and are responsible for the primary activity in tea fermentation in Ceylon.

Summary

The enzyme systems described in the first paper of this series have been found to be comparatively insensitive to KCN, being active up to KCN concentration of $M \times 10^{-3}$, and completely inhibited only at $M \times 10^{-2}$. The specificity for *o*-dihydroxy groupings in polyphenols has been further studied and it has been shown that the oxidation products of catechol (*o*-quinone) and white amorphous tea tannin preparation (most probably *o*-quinones) oxidize ascorbic acid, *p*-phenylenediamine and, apparently, quinol. These secondary reactions may appear confusing in certain cases where the enzymes in the presence of tea polyphenols give positive reactions, as for instance in the case of Nadi reagent. During the oxidation of catechol and tea extract substrates *in vitro* the peroxidase present in the enzyme preparation is not activated. When H_2O_2 is added to the enzyme preparations there is a marked increase in activity but a loss of specificity. Quinol is rapidly oxidized when H_2O_2 is added to the system and attention is drawn to a possibility that *o*-quinones may, in conjunction with H_2O_2, form powerful oxidizing agents giving apparent peroxidase activity.

The primary reaction under *in vitro* conditions is the oxidation of *o*-dihydroxy groupings in polyphenolic substrates and the enzyme system responsible appears to be characteristic in that it does not conform to the description of any other well-known oxidizing enzyme system. It is possible that the oxidizing enzyme system of tea leaf, which has an unusually high polyphenol content, is a specialized system.

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