REFERENCES

Abderhalden, E. & Blumberg, P. (1910). *Hoppe-Seyl. Z.* **65**, 318.
Adair, G. S. & Adair, M. E. (1934). *Biochem. J.* **28**, 1230.
Bailey, K., Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 360.
Blackburn, S., Carter, E. G. H. & Phillips, H. (1941). *Biochem. J.* **35**, 627.
Bowes, J. H. & Pleass, W. B. (1939*a*). *J. int. Soc. Leath. Chem.* **23**, 451.
Bowes, J. H. & Pleass, W. B. (1939*b*). *J. int. Soc. Leath. Chem.* **23**, 499.
Cherbuliez, E. & Stavritch, K. N. (1922). *Helv. chim. Acta*, **5**, 267.
Chibnall, A. C. (1942). *Proc. Roy. Soc.* B, **131**, 136.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943*a*). *Biochem. J.* **37**, 354.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943*b*). *Biochem. J.* **37**, 372.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Dakin, H. D. (1920). *J. biol. Chem.* **44**, 499.
Damodaran, N. (1932). *Biochem. J.* **26**, 135.
Foster, G. L. (1945). *J. biol. Chem.* **159**, 431.
Fraenkel-Conrat, H. (1943). *J. biol. Chem.* **148**, 453.
Fraenkel-Conrat, H., Cooper, M. & Olcott, H. S. (1945*a*). *J. Amer. chem. Soc.* **67**, 314.
Fraenkel-Conrat, H., Cooper, M. & Olcott, H. S. (1945*b*). *J. Amer. chem. Soc.* **67**, 950.
Fraenkel-Conrat, H. & Olcott, H. S. (1946). *J. Amer. chem. Soc.* **68**, 34.
French, D. & Edsall, J. T. (1945). *Advances in Protein Chemistry*, **2**, 277. New York: Academic Press.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
Hac, L. R. & Snell, E. E. (1945). *J. biol. Chem.* **159**, 291.
Highberger, J. H. & Retzsch, C. E. (1938). *J. Amer. Leath. Chem. Ass.* **33**, 341.
Jones, D. B. & Moeller, O. (1928). *J. biol. Chem.* **79**, 429.
Levy, M. & Silberman, D. E. (1937). *J. biol. Chem.* **118**, 723.
Lindley, H. (1946). Private communication.
Ma, T. S. & Zuazaga, G. (1942). *Industr. Engng Chem.* (Anal. ed.), **14**, 280.
Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.
Middlebrook, W. R. & Phillips, H. (1942). *Biochem. J.* **36**, 294.
Middlebrook, W. R. & Phillips, H. (1947). *Biochem. J.* **41**, 218.
Neuberger, A. (1944). *Biochem. J.* **38**, 309.
Nitschmann, H. & Hadorn, H. (1941). *Helv. chim. Acta*, **24**, 237.
Nitschmann, H., Hadorn, H. & Lauener, H. (1943). *Helv. chim. Acta*, **26**, 1069.
Peters, J. P. & Van Slyke, D. D. (1932). *Quantitative Clinical Chemistry*, **2**, 1st ed. London: Baillière, Tindall and Cox.
Plimmer, R. H. A. (1917). *The Chemical Constitution of the Proteins*, **1**. 3rd ed. London: Longmans, Green and Co.
Plimmer, R. H. A. (1925). *J. chem. Soc.* p. 2651.
Rees, M. W. (1946). *Biochem. J.* **40**, 632.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Schiff, H. (1900). *Liebigs Ann.* **310**, 25.
Shinohara, K. (1935). *J. biol. Chem.* **109**, 665.
Speakman, J. B. (1934). *J. Soc. Dy. Col., Bradford*, **50**, 341.
Speakman, J. B. (1936). *J. Soc. Dy. Col., Bradford*, **52**, 335.
Speakman, J. B. & Townend, F. (1937). *Nature, Lond.*, **139**, 411.
Steinhardt, J. & Fugitt, C. H. (1942). *Bur. Stand. J. Res., Wash.*, **29**, 315.
Stoves, J. L. (1943). *Trans. Faraday Soc.* **39**, 294.
Stoves, J. L. (1944). *Nature, Lond.*, **154**, 272.
Thomas, L. E. (1944). *Arch. Biochem.* **5**, 175.
Van Slyke, L. L. & Baker, J. C. (1918). *J. biol. Chem.* **35**, 127.
Wormell, R. L. & Kaye, M. A. G. (1945). *J. Soc. chem. Ind., Lond.*, **64**, 75.

Studies on the Fermentation of Ceylon Tea

8. FURTHER OBSERVATIONS ON THE RELATIONSHIP OF TEA FERMENTATION TO NORMAL RESPIRATION

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The original hypothesis of Roberts that all normal respiration ceases when tea leaf is rolled in the process of manufacture, and that the carbon dioxide observed during fermentation is due to a secondary carbohydrate oxidation by the *o*-quinones, has been further amplified in his two later communications (Roberts, 1941, 1943). This hypothesis, however, is in conflict with the finding in this laboratory (Sreerangachar, 1941) that when the leaf is mechanically damaged, as in tea rolling, a certain amount of normal respiration still persists, thus accounting for the carbon dioxide produced by the fermenting leaf. This difference of opinion makes a critical examination of the evidence presented on both sides desirable, especially since these investigations, seriously interrupted during the war, can now be continued.

The problem centres round two main issues. First, is there any residual normal respiration in the

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leaf which has suffered damage similar to that in tea rolling; and, secondly, do the tannin *o*-quinones play a role in the carbohydrate oxidation in fermenting leaf? The purpose of the present paper is to discuss the evidence relating to these two issues in the light of some fresh results obtained here.

EXPERIMENTAL AND RESULTS

Residual respiration in factory-rolled tea leaf

Residual respiration has been previously established in fresh or withered tea leaf after mincing but not in factory-rolled leaf. Whether or not respiration is completely suppressed in the rolled leaf depends on the nature and extent of the mechanical injury inflicted on the leaf tissues in rolling. The breaking up of leaf depends upon many factors such as its physical condition, the mechanics of rolling and so on; but even after the hardest rolling now in practice one can always pick out several green and undamaged pieces of tissue from a mass of rolled leaf. Complete rupture of all the cells does, no doubt, result in total suppression of respiration, but such extreme damage is never accomplished in a tea roller in Ceylon.

The most convincing proof of the presence of respiring tissue in rolled leaf is now furnished by direct experimentation. Measurements of respiration in this and subsequent experiments were done by conventional manometric methods using the Barcroft differential apparatus. Withered leaf, after one or more rolls of 30 min. in the factory, was well washed with water to remove the expressed polyphenols, both oxidized and unoxidized, which alone could provide material for Roberts's mechanism; 200 mg. of this tissue were suspended in 2 ml. of water and the respiratory CO_2 measured at 28°.

Table 1 gives results typical of those obtained in several experiments. Different leaf has been used in the different experiments shown in the table.

Polyphenol oxidation during tea fermentation

The fermentation period in the factory varies generally from 2·5 to 4 hr., and it is almost certain that the polyphenolic constituents are not completely oxidized within that period, as is shown by a typical case (Table 2).

Exp. 1 was carried out on factory samples whilst for exp. 2 withered leaf was minced in the laboratory. The total oxidizable matter, which was determined by the method of Shaw (1935), is proportional to the amount of polyphenols present (Eden, 1935), the extent of oxidation being indicated by the fall in total oxidizable matter. Part of this fall is probably due to condensation phenomena, but the fact that there is a correspondence between the fall in total oxidizable matter on the one hand, and the O_2 uptake (free from condensation) on the other, shows that the main conclusions are not much affected by such condensation changes. The O_2 uptake was determined by means of a macro-absorption apparatus using 20 g. of withered leaf. The degree of accuracy obtained with this apparatus is not as high as in the

Table 1. *Residual respiration in washed rolled leaf*

Time (min.)		15	30	60	90	120	150	180	210	240
CO_2 production (μl.).	Exp. 1. Withered leaf, 1 roll	11	20	47	60	75	86	98	102	111
	Exp. 2. Withered leaf, 3 rolls	14	22	35	44	54	60	63	—	—
Time (min.)		5	10	15	30	45	60	75	90	
CO_2 production (μl.).	Exp. 3. Withered leaf rolled:									
	1st dhool*	9	16	21	32	44	55	69	76	
	4th dhool	0	5	5	7	12	18	23	28	
	Withered leaf rolled in Clivemeare† roller	2	5	9	12	14	18	21	25	

* Rolled leaf sifted through a standard mesh is called a dhool. 4th dhool has had longer rolling than the 1st dhool.
† Clivemeare roller is a new type of roller which very quickly reduces green leaf almost to a pulp. It has not yet been adapted for commercial manufacture of black tea.

Table 2. *Oxidation of tea polyphenols during fermentation*

Period of fermentation (hr.)	Total oxidizable matter in terms of ml. 0·05 N-thiosulphate/g. dry matter		O_2-absorption during fermentation in exp. 2 (ml./g. dry wt.)
	Exp. 1	Exp. 2	
0	—	247	—
0·5	273	—	—
1	—	223	3·76
2	258	204	5·71
4	208	196	7·87
6	196	184	9·18
24	171	147	11·03

usual manometric experiments; but the results have a special value in that the conditions of leaf fermentation in this apparatus closely approximated to those in the factory.

It will be seen that at the end of 4 hr. fermentation, which is perhaps the maximum under factory conditions, the amount of oxidation is only 60–65 % of that attained after 24 hr. It is also clear that oxidation of polyphenols continues for periods much longer than the time usually allowed for fermentation in the factory.

Inhibition of respiration by tea-tannin oxidation products

In tea fermentation Roberts has observed that, after completion of tannin oxidation, CO_2 production falls to a very low level, and has, therefore, assumed a close dependence of the latter on the former. Evans (1928), however, found that the CO_2 production decreased only slightly from the first to the seventh hour of fermentation. My own results (Sreerangachar, 1941) have shown a slight fall in the CO_2 output towards the end of fermentation, but this is perhaps associated with the inhibitory effect of oxidized polyphenols on the normal respiration. Richter (1934) has shown that catechol oxidase is inhibited by *o*-quinone, and Boswell & Whiting (1938) have established the inhibitory nature of the quinonoid compounds on the normal respiratory system of potato. Other instances of oxidative quinone inhibitions are mentioned by Lipmann (1943). The possibility, therefore, exists that in tea fermentation a similar inhibition of respiration may occur.

In order to test this, the respiration of fresh leaf was measured in the presence of oxidized tea polyphenols and compared with the values obtained free from this influence. The figures in Table 3 represent the respiratory CO_2 of 200 mg. of fresh leaf suspended in 2 ml. of water in one case, and 2 ml. of black tea infusion (2·82 g./150 ml. water) in the other. The leaf samples were derived from the second leaf of a normal flush containing a bud and two leaves. The flushes employed in any one experiment were from the same bush. The results were confirmed by several similar experiments; they demonstrate that there is an inhibition of the normal respiration of the leaf by the tea-tannin oxidation products, and explain why there is a fall in respiratory rate towards the end of tea fermentation.

Stimulation of respiration by catechol

Boswell & Whiting (1938) have conclusively shown that on adding catechol to respiring potato slices both the O_2 uptake and the CO_2 output are increased. It must be emphasized that this stimulation of CO_2 output is not due to an *in vitro* reaction in which the *o*-quinone acts as H acceptor in carbohydrate oxidation, but that it is due to a stimulation of the *in vivo* respiratory system. Similarly, when catechol is added to the fermenting leaf-mince system, in which there is a certain amount of residual respiration, stimulation of the latter could naturally follow.

Results given in Fig. 1 afford confirmation of this hypothesis in that there is a stimulation of tea-leaf respiration by catechol. This has been investigated

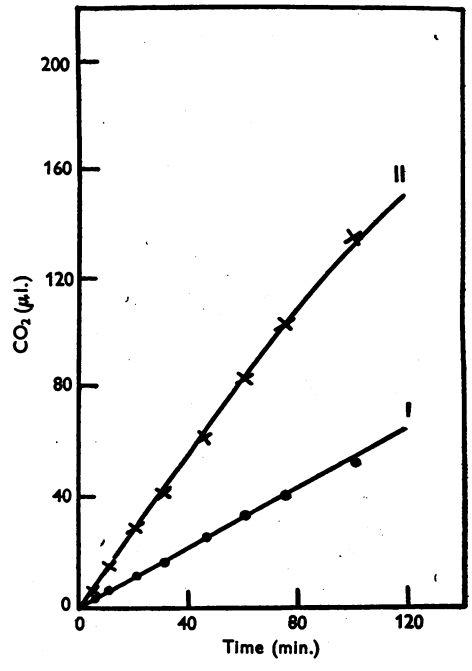

Fig. 1. Respiration of tea leaf. I, in water; II, in presence of 0·05 M-catechol.

by suspending tea leaf in 2 ml. of 0·05 M-solution of catechol and taking measurements in a Barcroft respirometer. To facilitate observation of results from the moment of polyphenol addition, catechol (0·5 ml., 0·2 M) was held in a dangling tube which was

Table 3. *Inhibition of respiration by oxidized tea polyphenols*

Time (min.)	15	30	45	60	75	90	105	120
Leaf suspended in water (µl. CO_2)	23	42	63	86	109	128	151	174
Leaf suspended in tea infusion (µl. CO_2)	16	30	46	62	79	95	104	120

dislodged and mixed with the contents of the flask (1·5 ml. of water) at zero time. As before, the leaf samples were derived from the second leaf of the flushes from the same bush. It is clear that the stimulation of CO_2 output, observed by Roberts when catechol was added to fermenting tea leaf, is due more to the stimulation of normal respiration in undamaged portions of the leaf than to a greater production of *o*-quinones causing a higher carbohydrate oxidation in the damaged tissue. Any possible interference in the above experiment by reactions at the cut ends of the leaf was eliminated by a prior washing of the cut pieces, and it was found by a control experiment that when the tissues were so washed direct oxidation of added catechol was negligible.

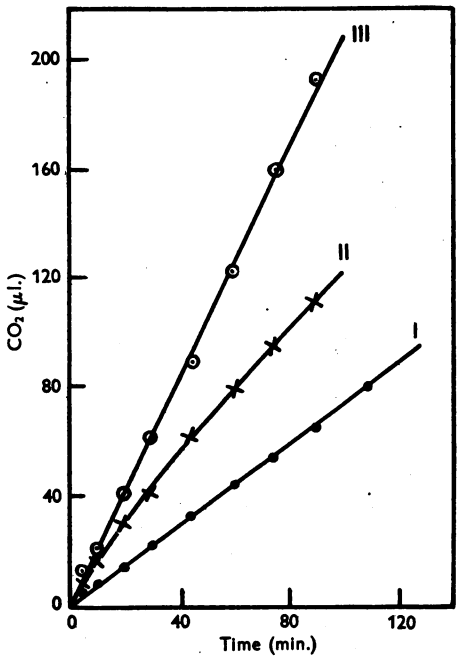

Fig. 2. Respiration of tea leaf. I, normal; II, in presence of 0·05 M-catechol, leaf cut into 5 pieces; III, as in II, but leaf cut into 28 pieces.

There is reason to believe, however, that the amount of the cut edges influences this stimulation of respiration by catechol. Thus when 200 mg. of leaf were cut into twenty-eight pieces the stimulation was higher than when the same amount of leaf was cut into five pieces (Fig. 2). It appears, therefore, reasonable to expect that, in the case of finely chopped tissues or of leaf mince such as that used by Roberts, the stimulatory effect would be still higher than that observed here. The exact mechanism of this stimulation calls for further study but the observation provides perhaps a more suitable explanation of Roberts's results.

DISCUSSION

By using an attachment which would grind nuts to a paste Roberts claims to have caused more extensive structural damage than I did, and he holds that factory rolling causes similar damage to the leaf. In his opinion the damage caused in both cases is, as already mentioned, sufficient to suppress completely all normal respiration. It can be shown, however, that even under these conditions of grinding there exists an appreciable amount of normal respiration in the finely minced tissues. Roberts & Sarma (1940) have given Q_{O_2} and R.Q. values for several plant tissues ground by Roberts's method. Let us first consider the two simple systems, e.g. the two non-tanniferous tissues *Tropaeolum majus* and *Hibiscus rosasinensis*. Calculated on the Q_{O_2} and the R.Q. figures these tissues will have a carbon dioxide output of 1·98 and 1·60 µl./mg. dry wt. of tissue/hr. respectively. These values are of the same order as that found by Jensen (1923) for *Tropaeolum majus* (1·29). As these tissues are non-tanniferous there is no question of polyphenol oxidation occurring after mincing, and since the R.Q. is very near unity the carbon dioxide is presumably due entirely to normal respiration of undamaged tissue.

Only four out of the 15 plant tissues for which Roberts & Sarma have given the R.Q. values seem to have carbon dioxide outputs outside the above range. It is quite possible that an explanation of their higher carbon dioxide outputs can be found on the basis of the wide variation of respiratory intensities in different plant species.

Roberts & Sarma (1940) place undue reliance on the correlation between tannin content and Q_{O_2}. This correlation $r = 0{\cdot}6160$ is vulnerable at two points. The mere establishment of statistical significance is no guarantee of an important degree of common causation between the variables. Translated into terms of the predictability index I (Treloar, 1936) the value is 0·2122, from which it is apparent that only 21 % predictability of the dependent variable is possible. Further, even if a high and significant value of r were shown, this would not establish the causal sequence between the *o*-quinone production and carbohydrate oxidation, for at no point do Roberts & Sarma differentiate between oxygen used for carbon dioxide production and oxygen used to produce *o*-quinone destined to form condensation products. This latter oxygen being the greater part (*vide* the low values of R.Q.) is undoubtedly capable of producing a correlation that will effectively mask any correlation between tannin and oxygen used for carbohydrate oxidation. Without the establishment of a partial correlation which cannot be calculated from the data, the existing

correlation is likely to be spurious as a basis for stipulating common causation.

The amount of carbon dioxide produced in the various tissues is a better indication of the role of tannins as H carriers in carbohydrate oxidation than the Q_{O_2} values. There appears, however, to be no correlation between the carbon dioxide output and the tannin content, as the correlation coefficient obtained ($r = 0{\cdot}29$) falls much short of significance (theoretical value $= 0{\cdot}5139$ for $P = 0{\cdot}05$). If the normal respiration has not completely ceased on grinding the non-tanniferous tissues, there is no reason why we should assume anything different in the case of tea leaf.

Perhaps the most important evidence which is considered to support Roberts's theory of the role of *o*-quinones in carbohydrate oxidation is the stimulation of the carbon dioxide output in the fermenting leaf by added polyphenols (catechol). It would indeed be difficult, as Roberts states, to explain this stimulation on my hypothesis, were it not that polyphenols like catechol are found to have a stimulating effect on the normal respiration of leaf (Fig. 1).

Roberts's (1941) picture of the equilibrium of the oxidation-reduction processes within the intact plant cell may be essentially correct, and it may also be possible that mechanical damage destroys the organization of the reactive surface, resulting in the cessation of normal respiration and anaerobic fermentation. The question, however, still remains as to whether all the active respiratory surface is completely disorganized by such mechanical injury as that occurring in a tea roller. As mentioned earlier, I have not reproduced exactly the conditions of the Tocklai experiments, nor is it possible to do so in the absence of detailed information about the grinding which is at present lacking; but this does not detract from the validity of my conclusion, especially since it can be shown from Roberts's own data that respiration was not completely suppressed in his experiments as well.

It has been made clear (Sreerangachar, 1943*a*) that the cytochrome theory of tea fermentation cannot be accepted in view of the spectroscopic and manometric findings. The nature of the enzyme responsible for fermentation has been further investigated, and it has been shown (Sreerangachar, 1943*b*) that tea polyphenol oxidase is also a copper-protein compound. The evidence presented here against Roberts's hypothesis of the relation between normal respiration and fermentation weakens yet another link in the mechanism of tea fermentation advanced by him.

SUMMARY

1. Residual respiration has been experimentally demonstrated in the factory-rolled withered tea leaf, thus establishing that normal respiration is not totally suppressed by the mechanical damage inflicted in rolling. Also the existence of residual respiration is inferred from Roberts's (1941, 1943) own data.

2. The main items of Roberts's evidence have been discussed and other explanations furnished for them in the light of the above observation.

3. Tea-tannin oxidation products produced during fermentation inhibit the normal respiration of the leaf, but addition of fresh polyphenols such as catechol has a stimulating effect on respiration. These observations explain the so-called dependence of carbohydrate oxidation on the tea-tannin *o*-quinones.

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REFERENCES

Boswell, J. G. & Whiting, G. C. (1938). *Ann. Bot., Lond.*, N.S. **2**, 847.

Eden, T. (1935). *Emp. J. exp. Agric.* **3**, 105.

Evans, D. I. (1928). *Bull. Tea Res. Inst., Ceylon*, **3**, 34.

Jensen, P. Boysen (1923). *K. danske vidensk. Vied. Selsk. Skr. Biol. Med.* **1**, 34. (Quoted from Stiles, W. (1936), *Introduction to Principles of Plant Physiology*, p. 146. London: Methuen.)

Lipman, F. (1943). *Ann. Rev. Biochem.* **12**, 1.

Richter, D. (1934). *Biochem. J.* **28**, 901.

Roberts, E. A. H. (1941). *Biochem. J.* **35**, 1219.

Roberts, E. A. H. (1943). *Nature, Lond.*, **152**, 77.

Roberts, E. A. H. & Sarma, S. N. (1940). *Biochem. J.* **34**, 1517.

Shaw, W. S. (1935). *Bull. Uni. Plant. Ass. South India*, **4***a*, 19.

Sreerangachar, H. B. (1941). *Biochem. J.* **35**, 1106.

Sreerangachar, H. B. (1943*a*). *Biochem. J.* **37**, 661.

Sreerangachar, H. B. (1943*b*). *Biochem. J.* **37**, 667.

Treloar, A. E. (1936). *An Outline of Biometric Analysis.* Minneapolis: Burgess.