SUMMARY

1. It has been shown, contrary to the results of Beavan & Brown (1949), that *Byssoschlamys fulva* produces pectin esterase and polygalacturonase.

2. It is suggested that the results of Beavan & Brown were due to the low activity of their enzyme preparations, which made the detection of pectin

esterase and polygalacturonase very difficult by the methods they used.

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The Fermentation Process in Tea Manufacture

12. THE ORIGIN OF CARBON DIOXIDE

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There are considerable differences of opinion on record regarding the origin of the carbon dioxide produced in fermentation, and the extent to which rolling and fine mincing destroy respiratory activity in the tea leaf.

Sreerangachar (1941, 1949) is of the opinion that there is much virtually undamaged tissue both in the fine minces used in manometric work, and in factory-rolled leaf, and that the carbon dioxide evolved during fermentation arises from normal respiration in such undamaged leaf. He produced experimental evidence which showed that the minces used in his manometric experiments contained appreciable amounts of intact tissue, but, as will be shown below, the same criteria when applied to our minces indicate a much greater degree of homogenization.

Roberts (1941c), on the other hand, concluded that fine mincing results in almost complete suppression of respiratory activity. This was indicated by the absence of anaerobic fermentation in a fine mince (Deb & Roberts, 1940), and by the low rates of oxygen uptake and carbon dioxide output observed in minces after polyphenol oxidation was complete (Roberts, 1939). It was pointed out that carbon dioxide output in a mince runs parallel with

oxygen uptake and that ascorbic acid, which reduces *o*-quinones, completely stops carbon dioxide output (Roberts, 1939). This output was believed, therefore, to depend upon oxidation of the polyphenols.

In the fermentation of factory-rolled leaf the Löwenthal non-tan titre was observed to decrease (Roberts & Sarma, 1938). The non-tans were then thought to include various respiratory substrates. A fall in reducing sugar content on fermentation was also recorded (Roberts, 1939) and it was suggested that reducing sugars and other respiratory substrates in the non-tan fraction were oxidized to carbon dioxide by the *o*-quinones produced in fermentation, the *o*-quinones functioning as H-acceptors in the dehydrogenations involved. Depletion of carbohydrates in the tea leaf by water culture in the dark for 3 days would therefore be expected to reduce carbon dioxide output in the fermentation of a leaf mince. As Sreerangachar (1941) has pointed out, the failure of Deb & Roberts (1940) to observe such an effect is inconsistent with the view that carbon dioxide is formed as a result of the oxidation by *o*-quinones of glucose and other respiratory substrates.

Roberts & Wood (1950) have shown that the polyphenols in the tea leaf yield considerable amounts of carbon dioxide on enzymic oxidation. This explains the parallelism of oxygen uptakes with carbon dioxide outputs, and also accounts satisfactorily for the amount of carbon dioxide produced, without having to postulate oxidation of substances such as glucose.

In this communication we present further evidence which establishes the almost complete absence of normally respiring tissue in our leaf minces, and the considerable inactivation of respiratory activity which occurs during tea manufacture as a result of the rolling process. The possible origin of carbon dioxide as a product of oxidation of glucose and other oxidizable substances in the leaf is considered, and shown to be extremely unlikely in a mince and of not more than secondary importance in the manufacturing process.

METHODS AND MATERIALS

Mincing of tea leaf samples. Tea leaf is minced in a domestic mincing machine using an attachment designed for grinding nuts to a paste. The fragments consist of two distinct categories. The smaller, numerically preponderant particles range from 100 μ . downwards, and consist almost entirely of cell debris, pieces of broken cell wall, and occasional chloroplasts. Most of these particles exhibit Brownian movement. The larger particles, which account for the greater proportion of the mass of the mince, range in size from 170 $\times$ 90 μ . up to 970 $\times$ 750 μ ., averaging 500 $\times$ 350 μ .

Manometric methods. For minces both the direct method of Warburg and the first method of Dickens and Šimer were employed (Dixon, 1934). For leaf disks the technique was as described by Deb & Roberts (1940). The extent of polyphenol oxidation in fermented leaf was determined by the residual uptake method (Roberts, 1941b, 1949), the total uptake recorded in 2 hr. giving an estimate of the polyphenols still unoxidized.

Enzyme preparations and substrates. These are as described by Roberts & Wood (1950).

Estimations of lipids. This procedure, which replaces methods involving extraction of the dried product in a Soxhlet with ether or light petroleum, is a combination of the methods described by Nielson & Bohart (1944) and Reichert (1944).

Finely minced leaf (10–20 g.) is refluxed for 15 min. with ethanol (50–100 ml.). After filtration the residue is washed twice with hot ethanol and the washings combined with the filtrate. The residue is dried and refluxed for 10 min. with light petroleum (60–80°) with which the tissue is allowed to remain in contact overnight. The solvent is removed by filtration and the residue washed with light petroleum. The washings are combined with the filtrate.

The residue from the light petroleum extraction is again refluxed with ethanol (25–50 ml.), filtered, washed with ethanol, and the extract and washings combined. The two ethanol extracts are mixed, NaCl (1 g.) added, and the mixture concentrated to a small volume, consisting essentially of an aqueous solution of ethanol-soluble matter with a layer of fats swimming on the surface. Subsequent treat-

ment with light petroleum and estimation of total lipid is as described by Nielson & Bohart (1944).

Estimation of carbon dioxide evolved from factory-treated leaf. This was estimated by a Pettenkofer method as described by Deb & Roberts (1940).

RESULTS

Determination of respiratory activity in fine minces

Washing of minced leaf. The effect of washing a fine mince of tea leaf is to reduce both oxygen uptake and carbon dioxide output, and the R.Q. is also appreciably diminished (Table 1). This contrasts strongly with the results of Sreerangachar (1941), and suggests that our mince, unlike that of Sreerangachar's, contains negligible proportions of intact, normally respiring tissue.

Table 1. *Effect of washing a fine mince*

(Minced tea leaf (2 g.) was washed twice with water (30 ml.) and respiratory activity determined on samples equivalent to 200 mg. of the original mince.)

	After 2 hr. shaking		
	(μ l. O ₂)	(μ l. CO ₂)	R.Q.
Original mince	460	175	0.38
Washed mince	47	10	0.21

Effect of glucose addition on fermentation. Addition of glucose to a leaf mince has no significant effect on the oxygen consumed in fermentation (Table 2). This is again in contrast with Sreerangachar's (1941) findings.

Table 2. *Effect of addition of glucose on fermentation*

(Minced tea leaf, 150 mg., suspended in water. Total volume 3 ml.)

Glucose concn. (M) ...	0.0	0.01	0.05	0.50
Time (min.)	O ₂ uptake (μ l.)			
10	108	106	111	105
30	204	198	204	204
60	231	225	228	229
90	237	230	239	234

Polyphenol oxidation in a fine mince. Oxygen uptakes were measured for systems consisting of tea leaf mince suspended in water, and of a 1 hr. infusion of the same mince in water, together with washed enzyme. The uptake with a 1 hr. infusion is about 77 % of that realized with the mince. Independent checks show that about 80 % of the water-soluble matter is extracted in 1 hr. when mince is infused with water under these conditions.

Fermentation in factory-rolled leaf

In north-east India it is customary to give the withered leaf two successive rolls of about 30 min. duration each. Table 3 shows residual oxygen uptakes determined for samples of leaf from a commercial factory, which had received one and two 30 min. rolls respectively. The second roll has resulted in very little extra fermentation.

Table 3. *Residual uptakes of fermented tea*

(Rolled leaf allowed to ferment for 3 hr. and then finely minced. 150 mg. portions suspended in 3 ml. water.)

Date of manufacture	Residual uptake in 2 hr. ($\mu\text{l. O}_2/\text{mg. dry wt.}$) No. of rolls	
	1	2
19. v. 49	2.84	2.27
31. v. 49	3.22	2.86
16. vi. 49	1.98	1.49
23. vi. 49	1.01	1.02

Polyphenol oxidation is still incomplete after a 3 hr. fermentation in the factory, and given sufficient time, further oxygen will be consumed. Judging from figures available for residual uptakes after different lengths of fermentation (Roberts, 1949) and for total uptakes for green leaf (Roberts, 1941c), 90% or more oxidation of the polyphenols will result eventually after one roll.

Carbon dioxide production in leaf exposed to chloroform vapour and in leaf warmed to 55°

When tea leaf is exposed to chloroform vapour, or maintained at a temperature of 50–55° (Roberts, 1941a) the polyphenols undergo irreversible oxidation and condensation in the same way as in fermentation, and the whole leaf assumes a uniform

Table 4. *Effects of chloroform vapour and of heating on respiratory activity of green leaf*

(Exp. 1, leaf disks (250 mg.) exposed to CHCl_3 vapour for 20 min. Exp. 2 leaf disks (200 mg.) in 3 ml. water maintained at 50–55° for 15 min.)

Time (min.)	Exp. 1		Exp. 2	
	($\mu\text{l. O}_2$)	($\mu\text{l. CO}_2$)	($\mu\text{l. O}_2$)	($\mu\text{l. CO}_2$)
15	38	18	66	12
30	71	36	124	20
60	135	66	207	37
120	200	100	323	60
180	237	118	403	76
240	256	129	—	—

brown colour. A manometric study has been made of the respiratory activity of leaf disks subjected to such treatments (Table 4), and it is found that the disks behave in much the same way as a fine mince. There is a period of rapid oxygen uptake followed by

a smooth die-away with the carbon dioxide output running approximately parallel with the oxygen uptake throughout the whole period of the experiment.

Total uptake after chloroform treatment is lower than after heat treatment. This is probably due to a quicker initiation of fermentation by chloroform so that appreciable fermentation has already taken place by the time that the chloroform-treated disks of leaf have been weighed out into the manometric vessels. This would obviously decrease the total uptake measured.

Association of carbon dioxide output with dehydrogenase activity

Effect of inhibitors. The effect of arsenite on the respiration of tea leaf is a significant depression of both oxygen uptake and carbon dioxide output, but this inhibitor is without significant effect upon the fermentation of a fine mince from the same bulk of leaf (Table 5). Iodoacetate (0.05M), malonate (0.05M) and 1-naphthol-2-sulphonic acid (0.01M) are all without significant effect on either oxygen uptake or carbon dioxide output of a leaf mince.

Table 5. *Effect of arsenite on respiration and fermentation*

(Fermentation 150 mg. mince; respiration, 300 mg. leaf disks.)

	After 1 hr. shaking			
	Fermentation		Respiration	
	($\mu\text{l. O}_2$)	($\mu\text{l. CO}_2$)	($\mu\text{l. O}_2$)	($\mu\text{l. CO}_2$)
Control	312	108	207	228
+0.01M-arsenite	302	95	111	144

Effect of cozymase. Addition of cozymase is without significant effect upon oxygen uptake or carbon dioxide output of minced tea leaf.

Table 6. *Effect of partial heat inactivation on R.Q.*

(Leaf fermented 3 hr. at 37°, finely minced and suspended in 3 ml. green leaf infusion, 20 g./200 ml.)

Time (min.)	($\mu\text{l. O}_2$)	($\mu\text{l. CO}_2$)
30	171	49
60	296	99
90	354	126
120	384	140
180	417	157

Differential inactivation by heat. Results presented in Table 6 show that when fresh green leaf infusion is added to a fine mince of previously fermented leaf, the R.Q. is 0.38 which is the same as for a mince of fresh green leaf.

Relation of R.Q. to complexity of system. Table 7 records oxygen uptakes and carbon dioxide outputs for systems consisting of (1) minced leaf, (2) washed enzyme and green leaf infusion, and (3) washed enzyme and a tea catechin preparation. No significant difference in the R.Q.'s is apparent, although the two latter systems are unlikely to contain the factors necessary to enable *o*-quinones to oxidize substances such as glucose.

Table 7. R.Q.'s of leaf mince and of enzyme and substrate systems

(O₂ uptakes and CO₂ outputs determined by method of Dickens & Šimer.)

	After 70 min.		R.Q.
	(μ l. O ₂)	(μ l. CO ₂)	
Leaf mince, 75 mg.	118	44	0.37
Washed enzyme, 40 mg. + green leaf infusion, 2 ml.	156	60	0.38
Washed enzyme, 40 mg. + tea catechins, 10 mg.	157	59	0.38

Oxidation of substances, other than polyphenols by o-quinones

Table 8 shows the amounts of carbon dioxide evolved when an infusion of green tea leaf, or an infusion from which polyphenols have been removed, is added to a mixture of tea oxidase and catechol. Refluxing an infusion with acid converts the catechins into reddish-brown insoluble products,

Table 8. Formation of carbon dioxide from non-polyphenols

(All systems contain washed enzyme, 40 mg. and catechol, 15 mg. at pH 5.6. 1 ml. of the solution under test is added in each case: Dickens & Šimer method.)

Solution added	(μ l. CO ₂)	Time of shaking (min.)
Nil	5	50
Green leaf infusion (20 g./200 ml.)	127	50
Same after hydrolysis with 2.5N-HCl and extraction with ether	11	50
Same after continuous extraction with ethyl acetate	29	70
<i>Hibiscus</i> infusion (20 g./100 ml.)	11	80

but also produces free gallic acid as a result of the hydrolysis of galloyl esters. This gallic acid is removed on continuous extraction with ether, and the resulting solution then yields comparatively little carbon dioxide when mixed with oxidase and catechol. Continuous extraction of the green leaf infusion with ethyl acetate removes all catechins, but not the polyphenolic acid believed to be *m*-digallic acid (Roberts & Wood, 1951*a*), and the effect of this removal of catechins is a substantial reduction in the output of carbon dioxide when the extracted infusion is treated with oxidase and catechol. The

table also shows a low carbon dioxide output for an infusion of *Hibiscus rosa-sinensis* leaf. This was shown by Roberts & Sarma (1940) to be of low polyphenol content.

There is no significant effect on total uptake when glucose, glucose-1-phosphate, caffeine, malic acid, citric acid, glycine, aspartic acid, glutamic acid and arginine are added either to a green leaf mince or to systems of washed enzyme and (+)-catechin buffered at pH 5.6. Data for glucose are given in Table 2, and the effects of additions of the other substances are essentially similar. None of these substances therefore appears to be oxidized by the *o*-quinones produced during tea fermentation.

In leaf fermented in the factory, fermentation appears to have no appreciable effect on the intensities of the amino-acid spots on two-way paper chromatograms (unpublished observations) so that appreciable oxidation of amino-acids during fermentation appears unlikely.

Previous estimations of ether-soluble matter (Roberts, 1942) indicated a relatively low lipid content (2%) in tea leaf, which was considerably reduced apparently as a result of fermentation. Using a revised procedure the lipid content is now found to be about 8%, but no significant reduction is observed as a result of fermentation.

Estimation of carbon dioxide output from factory-rolled leaf

Carbon dioxide evolution by rolled leaf continues for 5 hr. or more (Table 9), and this evolution is obviously not parallel with the oxygen uptake. The

Table 9. Evolution of carbon dioxide by fermenting leaf

(CO₂ evolution was determined on 100 g. samples of rolled leaf. Retention was estimated on 15 g. samples. Residual uptakes were determined with 150 gm. samples of a fine mince of the rolled leaf.)

Time (hr.)	CO ₂ output (μ l./mg. dry wt.)	Residual uptake (μ l. O ₂ /mg. dry wt.)	CO ₂ retention (μ l./mg. dry wt.)
0	—	5.13	1.42
1	0.305	—	—
2	0.752	1.54	2.22
3	1.130	—	—
4	1.370	1.10	—
5	1.528	—	—

average R.Q. for the first 2 hr. after rolling may be calculated from these results to be 0.21 and for the next 2 hr., 1.40. This latter figure suggests that some of the carbon dioxide initially produced is temporarily retained by the tissue, to be released slowly over a period of hours. Retention of carbon dioxide, as determined by the method of Wardlaw & Leonard (1939), is considerable and is also affected

by fermentation. This variable retention complicates estimations of carbon dioxide output and a proper study of this has yet to be made.

DISCUSSION

Respiratory activity in a fine mince

According to Sreerangachar (1941) even the finest mincing is unlikely to result in the disruption of every cell, so that some normal respiratory activity is likely to persist. It is not our belief, however, that such disruption, either in a mince or in factory-rolled leaf, is necessary to bring about fermentation. Rupture of the vacuolar membrane or an increase in its permeability to polyphenols is probably sufficient to initiate fermentation, and such damage may leave the outer cell walls intact. In leaf which has lost its turgor, by withering to 70 % moisture or less, we consider the twisting which can be imparted during rolling is sufficient to produce the necessary shearing stress required to rupture these membranes. In the fine mince the leaf has also been subjected to considerable shearing stresses, and it appears probable to us that the effects of this shear will not be restricted to cells actually cut, but will extend over a distance of several cell diameters. Few of the larger particles in our mince have a length greater than that corresponding with twenty cell diameters, and rupture of vacuolar membranes in practically all cells in particles of this size appears by no means unlikely.

There is little doubt that respiratory activity in our minces is negligible. The minces obtained by Sreerangachar (1941), however, are probably coarser and contain appreciable proportions of intact, normally respiring tissue. This essential difference is indicated by the different results obtained by Sreerangachar (1941) and ourselves, on washing a mince, and on adding glucose to a mince. The absence of normally respiring tissue from our minces is also indicated by the comparison between oxygen uptakes of the mince suspended in water, and of a mixture of washed enzyme and an infusion of the same mince. Allowing for the incomplete extraction of water-soluble matter when the mince is infused for 1 hr., the total oxygen uptakes of these two systems are comparable with each other. If the mince had contained any appreciable amount of undamaged tissue the polyphenols in this undamaged portion would not have become oxidized and the total oxygen uptake of the mince would have been lower than that of the infusion, incubated with enzyme. Our mince, therefore, probably approximates to a homogenate, and according to Bennet-Clark (1949) it is a characteristic of plant vacuolate tissue to show negligible total respiration in homogenates.

The total polyphenol content of Ceylon leaf (cf.

Lamb, 1936) is of the same order as that of leaf from Assam, so that total uptakes of green leaf minces from the two districts should be comparable. Comparing the published data for oxygen uptakes (Roberts, 1939, Fig. 8, Betjan leaf; and Sreerangachar 1941, Table 5) of Ceylon and Assam leaf, the much lower figures for the Ceylon leaf mince suggest that it contains a much higher proportion of undamaged tissue than minced Assam leaf.

Referring again to the data for Betjan leaf (Roberts, 1939) initial rates of oxygen uptake and carbon dioxide output are 3.7 and 1.2 $\mu\text{l./hr./mg.}$ wet weight. After 1 hr. shaking these rates have fallen to 0.2 $\mu\text{l.}$ oxygen and 0.08 $\mu\text{l.}$ carbon dioxide/hr./mg. wet weight respectively. With leaf disks suspended in water the rate of carbon dioxide output (Deb & Roberts, 1940) is about 0.8 $\mu\text{l.}$ carbon dioxide/hr./mg. wet weight. The leaf samples are reasonably comparable, so that it may be concluded that when polyphenol oxidation is nearly complete the rate of carbon dioxide output has fallen to about 10 % of its level in intact tissue. At this stage some polyphenol oxidation is still progressing, so that the data indicate at least 90 % inactivation of respiratory activity as a result of the fine mince. The low rate of oxygen uptake in leaf minces, obtained from species low in polyphenols, affords similar evidence, although Sreerangachar (1949) has queried these data and pointed out that the initial rate of oxygen uptake in a mince of *Tropaeolum majus* leaf (Roberts & Sarma, 1940) is of the same order as that recorded by Jensen (1923) for the intact tissue. We consider the initial rate represents a brief phase of irreversible oxidation of carriers and emphasize that after this short initial rapid uptake, lasting not longer than 15 min., the rate of oxygen uptake falls to less than 10 % of this level. It is this final low rate which was quoted as evidence for the destruction of respiratory activity by fine mincing.

Sreerangachar (1943, 1949) also suggests that low respiratory rates in leaf minces may be due to inhibition of respiration in intact tissue by polyphenol oxidation products. Our observations (Roberts & Wood, 1951*b*) show, however, that such inhibitions are negligible.

Carbon dioxide production in fine minces

Although it may be considered established that our fine minces contain practically no normally respiring tissue, the possibility remains that carbon dioxide might be formed as a result of oxidation of substances in the leaf by the *o*-quinones produced by enzymic oxidation of the polyphenols.

The fact that there is no inhibition of carbon dioxide output by arsenite, iodoacetate, malonate or 1-naphthol-2-sulphonic acid (cf. Axmacher & Ludwig, 1936), and no increase in carbon dioxide output on addition of cozymase, makes it unlikely

that the carbon dioxide originates from a normal glycolytic breakdown.

In view of the fact that high temperatures of fermentation result in appreciable inactivation of oxidase activity (Roberts, 1941*b*), such high temperature fermentation would be expected to affect the R.Q., as some differential effect on the extent of inactivation of the various enzymes involved would be expected. The absence of such an effect suggests that enzymes other than the oxidase are not involved in carbon dioxide production.

The identity of the R.Q.'s observed for minces and systems composed of enzyme preparation and tea catechins also suggests that the enzymes and co-enzymes concerned in glucose breakdown are not involved in the production of carbon dioxide in fermentation, as the acetone-treated tissue, constituting our enzyme preparation, can hardly contain the enzymes and coenzymes required for such glucose oxidation.

Enzymic oxidation of glucose, with *o*-quinones as H-acceptors is therefore probably eliminated, as an explanation of the origin of carbon dioxide in tea fermentation. We have also been unable to find any evidence for direct oxidation of substances such as sugars, amino-acids, etc. when these substances are added to systems producing *o*-quinones. Experiments with infusions of leaf, free or freed from polyphenols, also suggest that, apart from polyphenols of the type found in tea leaf, there are no substances present in such infusions capable of yielding carbon dioxide when oxidized by *o*-quinones.

The evidence available therefore indicates that almost the sole sources of carbon dioxide in fermenting leaf minces are the polyphenols themselves. Roberts & Wood (1950) have established that the polyphenols in tea leaf containing pyrogallol groupings, are oxidized enzymically with production of carbon dioxide. No other source of carbon dioxide in a mince has so far been demonstrated.

SUMMARY

1. The fine minces of tea leaf obtained in this laboratory are almost completely devoid of respiratory activity.
2. Carbon dioxide evolution in such minces is not associated with activity of any of the enzymes concerned with carbohydrate breakdown.
3. Oxidation of substances in the mince other than polyphenols is not detectable, so that carbon dioxide probably originates mainly from polyphenol oxidation.
4. Under factory conditions fermenting leaf in Assam shows little or no respiratory activity.
5. Carbon dioxide production in fermentation in the factory is also largely due to polyphenol oxidation, but there may be other subsidiary sources.

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