CIV. THE FERMENTATION PROCESS IN TEA MANUFACTURE

II. SOME PROPERTIES OF TEA PEROXIDASE III. THE MECHANISM OF FERMENTATION

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II. SOME PROPERTIES OF TEA PEROXIDASE

In the first paper on this subject [Roberts & Sarma, 1938] it was shown that during fermentation tea tannin was oxidized by peroxidase and H_2O_2 . Since this paper was written a considerable amount of experimental data on tea peroxidase has been accumulated from which it is possible to draw some conclusions as to the mechanism of the fermentation process.

Peroxidase content of green leaf

Peroxidase activities are determined by the method previously described. Except where otherwise stated, these activities are expressed in terms of so many indophenol units (I.U.) per g. dry weight of tissue and are determined using 10 g. samples of leaf. There are great variations in the activities of single shoots from the same bush, and in representative samples from different bushes in the same plot. Thus eight single shoots from one bush, weighing from 0.24 to 0.63 g., had peroxidase activities of 511, 549, 607, 710, 808, 880, 941 and 1040 I.U. Similarly the average peroxidase contents of each of 12 bushes selected at random from a small plot of tea were 218, 237, 250, 274, 278, 298, 311, 311, 314, 322, 385, 409 I.U. Despite these variations, if the leaf from a whole plot amounting to about 20–30 lb. is bulked, and 10 g. samples are taken for analysis, quite good replication is obtained. The mean activity of four such samples gives quite an accurate measure of the average peroxidase activity of leaf from the whole of a plot. It was rare in a whole season's observations that any one sample varied by more than 10% from the mean of the four and the average standard error of the mean was about 3%.

There are marked variations from week to week in the peroxidase activity of leaf plucked from the same plot over the whole manufacturing season as is shown in the curve (Fig. 1). The highest recorded value is 950 I.U. and the lowest 300 I.U. Despite these variations there is little or no change in the rate of fermentation, from which it follows that peroxidase activity is not the sole factor governing this rate. This seasonal curve for peroxidase activity falls into three well-defined parts, a period lasting till 20 June 1938 when peroxidase activity falls rapidly to a minimum, an intermediate period lasting until 3 October 1938 when the activity remains at a fairly constant level, and a final period where activity rises to a maximum of 757 I.U. and then falls steadily to

values of about 500 I.U. It is interesting to note that these three periods correspond closely with three quite sharply contrasted types of teas. The first period corresponds with the early "second flush" teas characterized by good quality, the second with the typical "rains" teas of no marked flavour and less strong liquoring properties, and the third with "autumnals". It is possible that the variations in peroxidase content are an index of physiological conditions of the leaf associated with these three types of teas.

Fig. 1. Seasonal variation of peroxidase activity.

Influence of variety on peroxidase content

Certain "jats"¹ of tea are characterized by a relatively slow rate of fermentation. Thus Kharikatia and Singlo leaf ferment more slowly than Betjan. The tannin content of all three varieties is much the same, so that it would seem quite likely that an enzyme deficiency would account for any slowness in fermenting. However, differences in peroxidase content are not big enough to account for these differences, as the following figures show.

Table I

	Betjan	Kharikatia
	I.U.	I.U.
27. vi. 38	465	480
4. vii. 38	521	419
22. viii. 38	552	523

It follows that peroxidase and tannin contents alone do not determine the rate at which fermentation proceeds. Further evidence for this is a complete lack of correlation between the strength of liquors in teas manufactured from single bushes and the peroxidase content of the green leaf from these bushes. If peroxidase content did determine the rate of fermentation some such correlation might be expected. The author is indebted to Dr Wight for much of the data for these comparisons.

Peroxidase content of different parts of the shoot

Shoots consisting of three leaves and a bud were plucked and the peroxidase contents of the various parts determined.

¹ A "jat" of tea is best defined as the progeny of the seed obtained from a commercial seed-garden. There is such complete hybridization nowadays that a pure line variety cannot be obtained.

Table II

	Peroxidase content I.U. per g. dry wt.
Bud	268
1st leaf	486
2nd leaf	556
3rd leaf	574
Stalk	493

The increase of peroxidase content with the size of the leaf confirms earlier findings that old leaf, too coarse for plucking, has a higher peroxidase activity than new shoots. Despite its lower peroxidase content, bud ferments at the same rate or even faster than the first leaf when these parts of the shoot are taken separately. This has been shown both by the rate of decrease in the tannin titre and, manometrically, by measuring the rate of oxygen uptake.

Properties of tea peroxidase

Temperature coefficient.

A series of peroxidase activity determinations was made, using the same solution of peroxidase, over a range of temperatures from 15° to 37°. A straight line relationship was found and the activity P_T at any temperature T was related to P_0 the activity at 25° by the equation

$$P_T = P_0 [1 + 0.02 (T - 25)].$$

The low value for the temperature coefficient is perhaps due to increasing competition at higher temperatures of the catalase, always present, for the H_2O_2 .

Inhibitors.

The effect of different concentrations of various inhibitors is recorded below:

Table III

NaF	I.U.	$C_6H_5NH.NH_2$	I.U.	Ascorbic acid	I.U.	N_2H_4	I.U.
Nil	581	Nil	581	Nil	743	Nil	542
M/200	196	M/2000	96	M/20	Nil	M/2500	542
M/50	143	M/500	Nil	—	—	M/1000	439
M/20	104	M/100	Nil	—	—	M/500	379
—	—	—	—	—	—	M/50	130

Despite the complete inhibition of peroxidase activity by low concentrations of phenylhydrazine, this inhibitor has very little effect on the rate of fermentation. There is a slight initial inhibition, after which fermentation proceeds at its normal rate. The phenylhydrazine is probably fixed by the carbohydrates present, after which it can exert no further inhibitory effect. Phenylhydrazine inhibition of peroxidase is reversible, judging by the initial inhibition and the complete recovery of the fermenting system from the effects of the inhibitor.

Freedom from oxidase.

A peroxidase preparation is always liable to show a slightly positive oxidase reaction if traces of H_2O_2 are present. A more specific test for oxidase is its ability to catalyse oxidation of catechol by atmospheric oxygen. Manometric experiments fail to show any O_2 uptake when catechol is acted upon by a tea peroxidase preparation. It may therefore be concluded that the tea leaf is free from polyphenol oxidase.

Increase of peroxidase during withering

The increase in peroxidase content of green leaf after withering, mentioned in the preceding communication, has been repeatedly confirmed. Loss of moisture alone is not responsible for this increase, as leaf kept in a closed vessel, in which the air is maintained at a 100 % relative humidity, still increases in peroxidase activity although not to the same extent as it does in a normal wither. The withering process itself therefore has some effect in determining the increase in peroxidase.

Table IV

Fresh leaf 76.82 % moisture	Leaf kept 24 hr. at 100% R.H.	Withered leaf 68.10 % moisture
I.U.	I.U.	I.U.
420	603	614
478	588	635
449	603	699
449	588	667
Mean 449	596	654

Four 10 g. samples for peroxidase activity determination were taken in each case.

Many experiments have been carried out in which the increase of peroxidase has been determined under varying conditions of wither. The rate of withering is determined largely by the temperature and humidity of the air. This withering process can be controlled by varying its duration and the thickness of spread of the leaf. Irrespective of the thickness of spread there is always an initial rise in peroxidase. Eventually a maximum peroxidase activity is reached, after which it falls again, this fall being most marked with very thin spreads. Results are quoted in Table V giving the variations in peroxidase activity of leaf withered for 18 and 42 hr. at different thicknesses of spread.

Table V

Lb. of leaf per sq. yard	Peroxidase content		Moisture content	
	18 hr.	42 hr.	18 hr. %	42 hr. %
$\frac{1}{2}$	665	237	59.56	24.13
$1\frac{1}{2}$	733	379	66.82	51.84
3	784	536	70.26	60.46

The original green leaf contained 570 I.U. per g. dry weight and had a moisture content of 74.30 %.

In experiments in which the rise in peroxidase was recorded at frequent intervals it was found that the rise was most rapid at the commencement of withering. Data of one such experiment, typical of several, are recorded in Table VI, and it will be seen that with a spread of 1 lb. per sq. yard the maximum activity is attained in 6 hr., while with a 3 lb. spread this maximum was not reached for 20-25 hr.

The peroxidase content begins to fall in both cases when the moisture content falls below 72 %. This latter figure must not be taken as a precise one, but there seems no doubt that the rise in peroxidase during withering is checked and then reversed when the moisture falls below a certain critical value.

To summarize these findings, loss of moisture up to a certain point accelerates the increase of peroxidase which takes place in leaf plucked from the bush, but

Table VI

Hours withering	1 lb. spread per sq. yard		3 lb. spread per sq. yard	
	Peroxidase	Moisture	Peroxidase	Moisture
0	423	79.37	423	79.37
2	480	77.54	552	78.52
4	548	77.14	692	78.45
6	618	76.25	716	77.75
8	578	75.54	—	—
12	585	72.56	—	—
15	591	72.26	—	—
24	472	66.95	781	75.34
30	—	—	724	71.80
51	—	—	650	70.70

this increase is checked by too rapid a loss of moisture. The maximum increases are observed when the loss of moisture is restrained by thick spreading of the leaf.

Effect of the increase in peroxidase during withering on the rate of fermentation

Were it not for the fact, already commented upon in this paper, that marked variations in the peroxidase activity of green leaf are generally without effect on the fermentation rate, it might be expected that withered leaf would undergo

Fig. 2. Tannin and non-tan titres during fermentation. I and III, fresh leaf; II and IV, withered leaf.

Fig. 3. I, Normal fermentation; II, Do. + 10 mg. tea tannin.

fermentation at a more rapid rate than green leaf. Determinations of the rate of fermentation in fresh and withered leaf were carried out both by measuring the rate of O_2 uptake and by the rate of decrease of the tannin titre. Typical results are illustrated in the accompanying curves (Figs. 2, 3). Details of the

manometric method will be given in Part III. In the tannin titration method a bulk of leaf was separated into two portions. One portion was spread to wither and the other passed through a mincing machine. Two 20 g. portions were at once infused with 500 ml. water and subsequently two such samples were taken and infused at intervals over a 4 hr. period. Tannins and non-tans (i.e. matter oxidized by KMnO_4 but not precipitated by gelatin and acid-salt) were determined in each of these infusions. The following day, after a 20 hr. wither in which the moisture fell from 75.40 to 58.80 %, a similar series of determinations was made after mincing the withered leaf.

There is no significant difference between the rates of O_2 uptake or of tannin oxidation in green and withered leaf. In both cases there is a slight separation between the two curves for green and withered leaf. This is not significant but might nevertheless be true, as during withering there is a loss of soluble matter by respiration which may amount to as much as 5 % of the total solid material of the leaf. This loss increases the amounts of other constituents of the leaf when they are expressed on a percentage basis.

During withering a certain amount of protein breakdown takes place. Protein-N in the leaf is determined by complete extraction of the non-protein nitrogenous matter with hot 85 % alcohol, followed by a Kjeldahl estimation of the residual N. This residual N is considered to be protein-N and decreases progressively throughout withering as shown in Table VII.

Table VII

Hours of wither 1½ lb. spread per sq. yard	Protein-N % on dry matter	Moisture content %
—	2.38 ± 0.02	74.30
18	2.19 ± 0.02	66.82
42	1.88 ± 0.05	51.84

The figures given for protein N represent the mean of six 10 g. samples of leaf.

As the decrease in protein-N is unaccompanied by any change in the rate of fermentation, the theory of Kursanov [1935] that protein degradation products protect peroxidase against the inactivating effect of tea tannin is effectively disproved.

DISCUSSION

It was concluded in the previous communication that H_2O_2 , and not an organic peroxide, is concerned in the oxidation of tea tannin by peroxidase. In the results presented here every line of evidence points to the rate of oxidation of tea tannin being completely independent of the peroxidase content between very wide limits. It must therefore be concluded that the peroxidase-catalysed oxidation of tea tannin in fermentation is preceded by a relatively much slower reaction, in which H_2O_2 is formed. Oxidation of tea tannin cannot proceed at a faster rate than the rate of formation of H_2O_2 , so that, as long as this latter reaction is unaffected, wide variations in peroxidase activity are without effect on the rate of oxidation of tea tannin.

H_2O_2 in respiring tissue is a normal by-product of aerobic dehydrogenations in which oxygen functions as the H-acceptor. During fermentation the non-tans undergo oxidation as well as tea tannin and it seems reasonable to suppose that the H_2O_2 required for tannin oxidation originates in this oxidation of non-tans. Since 1 mol. of H_2O_2 is needed to oxidize 1 mol. of tannin these non-tans should be present in at least equivalent proportions to the tannin, and further there should be approximate equivalence between the amount of tannin oxidized and

the extent of non-tan oxidation. Exact equivalence cannot be expected as the primary oxidation product of tannin may undergo further changes. These expectations are realized. The non-tans account for a substantial proportion of the Lowenthal total KMnO_4 titre, and frequently in the initial stages of fermentation the decreases in the tannin and non-tan figures are approximately equivalent. There is thus very strong evidence in favour of the following mechanism for the fermentation process.

The nature of these changes will be dealt with in Part III below.

SUMMARY

There is a marked seasonal variation in the peroxidase content of tea leaf, plucked at weekly intervals, from the same plot. The curve falls into three well-defined periods, each of which is associated with a particular type of manufactured tea.

Generally there is a complete lack of correlation between peroxidase activity and rate of oxidation of tea tannin in the fermentation process. It is concluded that the amount of peroxidase in the leaf is in excess of requirements. The controlling factor is the rate of production of H_2O_2 which is formed by the aerobic dehydrogenation of the non-tan oxidizable matter.

The increase in peroxidase content during the withering process is accelerated by loss of moisture from the leaf. Once the moisture falls below a critical level (72 %) peroxidase activity again declines. Protein-N decreases during withering and, as bruised withered leaf undergoes fermentation (tannin oxidation) at the same rate as bruised fresh leaf, the increase in the amount of protein degradation products has no protective effect against the inactivation of the enzymes by tea tannin.

III. THE MECHANISM OF FERMENTATION

The probable role of the non-tans in fermentation as the source of the H_2O_2 required for tea tannin oxidation has been pointed out above. Ascorbic acid suggests itself as the most likely substance to undergo such oxidation in view of the suggestions made by Huszák [1937] and Szent-Györgyi [1937] as to the respiratory mechanism of peroxidase plants generally. Ascorbic acid need not account for the whole of the non-tan titre as the dehydroascorbic acid formed by its oxidation will act as a H-acceptor in the oxidation of other metabolites, ascorbic acid itself being regenerated in this latter reaction. One molecule of ascorbic acid can in this way account for the oxidation of many molecules of such metabolites and there will be a large decrease in the amount of reducing substances (non-tans) with quite small amounts of ascorbic acid present.

Two lines of investigation are suggested by this hypothesis, the elucidation of the nature of the non-tans and the changes they undergo, and an enquiry into the role of ascorbic acid and ascorbic acid oxidase in the fermentation process.

The complete theory of the fermentation process must not only account for the chemical changes observed in fermentation but must also account for such

differences in the rate of fermentation as are observed with Betjan and Kharikatia leaf. Advantage was taken of this marked difference, experiments being made to decide the factor or factors responsible for this difference, in an attempt to gain some more insight into the nature of tea fermentation before proceeding with the lines of investigation just mentioned.

A comparison of the rates of fermentation in Betjan and Kharikatia leaf

Neither peroxidase nor tannin contents of Betjan and Kharikatia leaf are sufficiently different to account for any variation in the rate of fermentation. A greater toughness of Kharikatia leaf making it more resistant to the bruising effect of the rollers is also excluded, as with finely minced leaf where damage is more extensive the differences between the two persist as shown in Fig. 4.

Fig. 4. Tannin and non-tan titres during fermentation. I and III, Kharikatia; II and IV, Betjan.

Any possibility of a peroxidase inhibitor in the Kharikatia leaf has been excluded. Peroxidase inactivation is greater during the fermentation of Betjan leaf than it is with Kharikatia, owing probably to the greater enzymic-inhibiting powers of oxidized tea tannin. Further, a Betjan peroxidase preparation together with H₂O₂ oxidizes a green leaf infusion obtained from Kharikatia leaf at the same rate as an infusion of the same strength from Betjan leaf. Thus under conditions where H₂O₂ is introduced into both systems at the same rate the rate of fermentation is the same. It appears therefore highly probable that the difference in fermentation rate is due to a difference in the rate of production of H₂O₂ in the leaf. If the hypothesis is correct, that the production of H₂O₂ is due to an enzymic oxidation of ascorbic acid, then the differences between

Betjan and Kharikatia are understandable if the latter is deficient in ascorbic acid oxidase.

There is a certain amount of indirect evidence in favour of this hypothesis. The peroxidase preparation of Roberts & Sarma [1938] from tea leaf is almost without action on a green leaf infusion unless H_2O_2 be first added. In this enzyme preparation the green leaf has been exhaustively extracted with alcohol to free it from tannin, a procedure known to inactivate ascorbic acid oxidase [Srinivasan, 1935]. If the peroxidase be prepared from finely ground up tea leaf by the method of Manskaya [1935], in which tannin is adsorbed by hide powder, the enzyme preparation can oxidize a green leaf infusion directly without the addition of H_2O_2 . While not establishing that the factor producing H_2O_2 is ascorbic acid oxidase, this observation does indicate its possibility, and definitely establishes the presence of a non-tan oxidase. An enzyme preparation obtained in this way with hide powder from Kharikatia leaf oxidizes a green leaf infusion directly without the addition of H_2O_2 , but at a significantly lower rate than a similar preparation from Betjan leaf.

Manometric investigation of fermentation rate

Experimental technique.

In determining the rates of O_2 uptake of fermenting tea leaf by the Barcroft-Warburg method it is found most convenient to take 200 mg. portions of green leaf or 150 mg. portions of withered leaf. Such small samples of ordinary rolled leaf would be very unrepresentative. However, if samples of 40–50 g. are minced as finely and rapidly as possible, and the resulting mince well mixed, thoroughly representative samples of even less than 200 mg. can be taken. O_2 cannot diffuse into this fine mince, the surface layer only undergoing fermentation. O_2 uptake is however quite rapid if the leaf is suspended in 3 ml. water and the vessels are shaken at a speed of 90–120 oscillations per min. Until shaking starts O_2 diffuses very slowly into the leaf, so that during preparation of the vessels and attainment of temperature equilibrium comparatively little oxidation takes place. Unless otherwise stated, all experiments are carried out at 35.5°. Lower thermostat temperatures than this cannot be maintained during the Monsoon months. Uptakes are recorded in $\mu l.$ per 100 mg. tissue wet weight. Agreement between replicates is very good, the standard error of the mean with eight replications varying from 2 to 3% of the recorded uptakes. Normally all experiments are carried out in triplicate or quadruplicate, when differences of 10% or more are significant. The leaf itself contains sufficient buffering material to protect the system from any effects due to changes in pH. Comparable amounts of minced leaf were suspended in water alone and in buffers of pH 4.6, 5.6 and 6.6 respectively. In all four cases the rate of O_2 uptake was the same.

The completion of tannin oxidation in fermentation

O_2 uptake is initially quite rapid, Q_{O_2} may be as high as –20.0, but after $\frac{1}{2}$ hr. shaking the rate slows down considerably and after 1 hr. the uptake has come almost to a standstill. If fresh enzyme be added (the hide-powder peroxidase preparation containing “non-tan” oxidase) in amounts roughly equivalent to that originally present in the fermenting leaf, no further O_2 uptake is stimulated. On the other hand, as shown in Fig. 5, after addition of fresh substrate (a green leaf infusion), containing the same amount of tea tannin as was contained in the original portion of green leaf, there is a rapid O_2 uptake. The falling off in the rate of O_2 uptake is therefore due to the exhaustion of substrates. Fig. 6 shows

the greater uptakes recorded when 10 mg. of a tea-tannin preparation (86.4% pure) is added initially to the fermenting leaf. Fermentation does not come to a standstill till much later in this case, from which it can be concluded that the slowing down in the fermentation rate is due to the completion of the oxidation of the tannins. From the slope of the curve in Fig. 5 after the addition of fresh substrate it can be calculated that the enzymes in the fermented leaf retain about 80% of their activity.

Fig. 5. I, normal fermentation; II, fresh enzyme added; III, fresh substrate added.

Fig. 6. O₂ uptakes during fermentation. I, fresh leaf; II, withered leaf.

Production of CO₂

During fermentation a certain amount of CO₂ is produced as is shown by the lower net uptakes when no KOH is added to the central cups in the Warburg vessels. Very little CO₂ is retained by the suspension of fermenting leaf, so that the differences between the recorded uptakes with and without KOH gives an approximate figure for the CO₂ evolved if allowance is made for the differences in the constants of the vessels for O₂ and CO₂.

The CO₂ produced in the respiration of normal leaf is largely a product of carbohydrate oxidation, and it is not unreasonable to assume that in fermenting tea leaf it has a similar origin. The R.Q. of carbohydrate oxidation to CO₂ is 1.0, so that an estimate of the O₂ utilized in tannin oxidation is afforded by subtracting the CO₂ figures from the total O₂ uptake. Knowing the original amount of tea tannin in the tea leaf from its Löwenthal titre (1 ml. *N* KMnO₄ = 0.0416 g. tea tannin) and assuming that Tsujimura's [1930; 1931, 1, 2] structure of tea tannin, corresponding with a mol. wt. of 442 is correct, the number of atoms of O required by each molecule of tannin can be calculated from the O₂ consumed in tannin oxidation. To quote one case, one sample of leaf had a tannin titre of 130.4 ml. 0.04 *N* KMnO₄ per g. dry weight, and the total O₂ uptake recorded per g. dry weight in 1 hr. after subtracting the value for carbohydrate oxidation was

5584 μl . If the tea tannin requires 1 atom of O per molecule the uptake would be 5498 μl ., so that it can be concluded that in this particular case 1 atom of O only is required for complete oxidation. The average of six such calculations gives 1.08 ± 0.08 O which does not differ significantly from unity.

Fig. 7. I, total O_2 uptake; II, CO_2 output; III, O_2 consumed in tannin oxidation.

Fig. 8. I and III, Betjan; II and IV, Kharikatia.

There is always a marked parallelism between O_2 uptake and CO_2 output as is shown in Figs. 7 and 8. In the latter figure which gives the O_2 uptakes and CO_2 outputs of fermenting Betjan and Kharikatia leaf it will be seen that, with the slower-fermenting leaf, tannin takes longer to be completely oxidized and the rate of carbohydrate oxidation takes a correspondingly longer time to fall off. The ratios of the rates of tannin and carbohydrate oxidation are approximately the same for both types of leaf. This connexion between the rates of carbohydrate and tannin oxidation will have to find some explanation in any mechanism suggested for the fermentation process.

Identification of glucose as fermentable non-tan

The production of CO_2 in amounts very nearly equivalent to half the total O_2 uptake during fermentation suggests that the non-tans which are oxidized during fermentation are carbohydrates. The decrease in the KMnO_4 titre of non-tans and the amount of CO_2 produced in fermentation are approximately equivalent, as is shown by the equality of the ratios

$$\frac{\text{CO}_2 \text{ output}}{\text{O}_2 \text{ uptake}} = \frac{\text{Decrease in non-tan titre (in ml. KMnO}_4\text{)}}{\text{Decrease in tannin + non-tan titre}}$$

both of which are about 0.4 at the end of fermentation.

The majority of the non-tan oxidizable matter may be extracted from green leaf in the following manner, advantage being taken of the precipitation of tannins by lead acetate. The leaf is extracted with 80% alcohol and an aliquot of the extract representing 20 g. of green tissue concentrated on the water bath to 5–10 ml. 50 ml. of water are added and the solution washed into a 250 ml. flask. The extract is cleared by the addition of 3 ml. saturated neutral lead acetate after which it is made up to 250 ml. The solution is then filtered, de-lead with H_2S and again filtered. Aliquots of the clear filtrate are freed from H_2S

and are used for the determination of reducing sugars directly by the Shaffer-Hartmann [1925] method, results being expressed in mg. of glucose. Such an extract contains most of the oxidizable and all of the fermentable non-tan matter in green leaf, that is to say the whole of the non-tans oxidized during fermentation are present in this extract.

During fermentation there is a fall in the reducing sugar content of the leaf. Fresh green leaf in one experiment contained 3.12 % reducing sugar as glucose while the same leaf after fermentation contained only 1.40 % glucose. Both figures are expressed on a dry weight basis. Further evidence for the carbohydrate nature of the non-tan fermentable matter is its fermentation by yeast. CO_2 is produced at the same rate both from this extract and from a glucose solution of comparable strength. The reducing substance in the extract has been identified as glucose by the isolation in high yield of its osazone. It may therefore be concluded that the non-tan oxidizable matter which is oxidized in the fermentation of tea is glucose.

Ascorbic acid and tea fermentation

Tea tannin which seriously interferes with methods of ascorbic acid determination may be removed by addition of neutral lead acetate to a green leaf infusion. The ascorbic acid in the filtrate may then be determined by the method of Stevens [1938] in which 20 ml. of the solution are titrated with $N/100 \text{ I}_2$ after the addition of 4 ml. $12N \text{ H}_2\text{SO}_4$. Such estimations of the ascorbic acid content of green leaf indicate that 1 g. of fresh green leaf contains about 1 mg. ascorbic acid, but this must be regarded as a preliminary figure only. If excess ascorbic acid be added to the green leaf infusion the whole of this excess is found in the lead acetate filtrate, so that the above method can be employed to determine changes in ascorbic acid added to tea juice.

20 ml. of expressed tea leaf juice were mixed with 20 ml. of a 0.44 % solution of ascorbic acid and diluted to 100 ml. A further 20 ml. portion of juice was diluted with water and boiled for 5 min. after which 20 ml. of the ascorbic acid solution were added and the volume made up to 100 ml. Both mixtures were incubated for 1 hr. at room temperature (30°) with frequent shaking. Tea tannin was then removed by the addition of 5 ml. saturated lead acetate and ascorbic acid determined iodimetrically in 20 ml. portions of both solutions, and in a control portion of the original ascorbic acid solution treated in the same way with lead acetate.

Table VIII

	ml. $N/100 \text{ I}_2$
Control	19.1
Ascorbic acid + boiled tea juice	12.7
Ascorbic acid + unboiled tea juice	2.9

The unboiled tea juice brings about a much greater oxidation of ascorbic acid, which is evidence for the presence of a thermolabile catalyst of ascorbic acid oxidation in the tea leaf.

Addition of excess ascorbic acid to fermenting leaf has no significant effect on the rate of O_2 uptake, as is shown in Fig. 9. This rate however is maintained for a longer period and the total uptake is greater by an amount approximately equivalent to the ascorbic acid added. 1.60 mg. of ascorbic acid were added for each 100 mg. of fermenting leaf. This quantity of ascorbic acid requires $102 \mu\text{l. O}_2$ for complete oxidation and the increase recorded is $90 \mu\text{l.}$ which is in sufficiently close agreement.

For some 20–25 min. no CO_2 is produced whatever, indicating that no carbohydrate breakdown is taking place. During the same period the leaf remains bright green and there is no significant fall in its tannin titre. It was shown in the previous communication that such concentrations of ascorbic acid completely

Fig. 9. I and III, normal fermentation; II and IV, Do. + excess ascorbic acid.

inhibit peroxidase activity, so that it must be concluded that during this initial period of 20 min. the ascorbic acid inhibits both tannin and carbohydrate oxidation. The O_2 uptake during this period is 105 $\mu\text{l.}$, just equivalent to the excess ascorbic acid added, and one is justified in concluding that when such excess of ascorbic acid is present it is oxidized away, after which tannin and carbohydrate oxidation can take place as usual. During this period of ascorbic acid oxidation the rate of O_2 uptake is exactly the same as it is in normal fermentation. From this it can be concluded that the rate we are measuring in all manometric experiments on fermentation is that of the enzymic oxidation of ascorbic acid. The same experiment provides conclusive evidence of the role of ascorbic acid in fermentation and confirms the earlier conclusion that the rate of fermentation would prove to be the same as the rate of production of H_2O_2 . H_2O_2 is of course produced in the aerobic oxidation of ascorbic acid.

The hypothesis that Kharikatia leaf contains less ascorbic acid oxidase than Betjan can now be tested. As shown in Fig. 8, Kharikatia has a lower rate of O_2 uptake and hence it produces H_2O_2 at a lower rate. If the fermenting leaf from both "jats" be saturated with ascorbic acid (1.76 mg. per 100 mg. leaf) these differences in the rate of O_2 uptake should persist. The initial rates of O_2 uptake,

where the only reaction taking place is ascorbic acid oxidation, are quite different in the two cases, as shown in Fig. 10. As the substrate concentration is the same in both cases it must be concluded that the difference must lie in their ascorbic acid oxidase contents.

Fig. 10. I, Kharikatia + excess ascorbic acid; II, Betjan + excess ascorbic acid.

Variation of fermentation rate with amount of leaf

Normally when measuring the rate of O_2 uptake of respiring tissues the rate per unit weight of tissue is independent of the amount of tissue taken and of the volume of liquid in which the tissue is suspended as the reactions studied are surface reactions and not homogeneous.

In the particular case under consideration here, however, such variations have a significant effect on the rate of O_2 uptake. The smaller the amount of leaf taken the more rapid is the rate of the reaction per unit weight of tissue. It is therefore important to ensure that the same amount of leaf is taken for every experiment. Variations of 10 mg. in the amount of leaf weighed out are permissible, but greater deviations than this produce significant variations in the O_2 uptake.

Table IX gives the O_2 uptakes per 100 mg. leaf when quantities of 100 and 250 mg. of leaf undergo fermentation.

Table IX

Wt. of leaf mg.	15 min.	30 min.	45 min.	60 min.
250	99	182	204	213
100	132	190	204	209

Further investigation of this effect shows that, with smaller amounts of tissue, while tannin oxidation proceeds relatively faster carbohydrate oxidation is slowed down.

Table X

Wt. of leaf mg.		15 min.	30 min.	45 min.	60 min.
100	Tannin	84	110	107	111
	Carbohydrate	23	35	44	51
200	Tannin	60	102	117	122
	Carbohydrate	33	55	59	63

The figures give the O_2 uptakes in μ l. per 100 mg. fresh green leaf for tannin and carbohydrate oxidation respectively.

DISCUSSION

There seems to be no doubt that the first stage in the fermentation of tea is the enzymic oxidation of ascorbic acid, with the formation of dehydroascorbic acid and H_2O_2 . Peroxidase and H_2O_2 then oxidize the tea tannin. As each molecule of tea tannin takes up 1 atom of O only in its oxidation, and tea tannin contains the catechol grouping, the primary product of oxidation of tea tannin is most probably an *o*-quinone.

There is ample evidence that the primary product of oxidation undergoes irreversible condensations to form a series of products whose colours range from bright reddish-brown to dark brown. This subject will form the basis of a further communication at a later date. Meanwhile it can be taken that the *o*-quinone from tea tannin can be removed from the sphere of action by condensation.

The dehydroascorbic acid is an effective H-acceptor and will function as such in the dehydrogenations taking place during the oxidative breakdown of carbohydrates, the ascorbic acid being reformed in the process. A continuous regeneration of ascorbic acid must necessarily take place as about 20 mol. of tea tannin are oxidized for every mol. of ascorbic acid present in the leaf.

This scheme however fails to account for all the observed facts. There is no reason why carbohydrate oxidation should slow down when tannin oxidation has come to a standstill as the catalase in the leaf could deal with the H_2O_2 produced in the first stage of the process and prevent its rising to toxic concentrations. Further, all the reactions involved are enzymic and are thus heterogeneous, so that no explanation of the variations in fermentation rate with the amount of leaf taken can be advanced.

The simple scheme outlined above takes no account of the possible interaction between ascorbic acid and the primary oxidation product of tea tannin.

When this is considered it will be seen that two possible fates await the *o*-quinone on its formation. It can undergo an irreversible condensation or it can be reduced again to tea tannin by ascorbic acid.

The concentration at any moment of dehydroascorbic acid, on which the rate of carbohydrate oxidation depends, will therefore be determined partly by the concentration of the *o*-quinone. When tannin oxidation approaches completion the concentration of *o*-quinone will fall and with it the rate of oxidation of carbohydrate. The parallelism between tannin and carbohydrate oxidation can therefore be deduced as a necessary consequence of this reaction between ascorbic acid and the *o*-quinone.

This reaction is both homogeneous and bimolecular. If less than the normal weight of tissue be suspended in the normal volume of water in the Warburg

vessels the mass action effect will be to increase the relative concentration of the *o*-quinone and to decrease that of the dehydroascorbic acid. The net result will be to accelerate the complete transformation of tea tannin into its oxidized and condensed products and to decrease the rate of carbohydrate oxidation.

We may therefore write down the complete reaction scheme for fermentation as follows.

- (1) Ascorbic acid + O₂ $\xrightarrow{\text{ascorbic acid oxidase}}$ dehydroascorbic acid + H₂O₂.
- (2) H₂O₂ + tea tannin $\xrightarrow{\text{peroxidase}}$ tannin *o*-quinone.
- (3) *o*-Quinone → condensation products.
- (4) *o*-Quinone + ascorbic acid → tea tannin + dehydroascorbic acid.
- (5) Dehydroascorbic acid + glucose $\xrightarrow{\text{zymase}}$ CO₂ + ascorbic acid.

The "fermentation" of tea, like the browning of various other plant tissues on injury, is a case of decompensated respiration, and it should be possible to arrive at the mechanism of the true respiratory process in the tea leaf from a consideration of the "fermentation".

Respiration differs from "fermentation" in that the net change in the respiration process is the oxidation of carbohydrates to CO₂. No permanent changes in the tannins or other catechols take place.

If tea tannin can participate in normal respiration some explanation of its stability under these conditions must be found. While the R.Q. of respiring leaf is 1.0 that of fermenting leaf is initially about 0.3. Animal tissues show a similar fall in R.Q. after extensive damage. Thus the R.Q. of liver slices is 0.79, but finely minced ox-liver has an R.Q. of 0.37 as shown by Roberts [1936]. Further, the O₂ uptake of this finely minced liver was shown to be due almost entirely to purine base oxidation by xanthine oxidase. Unlike most dehydrogenases this enzyme can utilize molecular O₂ without the aid of a coenzyme, so that it would appear that the predominance of purine base oxidation in minced liver is due to a dispersal of coenzymes in the mincing process, leaving xanthine oxidase alone, with its full activity. A similar dispersion of coenzymes would be expected to follow the extensive damage done to tea leaf in the rollers or on mincing. The result of such a dispersion would be a slowing down of the dehydrogenations in which dehydroascorbic acid functions as the H-acceptor, and a consequent accumulation of dehydroascorbic acid in the system. This in its turn would involve a lower concentration of ascorbic acid and consequently a slower reduction of the *o*-quinone to tea tannin. If the *o*-quinone is not reduced as soon as it is formed it can then undergo further irreversible changes into condensation products and this process will continue until the whole of the tannin has been removed from the system.

Under this scheme respiration and fermentation differ only in the velocity of dehydrogenations taking place in carbohydrate breakdown.

The difficulty which stands in the way of accepting tea tannin as an O₂ carrier in normal respiration is its high concentration, accounting as it does for about 20 % of the total solid matter in the green leaf. Quercitrin also occurs in the tea leaf and in concentrations far more like those of O₂ carriers. In this case quercitrin would be the catechol compound oxidized by peroxidase in normal respiration, and tannin would be involved in the reactions only after damage to the tissue had permitted it to mingle with the other constituents of the respiration cycle.

It is at the moment impossible to decide between these two hypotheses but some observations of Mr C. J. Harrison favour the former. If green leaf is exposed to CHCl_3 vapour the leaf reddens and takes up O_2 very rapidly. This observation is readily explained by a greater sensitivity of dehydrogenases to the toxic effect of CHCl_3 . The consequent partial inhibition of carbohydrate oxidation would be expected on the above theory to lead to the production of condensation products of tannin and this is in fact observed. If green leaf is heated to 100° all the enzymes are inactivated and the leaf remains green. If, on the other hand, leaf is kept at $40\text{--}50^\circ$ it reddens. This reddening can be accounted for in the same way as the reddening after exposure to CHCl_3 vapour. The dehydrogenases are more susceptible to the inactivating effect of heat than the other enzymes concerned in respiration, so that moderately high temperatures, by a greater inhibition of carbohydrate oxidation, might be expected to cause some decompensation in respiration with a resulting formation of tannin condensation products.

SUMMARY

A complete reaction scheme for the fermentation process is deduced from the experimental data available. The H_2O_2 necessary for the oxidation of tea tannin by peroxidase originates in the aerobic oxidation of ascorbic acid, and this reaction controls the rate of the whole process. A shortage of ascorbic acid oxidase, as in the case of the Kharikatia leaf, results in a slower rate of fermentation.

The oxidizable non-tan which decreases during fermentation is identified with glucose. Dehydroascorbic acid functions as the H-acceptor in the oxidative breakdown of the latter.

One atom of O only is taken up per molecule of tea tannin during fermentation. The oxidation product then undergoes an irreversible change into condensation products.

The relation of the fermentation process to normal respiration is discussed.

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CORRECTION

In the first paper of this series *Biochem. J.* **32**, 1821, line 41, delete "infusion".