

ON THE NATURE OF THE ENZYME CATECHOL OXIDASE IN TEA PLANTS

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The enzyme catechol oxidase plays a key role in the manufacture of black tea. It is responsible for most, if not for all, of the chemical reactions which take place during tea fermentation. For this reason much research has been, and continues to be, devoted to the elucidation of the properties of this enzyme. Until recently, it was generally believed that the enzyme was insoluble in nature and the available evidence was interpreted to indicate that the enzyme was insoluble because it was bound to some cell particulates; probably the chloroplasts. A recent discovery has provided strong evidence which suggests that the enzyme is, in fact, soluble in nature. Further, it has been shown that the polyphenolic compounds present in the tissues of tea shoot tips cause all the soluble proteins (enzymes) to precipitate as soon as the organisation of these tissues is destroyed; and this is exactly what happens when these tissues are homogenized for the purpose of extracting the enzymes present in a cell free state or when flush is rolled in orthodox tea manufacture to bring about fermentation. These new findings suggest that the previous reports that the enzyme catechol oxidase was insoluble were due to the artifact caused by the precipitation of all soluble proteins in the tissues of tea shoot tips caused by the polyphenolic compounds which are present. A method for the extraction of soluble catechol oxidase from tea tissues has been developed and it is described in this report. The method is based on the inclusion of an insoluble polyphenol absorbant in the enzyme media. This additive is effective because it is able to absorb the polyphenols from solution *before* they are able to interact with the proteins present and cause them to precipitate.

Introduction

The enzyme catechol oxidase (also called polyphenol oxidase, polyphenolase, and phenolase) is of the utmost importance in tea manufacture because it is responsible directly or indirectly for most, if not for all, of the reactions which take place during fermentation. It is because of this fact that tea biochemists have paid so much attention to this enzyme in the past and it is why they continue to do so today.

The role of this enzyme¹ in tea fermentation may be summarized as follows: In undamaged tea flush the enzyme catechol oxidase is spatially separated from its substrates², the flavanols (also called catechins). That is, the enzyme is located in the cytoplasm of cells in the tea plant and the flavanols are located in the vacuoles of these cells. Separating the vacuole from the cytoplasm is a membrane called the tonoplast which prevents the free movement of substances between the two parts of the cell. Figure 1 illustrates the above points. No fermentation will occur in these tissues until the enzyme and the flavanols are brought into contact by mixing of the contents of the cells. This mixing of the cell contents is brought about by rolling in orthodox tea manufacture (Eden 1958; Keegel 1958; Harler 1963). Once the flavanols are brought into contact with the enzyme catechol oxidase, they are rapidly oxidized by oxygen from the atmosphere. This oxidation is shown diagrammatically in Figure 2. The oxidized flavanols can undergo condensation to form a variety of complex compounds; the major ones being called theaflavins and thearubigins (Roberts 1961; 1962). Furthermore, the oxidized flavanols are themselves very strong oxidizing agents which can cause non-enzymatic oxidation of other

¹Enzymes are the catalysts present in living organisms: All enzymes are proteins.

²A substrate is the substance on which an enzyme acts: The flavanols and oxygen are the substrates of catechol oxidase because their chemical interaction is catalyzed by this enzyme.

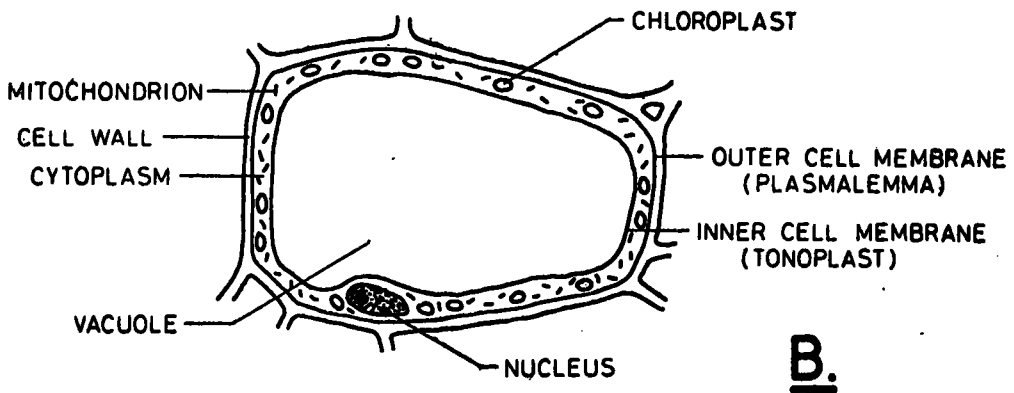
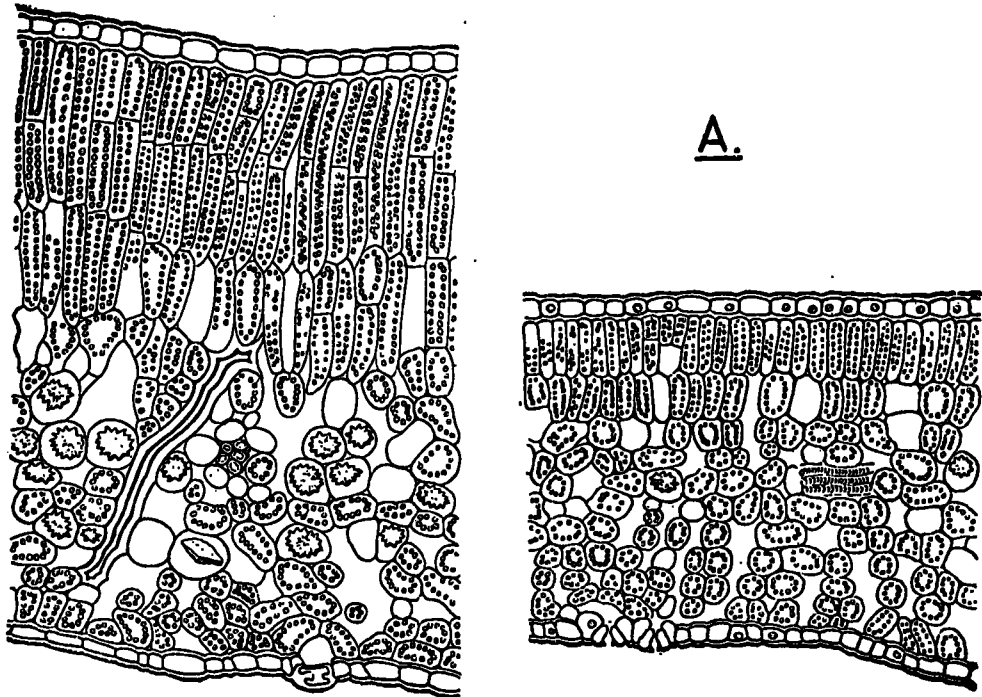


FIGURE 1 — *The inside of a tea leaf —*

A — Drawing of a portion of the cross section of a tea leaf showing the organization of the tissue — (Taken from de Haan, 1939)

B — Generalized drawing of a single leaf cell showing its main structural features

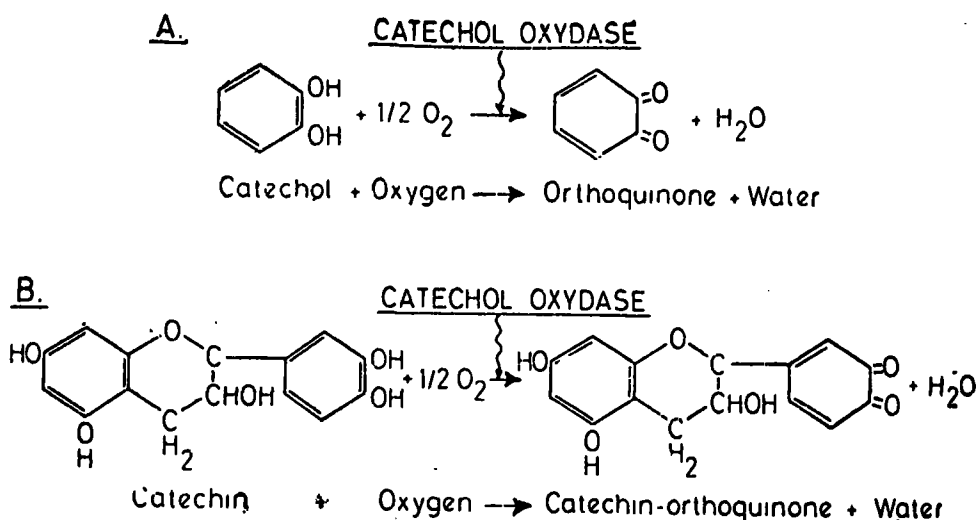


FIGURE 2—Diagrammatic representation of the oxidation of (A) catechol and (B) catechin by catechol oxidase—The following points should be noted: (1) The enzyme catechol oxidase acts on the orthodihydroxy grouping which is common to both catechol and catechin—All flavonols (catechins) possess this orthodihydroxy grouping and they are all oxidized in the same manner—(2) Oxygen is required: The enzyme cannot act in the absence of oxygen—(3) The enzyme catalyses the oxidation: In the absence of the enzyme the oxidation is many times (say 100 times) slower

substances in the flush. Quantitatively speaking, the formation of theaflavins and thearubigens is the most important reaction taking place during tea fermentation, but the oxidation of other substances by the oxidized flavanols is in all probability of primary importance in determining the quality of the manufactured tea (James *et al* 1948; Trautner & Roberts 1950; Popov 1956). These latter reactions are only poorly understood at the present time but they are under active investigation.

It can be seen from the above discussion that catechol oxidase plays an essential role in tea manufacture. A recent discovery (Sanderson 1964c) has altered the previously accepted ideas on the nature of this enzyme in tea (Roberts 1962; Stahl 1962; Bendall & Gregory 1963; Bhatia 1963) and it is the purpose of this paper to discuss these recent findings.

History of the problem

The enzymatic nature of tea fermentation was recognized at least as early as 1893 (Bamber 1893). While several early investigators purported to describe the characteristics of the enzyme in tea which caused fermentation, it remained for Sreerangachar and co-workers, working in the laboratories of the Tea Research Institute of Ceylon, to describe the enzyme accurately (Sreerangachar 1939; 1943a, b; Lamb & Sreerangachar 1940a, b). These investigators obtained the evidence which proved that this enzyme was a polyphenol oxidase and not peroxidase (Mann 1901; 1903; 1904; Manskaya 1935; Roberts & Sarma 1938; Roberts 1939a), ascorbic acid oxidase (Roberts 1939b), or cytochrome oxidase (Lamb & Roberts 1939; Roberts 1940) as had been suggested by other workers. However, the enzyme described by the Ceylon workers appeared to be insoluble^a in contrast to

^aThe enzyme was said to be insoluble because it could be separated as a precipitate from homogenates of tea tissues by filtering the homogenates through filter paper or by centrifuging them.

other polyphenol oxidases which had been described. This anomalous property of the tea enzyme led to a heated controversy (Roberts 1942; 1952) which delayed the general acceptance of these findings.

The issue seemed to be settled at last when in 1947 investigators working in California reported that the tea oxidase was located in the leaf chloroplasts (Li & Bonner 1947). The chloroplasts are relatively large particulates found in the cytoplasm of cells from green parts of plants (Figure 1). They are large enough to be withheld by the usual laboratory filters and so it appeared that the explanation for the insolubility of the enzyme had been found.

With this last piece of evidence the most outspoken critic of Sreerangachar's views was convinced that the enzyme of tea fermentation was a catechol oxidase (Roberts 1952) which brought about general agreement on the subject. However, with the passage of time came developments which made it clear that the work of Li and Bonner (1947) was not entirely satisfactory. The realization of this is indicated by the uncertainty expressed in recent reports about the exact particulate in which the catechol oxidase is located (Roberts 1962; Bendall & Gregory 1963). This was the situation at the time that the current series of investigations at the Tea Research Institute of Ceylon were initiated in 1963.

Recent Investigations

In our initial investigations use was made of acetone powders of tea shoot tips which were made essentially as described by Sreerangachar (1943a) as a source of enzymes (Sanderson 1963; 1964a, 1964b; Sanderson & Kanapathipillai 1964; Sanderson & Roberts 1964). The enzymes obtained by this extraction procedure were, without exception, insoluble in nature, but the possibility that this was an artifact due to an insolubilization of the enzyme proteins during the extraction process was clearly understood (Sanderson & Roberts 1964).

In early attempts to extract soluble enzymes from tea shoot tips, it was discovered that the usual methods of extracting enzymes from plant tissues (Colowick & Kaplan 1955) were not successful in extracting *any* soluble protein whatsoever from the tea plant material. This meant that either tea shoot tips were different from every other kind of plant tissue which had been investigated in that they contained no soluble protein *or* that the extraction methods in common use with other plants were not applicable to tea plants.

The solution to the problem came from the laboratory of Dr W. D. Loomis at Oregon State University (USA) when he and his co-worker discovered a method for the extraction of soluble enzymes from mint leaves (Loomis 1964; Loomis & Battaile 1964) which contain high levels of polyphenolic compounds as do tea leaves. This method depended on the inclusion of an insoluble substance with a strong affinity for polyphenolic compounds in the medium used in extracting the tissues.

The principles discovered by Loomis and his co-workers were tested on tea with success (Sanderson 1964c). Polycaprolaktam powder, an insoluble polyamide, was used as the insoluble polyphenol absorbant in these investigations. The results of these experiments are shown in Table 1 where it can be seen that as the level of the polyphenol absorbant in the extraction medium was raised the solubility of the enzyme catechol oxidase increased until when about one gram of polycaprolaktam powder per fresh weight of flush was used, virtually all of the enzyme was extracted in the soluble form. Further tests were made and it was found that as the level of polyphenol absorbant in the extraction medium was raised, the level of polyphenols in the soluble fraction of the homogenates decreased and the level of protein increased. This relationship is shown in Figure 3.

TABLE 1—The effect of polycaprolaktam powder on the extraction of soluble catechol oxidase from tea shoot tips

| Expt No | Level of polycaprolaktam powder in extraction media (g/g fresh wt tissue) | Catechol oxidase activity (μ moles catechol oxidized/g fresh wt tissue min) | | | Activity recovered in fractions (% activity in crude extract) |
|---------|---|--|-------------|-------------|---|
| | | Crude extract | Fractions | | |
| | | | Precipitate | Supernatant | |
| 1 | 0 | 61.5(100)* | 40.5 | 0 | 66 |
| 2 | 0 | 71.5(100) | 56.0 | 0 | 78 |
| 3 | 0 | 65.1(100) | 54.0 | 0 | 83 |
| 1 | 0.25 | 69.5(113) | 56.4 | 0 | 81 |
| 2 | 0.50 | 86.9(122) | 51.9 | 19.8 | 83 |
| 3 | 0.75 | 92.4(141) | 5.6 | 74.2 | 87 |
| 1 | 1.00 | 98.2(160) | 7.6 | 82.7 | 92 |
| 3 | 1.50 | 104.7(161) | 11.8 | 94.6 | 102 |

*Enzyme activity as percent of activity found when no polycaprolaktam powder was used in the extraction media.

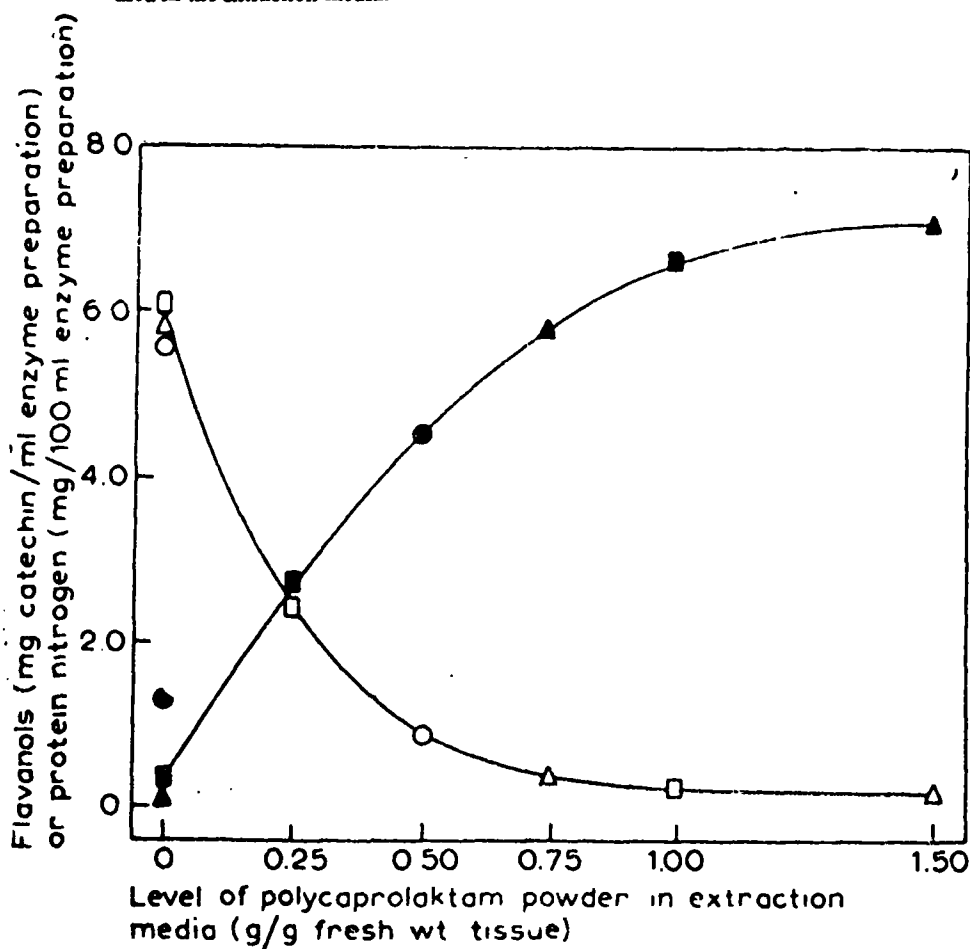


FIGURE 3—Effect of polycaprolaktam powder on the solubility of flavanols and protein in extracts of tea shoot tips—All analyses were made on the supernatant fractions described in Table 1—□, ○, △, total flavanols—■, ●, ▲, protein nitrogen (From Sanderson 1964c)

The ability of the polyphenols present in tea shoot tips to precipitate the enzyme catechol oxidase was shown in another experiment (Sanderson 1965). Here an extract of tea shoot tips containing polyphenols was added in graded amounts to an extract containing the enzyme, free of polyphenols. The mixtures were stored for different periods of time and then they were assayed for enzyme activity both before and after removal of insoluble material by centrifuging the mixtures at high speed (3,000 g for 10 min). The results obtained are shown in Figure 4. The results showed that the enzyme was not inactivated by storing with the tea leaf polyphenols since the activity of the enzyme was the same in all mixtures before the removal of the insoluble material by centrifugation, but that the polyphenols did cause the enzyme to precipitate, since there was a reduction in the activity remaining in the soluble fraction after centrifugation. Furthermore, the insolubilization was directly proportional to the amount of polyphenol extract in the mixtures.

The above results provide an adequate explanation for the apparent insolubility of the enzyme as reported by earlier investigators. That is, the tea leaf polyphenols cause a precipitation, or insolubilization, of the cytoplasmic proteins (enzymes) in tea tissues immediately upon their coming into contact with one another, and this did, in fact, happen with the enzyme extraction techniques used by the earlier investigators.

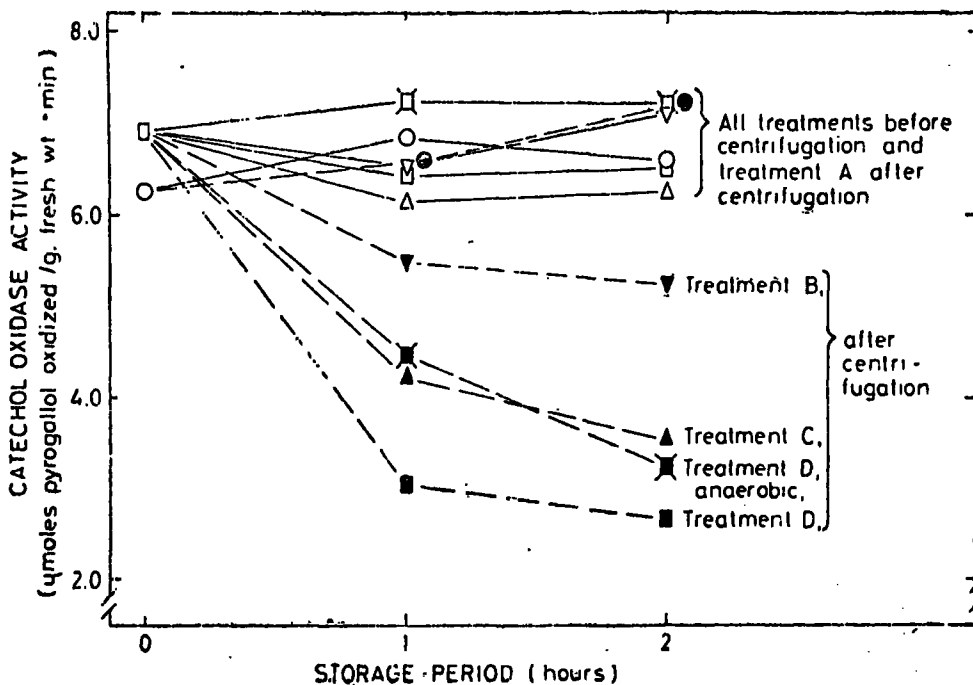


FIGURE 4—The effect of tea polyphenolic compounds on the enzyme catechol oxidase—Treatments consisted of mixing an extract of tea shoot tips containing soluble catechol oxidase with equal volumes of (A) distilled water or extracts of tea shoot tips containing the polyphenolic compounds of these tissues at (B) $\frac{1}{4}$, (C) $\frac{1}{2}$, and (D) full strength—All mixtures were stored aerobically (exposed to the atmosphere) except one set of mixtures (D) in which were stored anaerobically (in evacuated Thunberg tubes)—Open symbols indicate total enzyme activity (enzyme activity in mixtures before centrifuging)—Solid symbols indicate soluble enzyme activity (enzyme activity in mixtures which remains after removal of precipitated enzyme by centrifuging)—(From Sanderson 1965)

Description of the method for the extraction of soluble catechol oxidase from tea shoot tips. (Sanderson 1946c)

Tea shoot tips (5.0 g) are frozen in a deep freezer at 15°C. After freezing, the shoot tips are ground in a cold mortar (4°C) with an equal weight of polycaprolaktam powder (Polyclar AT or Polyamide Woelm can be substituted in one-half or the same amount, respectively), a small amount of acid washed sand (about 5g), and 10 ml of McIlvaine's buffer at pH 7.0 (McIlvaine 1921). After grinding, 30 ml of the same buffer is added to the homogenate. The homogenate is then strained through muslin cloth. The filtrate is centrifuged at 3000 g for 20 min. The supernatant containing the soluble catechol oxidase is decanted. The supernatant may be used directly in enzyme assays but if it is to be stored it should be treated once with a small amount of the polyphenol absorbant, say one-tenth the original amount, to remove any remaining polyphenols. For best results, all operations should be carried out in a cold room at 4°C using cold apparatus and reagents.

Summary and Conclusions

A method has been developed which makes it possible to extract the enzyme catechol oxidase from tea shoot tips in a soluble state. The experimental evidence discussed above, and elsewhere (Sanderson 1964c), establishes the soluble nature of the enzyme catechol oxidase in tea plants with considerable certainty. This is in contradiction to the previously generally held view that this enzyme was insoluble (particulate) in nature. It has been shown that the evidence for the insoluble nature of this enzyme was based on an artifact caused by the polyphenols present in tea leaf material. These polyphenols are efficient protein precipitants and they cause an insolubilization of the cytoplasmic proteins during the course of extraction when conventional extraction techniques are used.

The methods developed for the extraction of soluble catechol oxidase from tea shoot tips should prove useful to the tea biochemist by making it possible for him to investigate the properties of this enzyme, and others too, in their native state. Also, these new methods may find important applications in the future developments in tea technology; especially in the production of instant tea.

Acknowledgements

The very helpful discussion of his own work provided by Dr W. D. Loomis of Oregon State University (USA) is acknowledged with many thanks. The polycaprolaktam powder used in these investigations was supplied by Messrs Farbwerke Hoechst AG, Bobingen Germany.

References

- BAMBER, G. (1893) "*A Textbook on the Chemistry and Agriculture of Tea*". (Calcutta). Cited by Roberts (1942).
- BENDALL, D. S. & GREGORY, R. P. F. (1963) Purification of phenol oxidases, in "*Enzyme Chemistry of Phenolic Compounds*". Ed. J. B. Pridham, pp. 7-24. London, Pergamon Press.
- BHATIA, I. S. (1963) Chemical aspects of green leaf processing. *Two and a Bud*. 10 (2): 28-33.
- COLOWICK, S. P. & KAPLAN, N. O. editors (1955) "*Methods in Enzymology, Vol. 1*", pp. 1-146. New York, Academic Press.

- EDEN, T. (1958) "Tea", 201 pp. London, Longmans, Green & Co.
- DE HAAN, I (1939) De anatomische bouw van de theeplant. 1. Stengel en blad. *Arch. Theecult. Ned. Ind.* **13**: 318-343. (In Dutch.)
- HARLER, C. R. (1963) "Tea Manufacture". 126 pp. Oxford, University Press.
- JAMES W. O., ROBERTS, E. A. H., BEEVERS, H. & DE KOCK, P. (1948) The secondary oxidation of amino acids by the catechol oxidases of *Belladonna*. *Biochem. J.* **43**: 626-636.
- KEEGEL, E. L. (1958) "Tea Manufacture in Ceylon". 179 pp. Monographs on Tea Production in Ceylon, No. 4. The Tea Research Institute of Ceylon, Talawakelle.
- LAMB, J. & ROBERTS, E. A. H. (1939) Cytochrome oxidase in tea fermentation. *Nature, Lond.* **144**: 867-868.
- LAMB, J. & SREERANGACHAR, H. B. (1940a) Studies on the fermentation of Ceylon tea. 1. The nature of the enzyme system. *Biochem. J.* **34**: 1472-1485.
- LAMB, J. & SREERANGACHAR, H. B. (1940b) Studies on the fermentation of Ceylon tea. 2. Oxidising enzymes. *Biochem. J.* **34**: 1485-1492.
- Li, P. & BONNER, J. (1947) Experiments on the localization and nature of tea oxidase. *Biochem. J.* **41**: 105-110.
- LOOMIS, W. D. (1964) Personal communication.
- LOOMIS, W. D. & BATTLE, J. (1964) Cell free enzyme from tannin containing plant tissues. *Plant Physiol.* **39**: Suppl. xxi.
- McILVAINE, T. C. (1921) *J. Biol. Chem.* **49**: 183. (Described in Colowick & Kaplan (1955) p. 141.)
- MANN, H. H. (1901) The ferment of the tea leaf and its relation to quality in tea. *Bull. No. 2 of 1901*. 10 p. Calcutta: Indian Tea Association.
- MANN, H. H. (1903) The ferment of the tea leaf. Part 2. *Bull. No. 5 of 1903*, 8 p. Calcutta: Indian Tea Association.
- MANN, H. H. (1904) The ferment of the tea leaf. Part 3. *Bull. No. 2 of 1904*, 11 p. Calcutta: Indian Tea Association.
- MANSKAYA, C. M. (1935) The peroxidase of the tea leaf. *Biochem. Tea Prod.* **1**: 96-106. (in Russian).
- POPOV, V. P. (1956) Oxidation of amino acids in the presence of tannins and polyphenols of tea. *Biokhimiya* **21**: 383-387. (in Russian).
- ROBERTS, E. A. H. (1939a) The fermentation process in tea manufacture. 2. Some properties of tea peroxidase. *Biochem. J.* **33**: 836-842.
- ROBERTS, E. A. H. (1939b) The fermentation process in tea manufacture. 3. The mechanism of fermentation. *Biochem. J.* **33**: 842-852.
- ROBERTS, E. A. H. (1940) The fermentation process in tea manufacture. 5. Cytochrome oxidase and its probable role. *Biochem. J.* **34**: 500-506.

- ROBERTS, E. A. H. (1942) The chemistry of tea fermentation. *Advanc. Enzymol.* **2**: 113-133.
- ROBERTS, E. A. H. (1952) The chemistry of tea fermentation. *J. Sci. Fd. Agric.* **3**: 193-198.
- ROBERTS, E. A. H. (1958) The chemistry of tea fermentation. *J. Sci. Fd. Agric.* **9**: 381-390.
- ROBERTS, E. A. H. (1961) The nature of the phenolic oxidation products in manufactured black tea. *Tea Quart.* **33**: 190-200.
- ROBERTS, E. A. H. (1962) Economic importance of flavanoid substances: Tea fermentation, in "The Chemistry of Flavanoid Compounds" Ed. T. A. Giessman, p. 468-512. London, Pergamon Press.
- ROBERTS, E. A. H. & SARMA, S. N. (1938) The fermentation process in tea manufacture. 1. The role of peroxidase, *Biochem. J.* **32**: 1819-1828.
- SANDERSON, G. W. (1963) The chloroform test: A study of its suitability as a means of rapidly evaluating fermenting properties of clones. *Tea Quart.* **34**: 193-196.
- SANDERSON, G. W. (1964a) The chemical composition of fresh tea flush as affected by clone and climate. *Tea Quart.* **35**: 101-110.
- SANDERSON, G. W. (1964b) Changes in the level of polyphenol oxidase activity in tea flush on storage after plucking. *J. Sci. Fd. Agric.* **15**: 634-639.
- SANDERSON, G. W. (1964c) Extraction of soluble catechol oxidase from tea shoot tips. *Biochim. biophys. Acta.* **92**: 622-624.
- SANDERSON, G. W. (1965) The action of polyphenolic compounds on enzymes. *Biochem. J.* **95**: 24P-25P.
- SANDERSON, G. W. & KANAPATHIPILLAI, P. (1964) Further studies on the effect of climate on the chemical composition of fresh tea flush. *Tea Quart.* **35**: 222-229.
- SANDERSON, G. W. & ROBERTS, G. R. (1964) Peptidase activity in shoot tips of the tea plant (*Camellia sinensis*, L.). *Biochem. J.* **93**: 419-423.
- SREERANGACHAR, H. B. (1939). The endo-enzyme in tea fermentation. *Curr. Sci.* **8**: 13.
- SREERANGACHAR, H. B. (1943a) Studies on the fermentation of Ceylon tea. 4. Estimation of the oxidizing enzyme activity. *Biochem J.* **37**: 653-655.
- SREERANGACHAR, H. B. (1943b) Studies on the fermentation of Ceylon tea. 5. Comparative rates of oxidation of different polyphenolic substrates by tea oxidizing enzymes. *Biochem. J.* **37**: 656-660.
- STAHL, W. H. (1962) The chemistry of tea and tea manufacturing. *Advanc. Food Res.* **11**: 201-262.
- TRAUTNER, E. M. & ROBERTS, E. A. H. (1950) The chemical mechanism of the oxidative deamination of amino acids by catechol and polyphenolase. *Aust. J. Sci. Res. ser. B.* **3**: 356-380.