CCXXXVII. THE FERMENTATION PROCESS IN TEA MANUFACTURE

I. THE ROLE OF PEROXIDASE

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THE fermentation of the tea leaf is a stage in the manufacturing process of tea which has received but little attention in the past. Various qualitative studies have been made at the several Tea Research Institutes but no thorough investigation has been made apart from that of Oparin *et al.* [1935].

Results in this field have a considerable commercial use and they are also of interest 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.

Before dealing with the fermentation process itself it may be of advantage to outline the various stages in tea manufacture.

After plucking, the leaf is spread out in thin layers to undergo withering. During this process which usually takes about 18 hr. the leaf loses water and becomes flaccid. Unless this condition of flaccidity is produced the leaf is broken into flakes, instead of being twisted in the subsequent rolling process, and the resultant dry tea loses market value on account of its appearance. Not only is the withering process a physical preparation of the leaf for the further stages of manufacture but also some chemical changes may take place which partly determine the final quality of the manufactured tea. The nature of these changes however is still obscure.

The next stage in manufacture is rolling. The primary object of this process is to rupture the leaf-cells and to expose the expressed juices to the air. At the same time the tea receives its characteristic twist. As soon as the leaf tissue is in any way damaged fermentation starts, so that it is impossible to separate the rolling process from the next stage which is that of fermentation. After a period of about $1\frac{1}{2}$ hr. rolling the leaf is spread thinly on a fermenting floor, usually of polished concrete, where the oxidation process which commenced in the rollers is continued for perhaps another 2 hr., after which the tea is fired to stop fermentation and to dry the leaf.

During fermentation oxidative changes take place which largely determine the flavour and colour of the infused liquor. The amount of tannin extractable by hot water is considerably reduced, and some of the colourless tannin is transformed into red and brown products which arise from the original tannin, partly by oxidation and possibly partly by condensation. The precise mechanism of these changes is not yet properly understood.

Theories of the fermentation reaction

Although the fermentation process has been ascribed to simple oxidation, and, at a later date, to the activity of enzymes of micro-organisms on the leaf, there has been very little doubt since the work of Mann [1901; 1903; 1904] that

the oxidation is a process catalysed by the peroxidases of the leaf itself. The fermentation process is but another example of the general post-mortem changes that set in after damage to plant-tissues.

Fermentation will not take place *in vacuo* but starts as soon as oxygen is admitted into the system. The oxidative process takes place only within certain limits of acidity, the pH optimum being about 5–6. Acidification stops fermentation, while addition of alkali accelerates a non-enzymic autoxidation of the tannin bodies. Small amounts of KCN completely inhibit fermentation, as does a preliminary steaming of the leaf. All these observations are characteristic of an enzymic oxidation, and proof of this is afforded by the isolation of a peroxidase from the leaf which rapidly browns a green leaf infusion in the presence of H_2O_2 as is described later in this communication.

While there can be very little doubt that the oxidative process is catalysed by enzymes of the leaf, there is no doubt that micro-organisms do exert a very considerable influence on the quality of the final product. As yet only a lowering of quality is recognized as a result of the uncontrolled development of micro-organisms during fermentation, but this does not exclude the possibility that there may be some beneficial results.

Nature of the enzyme and substrate in the tea leaf

Having established with reasonable certainty that the fermentation of tea is an oxidative process catalysed by enzymes, the first thing to establish is the nature of the enzyme involved.

Using a press juice from fresh green leaf, artificial substrates such as the "Nadi" reagent do give a positive reaction for oxidase, but the development of colour as seen in a toluene extract is very much intensified by the addition of H_2O_2 . If the leaf is ground up with sand under alcohol the oxidase reaction becomes negligible while peroxidase activity is intensified. This intensification of peroxidase activity which is due to the removal of tannin will be discussed later. The disappearance of a weak oxidase reaction on washing with alcohol is probably due to removal of H_2O_2 formed in the leaf, small amounts of which are to be expected. Its presence would be revealed by a slight peroxidase reaction without the addition of further H_2O_2 . As a result, therefore, of qualitative tests oxidase cannot be detected in the tea leaf, while peroxidase activity is very marked. This confirms the observations of Manskaya [1935] who ascribed the oxidative processes in the tea leaf to peroxidase action; oxidase could only be found in negligible quantities. Any mechanism for the fermentation process must take account of this quantity of peroxidase and also a reaction involving oxygen uptake not catalysed by polyphenol oxidase must take place, as fermenting leaf takes up a considerable amount of oxygen.

The work of Szent-Györgyi [1937] and Huszák [1937] makes it very probable that in peroxidase plants oxygen is transferred to metabolites by the following reactions.

In normally respiring plant tissue the oxidation products are again reduced by activated H (dehydrogenase activity). It seems quite probable that the irreversible oxidations of damaged plant tissues are due to a dispersal of reducing

substances and coenzymes. Under these conditions the oxidation products formed in the above reactions find no activated H available and consequently undergo further transformations to form deeply coloured oxidation products of catechols.

It seems therefore very reasonable to suppose that in the fermentation of tea oxygen is absorbed, one of the products of oxidation being H_2O_2 , and that the tea tannin is then oxidized by the H_2O_2 with the catalytic assistance of the peroxidase. This theory has been tested experimentally and found to be absolutely correct.

EXPERIMENTAL

If tea leaf is finely minced it undergoes the fermentation reaction very readily even when suspended in water. If such a suspension of minced tea leaf is kept *in vacuo* no browning of the tissues is apparent for several days, whereas on exposure to oxygen the leaf assumes a brownish tinge throughout in 2 to 3 hr. If dilute H_2O_2 (such that the final concentration of H_2O_2 does not exceed $N/50$) is added slowly to the minced leaf suspension, stored *in vacuo*, fermentation starts at once, and the suspension soon becomes uniformly brown. Anaerobic fermentation is therefore possible in the presence of H_2O_2 . The same experiment shows that fermentation is probably catalysed by peroxidase.

It should be possible by mixing tea tannin with peroxidase and H_2O_2 to obtain the same chemical changes that occur in the actual fermentation process. Using a specimen of tea tannin kindly provided by Mr C. J. Harrison, and a tannin-free peroxidase extract from green tea leaf, the fermentation changes could not be simulated. Tannins, although tea tannin is not a true tannin, are known to have an inhibiting effect on peroxidase so that it was thought that the reason for this failure might be due to inactivation of the enzyme. Using a green leaf infusion instead of tea tannin, results indicating a satisfactory degree of oxidation were obtained. The infusion obviously contains substances which protect the enzyme from destruction by tea tannin.

On mixing the infusion, which, having been boiled, is free from enzymes, with a peroxidase solution, fermentation proceeds very slowly indeed, but on the addition of H_2O_2 the development of the brown fermentation colour is almost immediate. The optimum concentration of the H_2O_2 is about $N/200$. At higher concentrations the rate of fermentation is slowed down considerably and may even cease. To obtain the deepest brown colours it is best to add the H_2O_2 in small quantities at a time, never allowing it to accumulate in the system. This is in accordance with the well known susceptibility of peroxidase to H_2O_2 established by Willstätter & Weber [1926], Guthrie [1931], Mann [1931], and many others. H_2O_2 alone fails to oxidize tea tannin.

Tea tannin however only accounts for about 20% of the solid matter in a green leaf infusion, and the proper development of the fermentation colour in the experiment just described, while establishing the participation of peroxidase and H_2O_2 in the fermentation process, does not prove that it is the oxidation of the tannin to which this brown colour is due. Zender [1925] instances the oxidation of true tannins by oxidases so tea tannin is a likely substrate for peroxidase.

The same experiments were therefore carried out quantitatively, the tannin being titrated by KMnO_4 according to Löwenthal's method as modified by Carpenter & Harler [1922]. Incubation of a tea leaf infusion with peroxidase and H_2O_2 , added in successive small quantities, materially decreased the tannin titre, any excess H_2O_2 remaining being decomposed by the catalase also present in the peroxidase solution. Oxidation appeared to come to a standstill after the

addition of a certain amount of H_2O_2 (Table I), but the amount oxidized was increased either by doubling the amount of enzyme or by adding further enzyme and H_2O_2 when the oxidation was apparently complete (Table II). The apparent completion of oxidation was therefore due to destruction of the enzyme in the course of the fermentation, a point of practical importance when considering the optimal time of fermentation in the manufacturing process.

It will be noted, from the results of Table II, that when peroxidase is in excess, the decrease in the tannin titre is greater than that which corresponds to the amount of H_2O_2 added. This has been observed on each occasion that excess peroxidase was present, and it would seem to indicate that the enzymic oxidation product of tannin can undergo further oxidation without the assistance of H_2O_2 . It is well known that the oxidation of catechol derivatives rarely stops short at the first stage of oxidation, and subsequent stages of oxidation may take place readily without the aid of either enzyme or H_2O_2 .

KMnO_4 titration is not specific for tea tannin in an infusion and it is usual to subtract from these figures the results obtained for non-tans. The tannin, in a larger aliquot portion than that used for crude tannin estimation, is precipitated with gelatin, precipitation being aided by salting out with acid and NaCl . The KMnO_4 titre of an aliquot part of the filtrate gives the non-tan value. Determinations of crude tannin and non-tan after incubation with peroxidase and H_2O_2 show that non-tans are also oxidized to much the same extent as tea tannin itself (Table III). These non-tans may of course be catechol bodies. In these experiments a filtered peroxidase solution was used, as it was found that the end point with indigocarmine was much sharper than with the unfiltered solution.

Table I

ml. $N/20$ H_2O_2 added	Crude tannin titration ml. $N/20$ KMnO_4	Difference
—	9.45	—
2.0	7.65	1.80
4.0	6.20	3.25
6.0	6.05	3.40
8.0	5.85	3.60

An infusion of 10 g. green leaf in 100 ml. of water and a peroxidase extract of 100 ml. from a further 10 g. of leaf were prepared according to the directions given later in this paper. 10 ml. portions of these solutions were mixed and 0.5 ml. portions of $N/20$ H_2O_2 added at intervals of 10 min. After addition of the H_2O_2 the solution was titrated with $N/20$ KMnO_4 in the presence of indigocarmine.

Table II

	Fall in tannin ml. KMnO_4
A. 5 ml. infusion + 5 ml. peroxidase + 2 ml. $N/20$ H_2O_2	2.0 ml. $N/20$ KMnO_4
B. 5 ml. „ + 10 ml. „ + 2 ml. „	4.2 „
C. 5 ml. „ + two successive 5 ml. portions peroxidase and 2 ml. portions H_2O_2	5.0 „

Table III

	Tannin ml. $N/20$ KMnO_4	Non-tan ml. $N/20$ KMnO_4
Initially	7.5	4.55
At end of fermentation	4.8	2.1

5 ml. each of infusion and enzyme are mixed and four portions of 0.5 ml. $N/20$ H_2O_2 are added at 10 min. intervals. After the last addition another 5 ml. of enzyme and four more portions of H_2O_2 are added. For non-tan titrations five times these quantities are used.

In the actual fermentation process in manufacture both tannins and non-tans undergo oxidation but the oxidation of non-tan is relatively much less.

Table IV

	% Tannin	Non-tan*
Withered leaf	21.45	6.5 ml. $N/20$ $KMnO_4$
Leaf after 5 hr. rolling and fermentation	13.20	5.1 „

* As non-tans are ill-defined substances which have not as yet been separated they cannot be expressed on a percentage basis. They account for as much as 40% of the total permanganate titre of a tea extract. Shaw [1930] however is of the opinion that, in the gelatin precipitation, part of the tea-tannin molecule is split off and remains in the filtrate to be estimated as a non-tan.

The role of hydrogen peroxide

Reactions exactly analogous to those occurring in the fermentation process have been carried out *in vitro*, as just described, and the conclusion may be drawn that the browning of the tissue is due to peroxidase action on the tea tannin. A point unsettled in these experiments however is the participation of H_2O_2 . This substance can be found only in very small quantities indeed in the fermenting leaf, but as it would be continually used up by peroxidase as soon as it was produced, and as the catalase present effectively checks its accumulation, direct methods of estimation would not be expected to reveal its presence.

In the past in discussing the role of peroxidase in plant respiration there has been considerable talk of the possibility of organic peroxides taking the place of H_2O_2 in peroxidase catalysed reactions. This is the theory adopted by the Russian workers in this field throughout the whole of their work.

There is no experimental evidence in favour of this view, in fact all the available evidence contradicts it. Wieland & Sutter [1930] found that mono-substitution of the H atoms in H_2O_2 by organic radicals materially reduced its ability to function as an oxidizing agent in the presence of peroxidase. Disubstituted products were completely without effect. Böeseken [1930] confirmed these results and added organic per-acids to the list of substances incapable of replacing H_2O_2 . Szent-Györgyi [1925] has shown that *o*-quinones oxidize guaiacum without enzymic aid and we have shown that not only do *p*-quinones oxidize the Nadi reagent much more rapidly than the equivalent concentration of H_2O_2 in the presence of peroxidase, but that this oxidation by quinone is not accelerated by peroxidase. As there are several processes that can result in the formation of H_2O_2 in the leaf, and as the presence of catalase is a further indication of its presence, it will therefore be assumed that peroxidase oxidations in fermentation do actually utilize H_2O_2 and not some unspecified peroxide. The participation of H_2O_2 in the fermentation process is however capable of experimental verification. Catalase is a completely specific enzyme and if it could be shown that tannin oxidation is inhibited by catalase, it would prove that H_2O_2 is concerned in the fermentation process.

As shown before suspensions of minced leaf in water undergo fermentation. If excess of a liver catalase preparation is added to this suspension, fermentation is almost completely inhibited. If the catalase preparation is first inactivated by heat, fermentation proceeds unaffected.

It may therefore be concluded that H_2O_2 is concerned in the fermentation of tea, and as this fermentation is accompanied by a considerable uptake of O_2 it seems reasonable to ascribe the formation of H_2O_2 to this process. The nature of the O_2 uptake and the origin of the H_2O_2 will be investigated at a later date.

The oxidation of tannin is therefore a coupled oxidation such as that first established by Thurlow [1925]. Here secondary oxidation of guaiacum tincture by peroxidase was brought about in the presence of the H_2O_2 developed during the aerobic oxidation of hypoxanthine by xanthine oxidase. Kursanov's views [1935, 2], with the exception that he considers organic peroxides to be produced and not H_2O_2 , are identical with ours. The absorption of oxygen and the production of H_2O_2 whether it is enzymic or not is preliminary to fermentation and does not lead to any development of colour.

Estimation of peroxidase activity in tea leaf

Having proved the importance of peroxidase in fermentation it becomes necessary to have some method of determining its activity in the tea leaf. The purpurogallin method, although the simplest, cannot be used in Assam throughout the whole year owing to the volatility of ether in the hot weather. The method of Guthrie [1931], with suitable modifications, proved to be the best available.

Technique and preparation of substrate and enzyme

The substrate was made up exactly according to Guthrie. 2.1 g. cryst. citric acid were dissolved in 17 ml. *N* NaOH and diluted to 100 ml. To 50 ml. of this solution 50 ml. of water were added 0.25 g. of *p*-phenylenediamine hydrochloride and 5 ml. of a 4% solution of α -naphthol in 50% alcohol. The solution was then filtered. 10 ml. of this reagent were then mixed with the enzyme solution (usually 2 ml.) and 2 ml. of *N*/20 H_2O_2 were then added. After 10 min. incubation the reaction was stopped by the addition of 2 ml. 1% KCN. An aliquot portion, usually 3 ml., was taken and extracted with 25 ml. toluene and the depth of colour in the toluene layer estimated in a Lovibond tintometer. By a suitable mixture of reds and blues the colour could be matched exactly. A solution is said to have 6 Indophenol Units (I.U.) when the total reading of the red and blue slides is 6.0. The readings of the tintometer are not directly proportional to the concentration of indophenol blue, but a smooth calibration curve can easily be constructed. Determinations are accurate and reproducible to 0.1 I.U.

Tea juice or a suspension of the finely ground leaf in water cannot be used as the source of the enzyme as peroxidase activity is inhibited by tannin. Pretreatment of the leaf with alcohol seems to have no destructive effect on the enzyme, so the leaf sample is first ground up finely with sand under alcohol. The ground up tissue is filtered at the pump and the residue washed with alcohol until the washings are colourless. When all the chlorophyll has been removed the washings no longer show a positive tannin reaction.

After rapid drying *in vacuo*, to remove alcohol, the powdered residue is extracted with *M*/15 phosphate buffer (*pH* 7.0). A convenient enzyme solution is obtained by extracting the ground up residue from 10 g. leaf with 100 ml. phosphate. 1 ml. of the extract is then proportional to 0.1 g. of the original leaf. For determinations on single shoots (two leaves and a bud) the residue was extracted with 10 ml. phosphate, and an aliquot portion of this, 3–5 ml., was used in the estimation.

Testing of the method

The method described above must satisfy two conditions, the peroxidase must be extracted quantitatively from the leaf, and the amount of indophenol blue formed must be proportional to the enzyme concentration. That these conditions are fulfilled is shown in the following experiments.

Alcohol has very little effect on the activity of a peroxidase preparation even after 3 days' contact. Extraction of the peroxidase with the phosphate is quantitative, as if the tannin-free residue from 1 g. of leaf is extracted with 10 ml. of phosphate the activities of a 5 ml. portion of this extract and of the remaining 5 ml., together with the residue, are identical. Further, extractions for 5 and 15 min. gave enzyme solutions of identical activity.

The peroxidase activity of the final extract is much higher than that of an extract of fresh leaf owing to the tannin content of the latter. If to the final extract an amount of tea tannin equal to that originally present in the fresh leaf is added, it is found that the peroxidase activity of the extract is now depressed to the value it had originally in the fresh leaf. This has been established using both the indophenol blue and purpurogallin methods.

Fig. 1.

These experiments are considered to be sufficiently conclusive for it to be taken that extraction of the enzyme is quantitative.

The second requirement, that the extent of indophenol blue formation is proportional to enzyme concentration, is also fulfilled. The curve (Fig. 1) shows that the depth of colour is strictly proportional to enzyme concentration.

It is hoped to report the results obtained with this method in the study of fermentation at a later date.

Inhibition of peroxidase by tea tannin

Juice expressed from fresh tea leaf does not blue guaiacum even in the presence of H_2O_2 . It was suspected that this was due to the inhibiting effects of tea tannin, and confirmation of this was found by showing that the addition of tea tannin inhibited the blueing of guaiacum by potato juice. If the tannin

is removed from the tea leaf by extraction with alcohol a positive peroxidase reaction is then given with guaiacum.

Tea tannin inhibits the formation of indophenol blue and of purpurogallin by peroxidase, but apparently to different extents. Table V gives the relative amounts of these two pigments formed in the presence of varying amounts of tea tannin.

Table V.

	Indophenol blue	Purpurogallin
2 ml. enzyme	14.5	16.0
„ + 2 ml. 0.05 % tea tannin	7.6	14.1
„ + 2 ml. 0.5 % „	1.9	14.0
„ + 2 ml. 5 % „	0.5	9.5

The very marked inhibition of the indophenol blue reaction is partly apparent as tea tannin combines with indophenol blue and is not then extractable by toluene. If the tea tannin is added after the reaction has been stopped by KCN, most of the indophenol blue is fixed by the tannin and comparatively little is extracted by toluene. Acids have a similar effect so that the indophenol reaction must never be stopped by addition of acid as the weakly basic dyestuff is then retained in the aqueous phase on extraction with toluene. The inhibition of purpurogallin formation is a true enzymic inhibition; addition of tea tannin, after stopping the reaction, has no effect on the amount of purpurogallin formed.

In the above table, when 2 ml. 0.5 % tannin are added to the enzyme extract, tea tannin and peroxidase are in approximately the same proportions as they are in the leaf. The importance of removing tea tannin before estimating the peroxidase activity of a sample of leaf is at once apparent. True inhibition by tannin is not very high at this concentration but the degree of combination with indophenol blue is sufficient to make the results highly erroneous.

The theory has been advanced by Kursanov & Oparin [1929] that the inactivation of peroxidase by tea tannin can be minimized by protein decomposition products. In the leaf these were supposed by Kursanov [1935, 1] to originate by proteolysis during the withering stage of the manufacturing process. There seems no doubt that a green leaf infusion does contain protective substances as such an infusion is oxidized by peroxidase whilst tea tannin itself is not. On the other hand the evidence that these protective substances are protein decomposition products is slight.

To investigate this possibility experiments have been made using bacteriological peptone, which gives a precipitate with tea tannin, and a mixture of amino-acids. Neither had any effect over a whole range of concentrations on the formation of indophenol blue in the presence or absence of tannin. On the other hand both substances inhibited purpurogallin formation, presumably by combining with the quinone initially formed from pyrogallol, but had no effect on tannin inhibition. The effects of peptone (or amino-acid) and tannin were additive.

The suggested role of protein breakdown products in promoting fermentation receives therefore no confirmation.

Variation of peroxidase throughout manufacture

Some preliminary results are recorded here on the variation in peroxidase content of the leaf in the various stages of manufacture from green leaf to the final manufactured tea.

In each experiment 50 lb. of green leaf were manufactured. Samples were taken in duplicate of the green and withered leaf, at two different stages of rolling and at intervals throughout fermentation. When the fermented leaf was fired, part was retained to undergo further fermentation up to a period of 25 hr. The fired tea was also estimated for peroxidase activity. In cases where peroxidase activity was low, the amount of indophenol blue formed was increased either by increasing the amount of enzyme solution or by increasing the aliquot portion of the final incubation mixture. For fired tea both enzyme and aliquot had to be increased. Enzyme determinations were performed in duplicate. The sampling error was not great, only once did duplicates differ by more than 5% and this was with leaf fermented for 25 hr. where bacterial development was very marked.

For the sake of economy in space the results obtained are not quoted in full. Enzyme activities are expressed in terms of I.U. per g. of dry tissue. The results obtained for a normal manufacture and for a protracted fermentation are tabulated in Tables VI and VII respectively.

Table VI

Green leaf	894	I.U. per g. dry wt. of tissue
Withered leaf	1293	" "
40 min. roll	780	" "
90 "	685	" "
2½ hr. fermentation	647	" "
4 "	536	" "
Fired tea	7	" "

Table VII

Withered leaf	1051	I.U. per g. dry wt. of tissue
90 min. roll	652	" "
3¼ hr. fermentation	617	" "
5 "	178	" "
6½ "	115	" "
25 "	132	" "

As has been noted before by Mann [1901; 1903; 1904] and Manskaya [1935] there is a definite increase in activity during withering. We also find a definite decrease in peroxidase activity during the first roll, after which the fall, although regular, is comparatively slight up to the point where fermentation is stopped by the firing process. If fermentation is prolonged beyond 4 hr. peroxidase activity again falls rapidly.

The earlier experiments described in this paper established the toxic effects of both tannin and H_2O_2 on the enzyme causing fermentation; in fact the enzyme could be completely inactivated. These factors are not so important in actual manufacture, as not only is H_2O_2 present in very low concentration but also resistance to tannin inactivation is much greater, owing presumably to similar protective bodies to those shown likely to be present in the green leaf infusion.

It is found that the firing process does not completely inactivate the peroxidase, 0.5 to 1.0% of the original activity of the withered leaf being retained. Some of this activity is retained for at least 6 months. It is suggested that this residual activity may account in part for the post-fermentative changes which occur in the maturing of tea.

SUMMARY

A brief outline is given of the various stages in the manufacture of tea.

Oxidase is present in negligible quantities in the tea leaf and the fermentation process is brought about by peroxidase. The first stage in fermentation is an

uptake of oxygen leading to the oxidation of some as yet unidentified substance, and the production of H_2O_2 . This oxidation is not necessarily an enzymic process and may be an autoxidation. The peroxidase then catalyses the oxidation of the tea tannin by H_2O_2 leading to the production of brown pigments which impart the characteristic colour to a tea infusion. Substances, grouped as non-tans, may also undergo oxidation.

No evidence is available for the utilization of organic peroxides by peroxidase, and in the fermentation of tea, decomposition of H_2O_2 by excess catalase inhibits tannin oxidation almost completely.

A quantitative method for the estimation of peroxidase in the tea leaf is described.

Peroxidase is inhibited by tea tannin although substances present in the leaf exert some protective influence against its effect. The theory that these protective substances are protein degradation products, formed during withering, finds no support.

An outline is given of the changes in peroxidase content of the leaf throughout the various stages in manufacture.

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