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VARIOUS aspects of tea-fermentation have been discussed in a series of papers from this laboratory [Roberts & Sarma, 1938; Roberts, 1939, 1, 2; Harrison & Roberts, 1939; Roberts, 1940, 1, 2; Deb & Roberts, 1940; Barua & Roberts, 1940; Roberts & Sarma, 1940; Roberts, 1941]. During the course of this work views have naturally been modified in the light of fresh data, and several statements stand in the literature which the author feels need some amendment, while many of the earlier observations are now seen to have fresh implications. A critical summing up of the work so far published therefore seems to be called for. Additional reason for such a step is furnished by the publication of the results of the Ceylon workers in this field [Lamb & Sreerangachar, 1940]. While agreeing with the Ceylon workers on many points we do not consider that all their experimental methods are completely valid, while some differences that apparently exist may possibly be due to variations in the nature of the material under investigation.

Two main problems seem to be inherent in these investigations, the nature of the oxidase system responsible for tannin oxidation, and the relation of tea-fermentation to normal respiration. There are distinct differences in opinion between Ceylon and Tocklai as to the nature of the oxidase system concerned in tea-fermentation, but evidence will be presented which accounts satisfactorily, if not conclusively, for such differences. As far as the second problem is concerned, in order to avoid misunderstandings our conclusions, considerably strengthened by much unpublished work, will be set out in some detail.

8. THE NATURE OF THE OXIDASE SYSTEM

It was originally claimed [Roberts & Sarma, 1938] that the enzyme responsible for tannin oxidation in tea-fermentation was peroxidase. The main evidence for this consisted in the observation that a strong solution of catalase added to fermenting tea-leaf, damped with an equal quantity of water, strongly depressed the fermentation rate. Balls & Hale [1935] made a similar observation of the effect of catalase on the darkening at the freshly cut surface of the apple. It was suggested by Lamb & Sreerangachar [1940] that this effect in the case of tea

might be due to protease action on oxidizing enzymes, but this criticism does not appear to be valid. Destruction of oxidizing enzymes by protease would have to be very rapid to inhibit the browning of the green tea-leaf after damage, and the author's own experiments [Roberts, 1936] show that xanthine oxidase and other enzymes in the liver extract used preserved their activity for several hours without loss.

I agree with Lamb & Sreerangachar [1940] that peroxidase appears to play no important part in tea-fermentation. This conclusion was arrived at independently before the Ceylon workers produced their evidence to show that leaf could contain both tannin and peroxidase and yet be unable to undergo fermentation.

The role if any of peroxidase in the tea-leaf therefore remains a mystery, nor are we able to account satisfactorily for the inhibition of fermentation by catalase. It is interesting to recall that in dilute suspensions of tea leaf tissue catalase has no inhibitory effect [Roberts, 1940, 2].

The possibility that ascorbic acid oxidase was concerned in tea-fermentation was seriously entertained, but later results showed that the oxidation of ascorbic acid was secondary [Roberts, 1940, 1]. The criticisms of Lamb & Sreerangachar on this point, therefore, only confirm this finding.

Temporary agreement was reached when Lamb & Roberts [1939] suggested that cytochrome oxidase was the enzyme oxidizing tannin during fermentation, but of late our views have again diverged. In the following pages results from both Tocklai and Ceylon will be discussed and an attempt made to account for any outstanding differences.

(1) *Experimental methods*

To a certain extent the inconsistencies between the work on tea-fermentation carried out in the two laboratories must be put down to different methods of attack, and the employment of different experimental techniques. At Tocklai we have carried out our experiments as far as possible under conditions closely resembling those *in vivo*, and have not undertaken any extensive work on the preparation of enzymes from the tea-leaf. Lamb & Sreerangachar have taken the opposite view and have attempted a study of the simpler component reactions of tea-fermentation before embarking on the much more complicated *in vivo* system. It is of course desirable that both aspects of this work should receive attention and it is not surprising that we are unable to reach complete agreement under the circumstances.

As far as techniques are concerned we have largely relied upon manometric methods, paying careful attention to the possible sources of error mentioned by Dixon [1934]. Titrimetric methods have been found to be misleading [Barua & Roberts, 1940] and have not been extensively used. Lamb & Sreerangachar on the other hand have relied almost entirely on the iodine method for polyphenol estimations, although some use has been made of a crude form of respirometer. They admit that the iodine method is not specific for polyphenols, but there is a further drawback in that the condensation of tannins following enzymic oxidation materially affects the titre [Barua & Roberts, 1940].

We have evidence, shortly to be published, which goes to show that whereas oxidation of tannins by the tea oxidase reaches its maximum rate at about 29°, condensation of the tannins accelerates continuously with temperature. At the higher temperatures of fermentation, therefore, extent of condensation is no longer primarily determined by the rate of oxidation and if the iodine titre is affected by condensations the fall in iodine titre is not a true index of tannin

oxidation. It is also likely that the effect of *pH* variance on oxidation and condensation may differ. Before Lamb & Sreerangachar's results on the kinetics of tea oxidase systems can be accepted I therefore feel it is incumbent on these workers to repeat their results using methods less subject to error.

It is quite likely that the errors in the iodine titres due to condensation are small, as the tannin solutions are relatively dilute when compared with fermenting tea-leaf. The concentration of tannin in the juice of leaf withered to 60 % moisture is about 13 %, which is nearly ten times that in the enzyme-substrate mixtures used by Lamb & Sreerangachar. This dilution must result in a very considerable reduction in the extent of condensation. In confirmation of this it has been shown that the Lowenthal permanganate or iodine titres of juice from fermented tea-leaf are considerably reduced if the juice is boiled, but if the juice is diluted 20-fold with boiling water and maintained at the boiling point for 1 hr. the titre is relatively little affected. This experiment indicates a continuance of condensation in the juice at the boiling-point, but not after dilution, where mass-action effects considerably lessen the chances of the necessary molecular collisions.

Results obtained with the crude respirometer by Lamb & Sreerangachar are of doubtful value, but no important findings are based upon its use.

(2) *Soluble and insoluble forms of the tea-oxidase and their kinetics*

Perhaps the most interesting of Lamb & Sreerangachar's findings concern the soluble and insoluble forms of the tea-oxidase. It would appear from the increase in the water-soluble form of the enzyme, after withering the leaf, that the water-soluble enzyme is another form of the insoluble enzyme and not an entirely different substance. It may be recalled that my previous results [Roberts, 1941] show that the normally water-insoluble oxidase becomes soluble as a result of withering. The soluble and insoluble forms have different kinetics, their temperature optima in Ceylon leaf are 49° and 27° respectively, and the optimum *pH* values are 6.8 and 5.4.

The relations of these two forms of the tea-oxidase are somewhat similar to those existing between coenzyme factor (diaphorase) and heart flavoprotein [Corran *et al.* 1939], in which the former is associated with insoluble particles and is inactivated by heat at 55°, whereas the water-soluble heart-flavoprotein must be warmed to 85° for inactivation. As the water-soluble tea oxidase is the more susceptible to inactivation by high concentrations of tannin, its different *pH* optimum may be due to the tannins having a greater inactivating effect at lower *pH* values.

The observation that the water-soluble oxidase is readily inactivated by tannins in high concentration suggests that it plays a comparatively unimportant part in tea-fermentation *in vivo*, for the tannin concentration is high in fermenting leaf. My own observations on the kinetics of tea-fermentation, which follow below, confirm this.

As already shown [Roberts, 1941], the optimum temperature for fermentation is about 29°, in good agreement with the value of 27° for the insoluble oxidase in Ceylon leaf. I have also carried out a series of manometric determinations of the variation of fermentation rate with *pH*. 200 mg. portions of finely ground tea-leaf were suspended in 2 ml. water + 1 ml. buffer (phosphate or phthalate) and the *pH* of the mixture determined with the Beckmann glass electrode *pH*-meter. Autoxidation was apparent at *pH* 6.5 and controls were therefore carried out with boiled tissue suspensions. Fig. 1 shows the relation between the *pH* of the system and the uptakes for the first 20 min. after subtracting the

autoxidation figures. The pH optimum is very close to that found by the Ceylon workers for their insoluble oxidase. As both temperature and pH optima for the insoluble oxidase agree very closely with the optima found for fermenting tea-leaf there is some foundation for the belief that the water-insoluble oxidase is largely responsible for the oxidation of tannins during fermentation.

Fig. 1.

Fig. 2.

The uptakes at pH 3.5 slow down considerably after the first 10 min. and the colour of the suspension remains greenish with only a slight tinge of brown. Addition of phosphate buffer pH 8, which brings up the pH to 5.2, leads only to a slight increase in the rate of uptake, and it is clear that incubation of the tea oxidase at pH 3.5 for 20 min. results in a considerable inactivation of the enzyme. On the other hand the enzyme is not destroyed at pH 7.2. Adjustment to pH 5.4 results in a complete recovery of oxidase activity.

As already stated autoxidation is observed at pH values >6.5 . The end-product of tannin oxidation under such conditions is no longer the *o*-quinone, and the more alkaline the solution the greater becomes the total uptake of O_2 . Fig. 2 represents the total uptakes after 90 min. over a range of pH from 3.5 to 7.3. At a low pH the total uptake is low because of inactivation of the enzyme. From pH 4.5 to 6.4 there is little if any effect on the total uptake, but at pH >6.5 , where autoxidation is appreciable, the total uptake increases rapidly with the pH .

(3) Substrate specificity

Perhaps the greatest differences between our results and those of the Ceylon workers concern the substrate specificity of the enzyme preparations. Lamb & Sreerangachar [1940] deny any oxidation by tea oxidases of quinol and *p*-phenylenediamine, and claim that the observed oxidation of ascorbic acid is due to adsorbed polyphenols. The reasoning in this latter case is not quite convincing, for if, as was shown by Deb & Roberts [1940], catechol is oxidized at

a higher rate than ascorbic acid by the tea oxidase, addition of catechol to enzyme + ascorbic acid must accelerate oxidation of the latter substrate as the *o*-quinone itself can oxidize ascorbic acid.

It is dangerous to draw conclusions from observations on the oxidation of substrates by crude enzyme preparations and the differences between our results here may not be so serious as at first appears. However it is necessary for the cytochrome theory of tea-fermentation for substrates other than *o*-dihydroxyphenols to be oxidized and hence the Ceylon findings are rather disturbing. In view of the probable inactivation by organic solvents of the cytochrome system during preparation, however, these observations may have little significance.

The existence of a cytochrome system in fermented tea-leaf is strongly indicated by the finding that addition of substrates such as ascorbic acid and *p*-phenylenediamine to fermented tea-leaf suspensions, after oxidation of tannins has sunk to low levels (impoverishment), does result in a stimulation of the O_2 uptake. The increase in the oxidation rate shows that these substrates are oxidized directly and not through a tannin or other polyphenol as a carrier. It will be observed that the rate of oxidation of these substrates by 'impoverished' tea-leaf is much lower than that of tea-tannin (Table 1). The results are therefore not completely satisfactory as they might possibly be due to a heavy metal plus protein functioning as a pseudo-oxidase.

Table 1. O_2 uptakes in $\mu l.$ for 100 mg. fermented tea-leaf

	min.	...	15	30	45	60	75
1. Control			7	14	22	26	—
Control + P.P.D.			47	88	128	162	—
2. Control			15	26	—	42	50
Control + ascorbic acid			23	46	—	83	113

In the second experiment the uptake of O_2 was 17 $\mu l.$ in the 15 min. immediately preceding addition of ascorbic acid.

(4) *Effect of organic solvents on tea oxidase*

The very considerable extent of inactivation of the oxidase in Assam leaf that attends the washing of tea-leaf tissue with acetone or alcohol first suggested that a cytochrome system was operative in tea-fermentation. Even if the tissue is cooled in ice-salt mixtures during treatment with these solvents inactivation is considerable. The same is true of enzyme preparations obtained by precipitating tea-leaf juice with acetone. The resulting enzyme has only a fraction of the original activity of the juice. For this reason it is unwise to infer the properties of the tea oxidase from those of its preparations obtained by treatment of tea-leaf tissue with acetone or alcohol.

The effects of organic solvents found in our experiments apparently stand in strong contrast to those found by the Ceylon workers. The enzyme preparations obtained in Ceylon all involve the use of alcohol or acetone and are carried out at room temperatures presumably not far from 20°. In view of the strong activities claimed by Lamb & Sreerangachar, who consider that their enzyme preparations contain the greater part of the original oxidase activity of the green leaf, it would appear that the effect of organic solvents is considerably less marked in Ceylon than it is in our case. This may represent a very real difference between Ceylon, and Assam materials.

In view of the above possibility it is perhaps unfortunate that the Ceylon experiments appear to be incompletely controlled. A comparison is made between the fermentation of freshly rolled leaf and of an enzyme-substrate mixture diluted with a considerable amount of water or buffer-solution. Such dilution may result in as much as a threefold increase in the rate of O_2 uptake [Roberts, 1940, 2], in which case the above results would show that a very substantial loss of activity attended the acetone treatment.

(5) *Carrier-nature of the tea oxidase*

At various times the non-identity with polyphenol oxidase of the main enzyme concerned in tannin oxidation has been stressed [Roberts, 1940, 1]. Apart from questions of solubility and inactivation by alcohol or acetone, the enzyme appears to bring about oxidation of polyphenols through a carrier. Two independent series of observations have led to this conclusion.

First it was observed that although catechol is oxidized by the oxidase in finely ground tea-leaf at a slower rate than tea-tannin, addition of this substrate to fermenting tea-leaf did not depress the rate of O_2 uptake as it would be expected to if catechol and tannin were in competition for the enzyme. Addition of catechol produced no immediate effect on the uptake of O_2 (Fig. 3), but after a time equivalent to that required for the oxidation of the greater part of the tannin, the rate persisted at a level approximately equal to that of catechol oxidation. This probably means that tannin oxidation proceeds nearly to completion before catechol oxidation begins. Such behaviour is consistent with the oxidation of substances by the oxidized form of a carrier, where oxidation-reduction potentials decide the order of oxidation, but is not generally observed when two substrates are in competition for the same enzyme.

Fig. 3. I, control. II, tea-fermentation plus added catechol ($M/100$).

The second series of observations concerns the variation of the rate of O_2 uptake in fermenting tea-leaf suspension with the degree of dilution of the tissue [Roberts, 1940, 2]. Differences in diffusion rate and variations in substrate concentration cannot explain the increase of Q_{O_2} with the dilution. The best explanation seems to be that at the higher dilutions there is a relatively greater percentage of the carrier in the reduced form, and that with the consequent

increase in true substrate concentration the uptake of O_2 becomes faster. Although the mechanism of this process remains obscure an explanation is to be found in the possible interaction between tannin and cytochrome. This will be considered in a later section.

(6) *Identity of the oxidase in Assam leaf with the cytochrome system*

It is clear from the results quoted by Roberts [1940, 1] and by Deb & Roberts [1940] that the oxidase in tea-leaf responsible for fermentation has many resemblances to the cytochrome system. The tea oxidase however has some properties which appear to distinguish it from the cytochrome oxidase of animal tissues. Thus it undergoes readier peptization and consequently can be freed to a certain extent from insoluble particles. Oxidation of such substrates as quinol and *p*-phenylenediamine is less marked than that of *o*-dihydroxyphenols. This finding may be compared with the statement of Okunuki [1939] that the indophenol (cytochrome) oxidase of lily pollen oxidizes cytochrome-*c* but not quinol. The tea oxidase appears less susceptible to organic solvents, for preparations involving the use of acetone can be employed to effect the oxidation of quinol, *p*-phenylenediamine and the Nadi reagent. The latter oxidation is inhibited by CO but this inhibition is reversed in the light. As such behaviour is typical of cytochrome oxidase it indicates that in plant tissues the enzyme may be only partially inactivated by organic solvents. The tea oxidase is comparatively insensitive to cyanide, $10^{-2} M$ HCN being required for maximum inhibition. Finally the enzyme has a lower optimum temperature and shows its maximum activity at pH 5.4. It is inactive at pH 3.5 and 7.2.

These very marked differences, however, do not justify us in rejecting the idea that a cytochrome system is responsible for tea-fermentation. Although Bhagvat [1939] has shown the wide distribution of the cytochrome system in plant tissues, there is comparatively little information available as to its properties and we certainly have no right to assume that the properties of the enzyme in the plant will agree closely with those in animal tissues.

Unfortunately the determination of cytochrome and its oxidase in the leaf has special difficulties. Spectrometric investigation is ruled out owing to the masking of the cytochrome spectrum by the chlorophyll. Removal of the chlorophyll involves washing with solvents which largely inactivate the oxidase, and mechanical separation seems of little effect owing to the association of the oxidase activity with chloroplastic material [Roberts, 1941]. Addition of cytochrome-*c* prepared from ox-heart has a slight retarding effect upon the oxidation of tannins by finely ground tea-leaf tissue. This failure to accelerate tannin oxidation however cannot be taken as proof that a cytochrome system is not responsible for tea-fermentation. The possibility arises that the system already contains sufficient cytochrome to bring about the maximum rate of tannin oxidation. It has also to be borne in mind that we are adding a foreign protein to the tea-leaf, and we already have evidence [Roberts & Sarma, 1940] which shows that tea-tannin has a marked inhibitory effect on catalytically active proteins (i.e. enzymes) from other plant sources. As the enzymes of the tea-leaf itself are but little affected by tea-tannin it appears that they possess some type of immunity towards tea-tannin. It is therefore not unlikely that cytochrome-*c* from ox-heart may be inactivated by tea-tannin.

Although we have been unable to produce direct evidence of the presence of cytochrome in the tea-leaf it has been possible to demonstrate its presence in the basal portion of the stem. Thanks are due to Miss K. Bhagvat who undertook a spectroscopic investigation of this tissue for us. An absorption band

corresponding to the *b* band of cytochrome was observed in the green region of the spectrum. On adding succinate and cytochrome-*c* to the finely ground tissue the bands of reduced cytochrome were visible but disappeared on vigorous shaking. Unfortunately the oxidase activity of the tissue was too low to confirm the presence of the cytochrome system manometrically, but the spectroscopic results give clear evidence of the presence of both cytochrome oxidase and succinic dehydrogenase in the tea-plant. Efforts are now being made to observe the spectrum in etiolated tea seedlings.

Perhaps the most serious objection to the cytochrome theory of tea-fermentation is to be found in the very low cyanide sensitivity of the tea-oxidase. Deb & Roberts [1940] have attempted to explain this in terms of competition between cytochrome and other iron compounds for the cyanide, but the observations of Lamb & Sreerangachar [1940], who show that peroxidase activity in fermenting tea-leaf is inhibited by 10^{-5} *M* HCN, render this hypothesis an unlikely one, for the sensitivities of cytochrome oxidase and peroxidase to cyanide are generally observed to be of the same order. It therefore appears that the tea oxidase has a relatively low sensitivity to cyanide. The few data available on other plant tissues [see Deb & Roberts, 1940] show that low sensitivity to cyanide is not confined to the tea oxidase. It seemed desirable to extend these observations, and Table 2 records the inhibition of various plant oxidase systems by HCN. It will be noted that maize embryos retain some oxidase activity in the presence of 10^{-2} *M* HCN. It has been shown by Bhagvat [1939] that germinating maize contains a relatively powerful cytochrome oxidase, so the possibility that plant cytochrome systems have a low cyanide sensitivity becomes much more likely.

The low sensitivity to cyanide may of course be due to impurities associated with the enzyme preparations, although the suppression of peroxidase activity in tea-leaf by 10^{-5} *M* HCN makes this unlikely. Further, Wieland & Sutter [1930] have shown that the potato oxidase (polyphenol oxidase) is 50% inhibited by 5×10^{-4} *M* HCN. The balance of evidence therefore suggests that respiratory oxidases in plant tissues other than polyphenol oxidase and peroxidase require about 10^{-2} *M* HCN for maximum inhibition.

Table 2. *Inhibition of plant oxidases by HCN*

Cyanide conc. ...	<i>M</i> /2000	<i>M</i> /1000	<i>M</i> /500	<i>M</i> /100
	%	%	%	%
Tea (fermentation)	45	—	72	85
Tea (respiration)	65	—	73	78
Tobacco (respiration)	—	—	34	58
Maize embryos (respiration)	—	61	—	75
<i>Eugenia balsamea</i> (fermentation) (i.e. tannin oxidation in finely ground leaf tissue suspensions)	—	25	—	77

The percentage inhibition in nearly all the above cases increases with time.

There is no information available as to the *pH* optima of cytochrome systems in plant tissues, although Gräff [1922] has shown that the optimum *pH* range for the Nadi-reaction is on the acid side of neutrality for leaf-tissue, but on the alkaline side for animal tissues. It has also been shown in this laboratory that the oxidation of tannins in a fine mince of *E. balsamea* is optimal at *pH* 5.5. The reversibility of the inhibition at *pH* 7.2 of the tea oxidase suggests that with substrates other than tea-tannin the enzyme might be active at higher *pH* values.

It will be seen that what little information there is on cytochrome systems in the leaf, or on systems which may possibly be identified with the cytochrome system, does indicate that there are some differences in its properties when compared with those of the animal cytochrome system. More information is needed on plant oxidase systems, but in the meantime it is claimed that the properties of the tea oxidase are such as to justify its tentative identification with a cytochrome oxidase.

In conclusion the assumption that cytochrome acts as a carrier in the oxidation processes in the tea-leaf enables a new explanation of the concentration effect, described by Roberts [1940, 2], to be made. It has already been suggested that tea-tannin may combine with the cytochrome-*c* of ox-heart and so render it ineffective as a carrier. Combination of tea-tannin with the cytochrome of the tea-leaf is also a possibility, but is not likely to be so complete, by analogy with the effect of tannins on plant oxidases. The amount of free, uncombined cytochrome in suspensions of finely ground tea-leaf may therefore be determined by mass action effects, and in the more dilute suspensions its concentration would be expected to be relatively higher. On this hypothesis, as dilution increases the amount of free cytochrome per unit quantity of tissue also increases, and the initial rate of O_2 consumption may also be expected to increase.

(7) *The oxidase system in Ceylon leaf*

Lamb & Sreerangachar [1940] have amassed a considerable body of evidence which seems at first sight to make it unlikely that cytochrome oxidase is responsible for tannin oxidation in their leaf.

Some of their observations, notably the failure of cytochrome-*c* from ox-heart to promote more rapid tannin oxidation, and the low sensitivity of tannin oxidation to cyanide, have been confirmed, but it is not considered that these facts are incompatible with the functioning of a cytochrome system in tea-fermentation, as was shown in the preceding section.

It must be remembered that all the Ceylon workers' results were obtained with enzyme preparations in which the tissue had been treated with acetone or alcohol at a temperature of probably about 20° . This treatment would have led to a marked decrease of cytochrome oxidase activity, despite the apparent resistance of the plant oxidase to destruction by organic solvents. The failure to obtain evidence for the oxidation of quinol and *p*-phenylenediamine by their enzyme preparations is therefore not surprising. Using the sensitive manometric method we obtain only weak activity towards these substrates with our preparations, where the treatments with alcohol or acetone have been carried out at 0° .

I have already cast doubt on the claim made by Lamb & Sreerangachar that their enzyme preparations contain practically the whole of the original oxidase activity of the tissue. However, it does not appear unlikely that the enzymes of the Ceylon leaf are rather more resistant to organic solvents than those in Assam leaf, and from the figures quoted by Lamb & Sreerangachar there is reason to believe that catechol may be oxidized by the Ceylon preparations at a higher rate than tannin, although this requires further confirmation. With our preparations the reverse is the case.

This could be taken to imply that different oxidase systems are concerned in the tea-leaf from Ceylon and Assam respectively, but I am reluctant to advance so extreme a hypothesis.

On the other hand we know from the work of Boswell & Whiting [1938] and Steward *et al.* [1940] that other respiratory enzymes in addition to the polyphenol

oxidase are concerned in the respiration of the potato tuber, and in many animal tissues it is impossible to make cytochrome oxidase responsible for the total consumption of O_2 . Now the conditions under which tea is grown vary considerably, and in the high altitudes in Ceylon and the plains of Assam not only are there taxonomic differences in the bushes to start with, but there are also considerable differences in environment, soils and the climatic conditions. In Assam the bushes are pruned annually and for three months in the year there is a dormant period in the growth. In Ceylon, on the other hand, growth is fairly continuous throughout the year and the bushes are pruned at three- or four-year intervals. Such differences may be expected to have their effect on the relative proportions of oxidizing enzymes in the leaf, and it is possible that the cytochrome oxidase plays a rather less dominant role in Ceylon leaf than it does under our conditions. The existence of a bush, whose leaves fail to undergo the colour changes characteristic of fermentation unless a tea-oxidase from other bushes is added to them, shows that the oxidase responsible for tannin oxidation is not the only one concerned in the respiration of the tea-leaf. This observation, in my opinion, provides convincing evidence of the existence of more than one oxidase system in the tea-leaf.

The suggestions made are tentative only, and the question of the true nature of the tea oxidase is left an open one. It may be thought that the evidence for a difference between Assam and Ceylon leaves is rather tenuous. My intention is merely to point out that such differences may possibly be expected and that there are already indications which point in this direction. It is clear that much further work both on the tea oxidase or oxidases and on plant oxidases in general is needed. One additional point however may be made. It has recently been shown by Tuzimura *et al.* [1940] that riboflavin occurs in Japanese green teas. This is a strong point in favour of the presence of cytochrome in the tea-leaf and it suggests that the normal cytochrome-flavin-phosphopyridine chain is concerned in the transfer of O_2 to the respiratory substrates.

DISCUSSION

From consideration of the results obtained in this laboratory only, the available evidence goes to show that the oxidase responsible for tea-fermentation is probably to be identified with a cytochrome system. Differences in the properties of this enzyme from those of the cytochrome oxidase of animal tissues exist, but it must be emphasized that little is known to date of cytochrome oxidase in plant tissues, and almost nothing of the oxidase in the leaf.

The results obtained in Ceylon do not differ seriously from our own except in the matter of substrate specificity. The data also suggest that catechol is oxidized faster than tannin by the enzyme in Ceylon leaf, and that organic solvents have a less pronounced effect on the activity of the oxidase than in our experiments. These differences if they are confirmed would suggest that at least two oxidases are concerned in the oxidation of tea-tannin, and that the relative proportions of these oxidases may be affected by differences in cultural and climatic conditions such as exist between the plains of Assam and the hills of Ceylon. Although this possibility must be borne in mind the evidence is by no means conclusive and the observed differences may be a consequence of the use of different techniques in the estimation of enzymic activity.

SUMMARY

The activity of the oxidase in fermenting tea-leaf is maximal at pH 5.4. The enzyme is destroyed at pH 3.5, but although activity at pH 7.2 is low, readjustment to pH 5.4 restores the full activity.

The properties of the tea oxidase are compared with those of cytochrome oxidase and reasons are advanced for supposing that cytochrome systems in plant tissues differ in several important respects from those in animal tissues.

There may be more than one oxidase in the tea-leaf, and it is suggested that variations in the relative amounts of these oxidases under varying conditions of culture may account for some of the possible differences in properties found in Assam and Ceylon leaf.

9. THE RELATION OF TEA-FERMENTATION TO RESPIRATION

BEFORE discussing the mechanism of the tea-fermentation process and its relation to respiration it is necessary to deal with the extent of oxidation of the tannins that takes place as the result of fermentation, and to consider the extent of mechanical damage to the tissue.

Statements have been made in earlier papers [Roberts, 1939, 2; Harrison & Roberts, 1939] as to the amount of O_2 consumed in tannin oxidation when fermentation is complete. These may now be amplified.

The total amount of O_2 consumed in fermentation may be determined manometrically; after 60 min. shaking the rate of uptake is low and the total uptake after 90 min. is very little lower than the maximum attainable. Total uptakes have been determined in 52 different experiments, each in triplicate or quadruplicate, and the average value from all these results is $11.85 \mu\text{l./mg. dry weight}$ with a standard error of $0.11 \mu\text{l.}$ only. Some of this O_2 is used up in carbohydrate oxidation and assuming the oxidation to follow the conventional course, $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$, the amount of O_2 used up in oxidizing tannins may be obtained by multiplying the total uptake by $(1-x)$ where x is the R.Q. From 22 separate experiments the R.Q., when 200 mg. of finely ground tea-leaf are suspended in 3 ml. water at 36° , is 0.367 ± 0.007 . The average consumption of O_2 in tannin oxidation is therefore 11.85×0.633 which amounts to $7.5 \mu\text{l./mg. dry weight}$.

Several thousand tannin contents of green tea-leaf have been determined at Tocklai in the last 20 years and the average figure 22% or $0.22 \text{ mg./mg. dry weight}$ has an entirely negligible standard error. Assuming a molecular weight of 300 for tea-tannin [Harrison & Roberts, 1939] this is equivalent to an O_2 consumption of 0.91 atom per mol. In view of the assumptions made we can say with some confidence that 1 atom of O_2 is consumed for every molecule of tea-tannin.

In these manometric experiments the leaf is ground to a fine paste. The point has been raised that we cannot be certain that a considerable proportion of the tissue has not escaped damage. The CO_2 produced could then be considered as due to the respiration of intact cells, in which case it must be assumed that only 63.3% of the cells have been ruptured. Our calculation now leads to the conclusion that 1.44 atoms of O_2 are consumed per mol. of tannin. This is highly improbable and could only be accounted for if our estimate of the mol. weight of tannin were seriously at fault.

The supposition is made unlikely when we consider the extent of tannin oxidation in fermenting tea-leaf under factory conditions. With the twisted leaf obtained after mechanical rolling in the factory, fermentation slows down considerably after 2–3 hr. although enzymic activity is still high. This suggests completion of oxidation of the substrate. If the fermented leaf be finely ground to a paste and then suspended in water there is a small further uptake of O_2 amounting to 0.6 to 3.0 μ l. O_2 /mg. dry weight. Assuming a round figure of 12 μ l. O_2 /mg. dry weight for complete fermentation this is equivalent to an uptake of 9.0–11.4 μ l. O_2 or 75–95 % completion of tannin oxidation in the fermented leaf. The same answer may be obtained in an entirely different way. Extraction of the tissue with hot water for 1 hr. results in almost quantitative removal of the unoxidized tannins, and if such an extract be added to finely ground fresh tea-leaf tissue, the O_2 consumed after 90 min. by the tissue is increased by an amount equivalent to the unoxidized tannin in this extract. It is always found that the observed increase agrees closely with the residual uptakes obtained by a fine grinding of the fermented leaf.

This close agreement not only proves that the leaf is sufficiently damaged in the mechanical rolling to realize approximate completion of the fermentation process, i.e. from 75 to 95 % oxidation of the tannins, but also shows that the fine grinding results in the oxidation of all tannins extractable by boiling water. It also follows that the damage inflicted on the tissue need not be sufficient to rupture each individual cell. Microscopic examination of well fermented tea-leaf reveals that the majority of the outer cell walls are intact. It is therefore probable that the effect of the twisting of the leaf in inducing fermentation is upon the internal arrangement of the cell.

The persistence of carbohydrate oxidation and production of CO_2 during fermentation might be considered to be due to normal respiration, but below we offer clear evidence to show that damage to the tissue, sufficient to bring about complete oxidation of the tannins, is also sufficient to cause complete suppression of respiratory activity. The carbohydrate oxidation observed in tea-fermentation is to be considered a secondary oxidation by the tannin oxidation products.

After completion of tannin oxidation in tea-fermentation, CO_2 production falls to a very low level, and in non-tanniferous leaves such as *Tropaeolum* [Roberts & Sarma, 1940] fine grinding of the tissue leads to almost complete inhibition of O_2 uptake. The most convincing evidence for the suppression of normal respiration by mechanical damage however is provided by measurements of the anaerobic fermentation [Deb & Roberts, 1940]. Production of CO_2 under anaerobic conditions can readily be demonstrated for the whole tea-leaf, but in the ground up paste used in manometric experiments no CO_2 production can be detected when the suspension is shaken in an atmosphere free from O_2 . Similarly, with well-twisted leaf such as is obtained by mechanical rolling in the tea factory, up to 98 % inhibition of anaerobic fermentation is observed. On the other hand, leaf passed through a coarse meat chopper retains about 30 % of its anaerobic fermentative powers and many green, undamaged pieces of tissue can be detected in the coarse mince which do not undergo the characteristic tea-fermentation without further bruising.

The secondary nature of the oxidation of carbohydrates in tea-fermentation is clear from the strict dependence of the rate of CO_2 production on the rate of tannin oxidation and from the suppression of carbohydrate oxidation by the addition of ascorbic acid, which reduces o-quinones as soon as they are formed.

It is perhaps desirable to present a balance sheet for the oxidation of carbohydrate during fermentation which shows that our assumption of the origin of CO_2 during this process is a valid one. To quote a typical experiment, the reducing sugars expressed as glucose fell from 2.32 to 1.13 % as a result of a 3-hr. fermentation. (The original withered leaf was free of starch and hydrolysable non-reducing sugars.) The complete oxidation of this quantity of glucose would be equivalent to an output of 8.89 $\mu\text{l. CO}_2/\text{mg. dry weight}$. Figures for the CO_2 output of fermenting leaf are available only for suspensions of finely ground tissue in water, but the amount of CO_2 evolved where the volume of the suspending fluid is zero can be obtained by extrapolation [Roberts, 1940, 2] and amounts to 8.90 $\mu\text{l. CO}_2/\text{mg. dry weight}$. The excellent agreement is probably fortuitous. However, in several experiments the mean fall in reducing sugars as a result of fermentation amounts to 1.20 % of the total dry weight, so that we are certainly justified in concluding that the CO_2 produced in fermentation comes largely from carbohydrate breakdown.

Recapitulating the above arguments the following experimental facts emerge.

- (1) In the course of tea-fermentation nearly the whole of the tannins is oxidized.
- (2) The mechanical damage necessary to cause complete oxidation of the tannins results in a complete suppression of normal respiration.
- (3) Such carbohydrate oxidation as occurs is a secondary oxidation with the *o*-quinone of tea-tannin as the H-acceptor.
- (4) After correcting for the amount of O_2 consumed in oxidizing carbohydrates, the uptake of O_2 corresponds to a consumption of 1 atom per molecule of tannin.

The *o*-quinone of tea-tannin if not reduced again is removed irreversibly from the system by condensation to form bodies with more marked tannin properties [Harrison & Roberts, 1939]. The main chemical changes in tea-fermentation may therefore be summarized:

or more shortly

and the process will continue until the whole of the tea-tannin has been converted into the oxidized-condensation products found in 'black' tea.

How are we to account for this effect of extensive mechanical damage on the respiratory mechanism of the plant cell? Reaction (4) emphasizes the fact that both oxidase and dehydrogenase activities are relatively unimpaired. Partial inactivation of dehydrogenases cannot account for the phenomena observed, as anaerobic fermentation in the bruised tea-leaf and respiratory activity in crushed non-tanniferous leaves would otherwise be noticed. It therefore appears that carriers between oxidase and dehydrogenase are affected by mechanical damage. As the phenomenon of pigmentation after damage is observed in systems containing the polyphenol oxidase, which do not require the assistance of a flavin in the oxidation of substrates, it is probable that some form of coenzyme inactivation is responsible, this being the carrier common to polyphenol and cytochrome oxidase systems.

There is a difficulty in accepting this mechanism at first sight. The complete suppression of anaerobic fermentation by extensive damage requires complete destruction, or rather dispersal, of coenzymes, whereas the relation of carbohydrate oxidation to *o*-quinone concentration requires some coenzyme activity. It was suggested by Roberts & Sarma [1940] that the *o*-quinone of tea-tannin might function as a H-acceptor for dehydrogenase action without the need for a coenzyme. However, it has now been shown that addition of catechol or pyrogallol to fermenting tea-leaf materially increases the CO₂ production (Fig. 4). As Dixon & Zerfas [1940] have shown that *o*-quinone itself will not function as a H-acceptor in the absence of a coenzyme it is evident that the concentration of coenzymes in fermenting tea-leaf is sufficient to ensure secondary oxidation of carbohydrates by the *o*-quinones of tea-catechins.

Fig. 4. I, O₂ uptake, control fermentation. II, CO₂ output, control fermentation. III, O₂ uptake with added catechol (*M*/100). IV, CO₂ output with added catechol.

It is now possible to account for this apparent inconsistency. In the intact cell oxidation of substrates probably takes place at interfaces consisting of a complex mosaic of protein and phosphatide molecules. Adsorption of coenzymes at specific points adjacent to both oxidase and dehydrogenase is necessary before substrate oxidation can occur. The organization of such an active surface can be destroyed by a shearing stress quite inadequate to rupture the comparatively robust cell wall. In this connexion it may be remembered that Phillis & Mason [1937] have shown that the result of a slight shearing stress on leaf tissue has a very marked effect on the composition of the continuous phase in the cytoplasm, and hence on the permeability to solutes of the cytoplasm. With the organization destroyed the chances of oxidase, carrier, substrate and dehydrogenase all being in sufficiently close contact to effect oxidation of the substrate are very considerably reduced and respiratory activity in consequence is largely inhibited.

The fermentation process however does not need this organization. After mechanical damage to the cell the tannin is no longer confined to the vacuole but penetrates into the cytoplasm so that it is in intimate contact with the oxidase system, and *o*-quinone formation will in consequence be rapid. The coenzymes like the tannins are probably in homogeneous solution and must be largely

oxidized by the *o*-quinones and will be able to function as H-acceptors in substrate oxidation by the dehydrogenases. The presence in the system of a relatively high concentration of *o*-quinones provides the necessary link between oxidase and dehydrogenase which was lost when the organization of the cell was destroyed. Under these conditions some dehydrogenase activity will be apparent until the *o*-quinones have been irreversibly lost by condensation.

That *o*-quinone concentration is correlated with the rate of carbohydrate oxidation is shown by the variation of the R.Q. with the dilution of tissue suspensions [Roberts, 1940, 2]. As the dilution increases, the concentration of *o*-quinone and hence also of the oxidized form of the coenzymes decreases, so that carbohydrate oxidation becomes relatively less rapid.

The effect of withering on the R.Q. of fermenting tea-leaf

Measurements of the R.Q. of both fresh and withered tea-leaf have shown that the R.Q. of the undamaged tissue is 1.0 within the limits of experimental error. The R.Q. for a suspension of finely ground tissue equivalent to 15 mg. dry weight per ml. is 0.37 for both fresh and withered leaf. The variation of the fermentation rate with dilution [Roberts, 1940, 2] is also the same for both fresh and withered leaf.

These experimental facts are re-emphasized in view of the statements made by the Ceylon workers. It is as easy to obtain a good fermentation with unwithered leaf as it is with withered leaf, in fact in some parts of North-east India where conditions for satisfactory withering are rarely obtained the withering process is omitted. It is certainly easier to produce the necessary damage to withered leaf, but fresh leaf despite its turgor can be dealt with satisfactorily and the fermentation times required are if anything somewhat shorter than after withering.

It is considered that withering has an effect, although a small one, on the rate of fermentation [Roberts, 1941], but none of our results indicates the profound effects hinted at by Lamb & Sreerangachar [1940]. The results of Evans [1928] quoted by these authors cannot be accepted. The method by which these figures were obtained is not disclosed, but it is well known [Dixon, 1934] that measurements of gaseous interchanges are liable to very serious errors if gaseous diffusion is not very carefully controlled.

SUMMARY

The effect of mechanical damage to the tea-leaf is to suppress normal respiration and anaerobic fermentation almost completely. This damage need not involve mechanical rupture of each individual cell. With a suitable shearing stress the organization within the cell is so destroyed that the coenzymes can no longer function as links between oxidase and dehydrogenase. A second consequence of the destruction of the organization of the cell is the penetration of the cytoplasm by the tannins.

The tannins are now oxidized directly by the oxidase system. Oxidation to the *o*-quinone stage is complete and condensation following this oxidation converts the tannins into a series of brownish-red pigments. When in the *o*-quinone stage the tannins can effect secondary oxidation of carbohydrates with the aid of the coenzymes dispersed through the tissue. During fermentation carbohydrate oxidation is only observed while *o*-quinones are being formed, and is stopped by removal of the *o*-quinones by condensation or reduction with ascorbic acid.

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