differences have been satisfactorily accounted for. From the evidence presented, Roberts's cytochrome theory of tea fermentation cannot be considered valid.

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— Studies on the 'Fermentation' of Ceylon Tea

7. THE PROSTHETIC GROUP OF TEA OXIDASE

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Earlier investigations in this laboratory on the specific nature of the oxidizing enzymes in tea [Lamb & Sreerangachar, 1940; Sreerangachar, 1943] have shown that the enzyme belongs to the family of polyphenol oxidases of which the potato oxidase and the mushroom oxidase are well-known members. The work of Kubowitz [1937; 1938] and of Keilin & Mann [1938] respectively on these enzymes has established that these polyphenol oxidases are Cu-protein compounds, and undergo a cycle of transformation from cupric to cupro form and vice versa, in the course of their catalytic activity. Cu is also found in the prosthetic groups of other oxidative enzymes such as laccase, ascorbic acid oxidase and tyrosinase, but the specific protein carrier in each of these enzymes is considered to be different. The Cu-proteins, unlike the Fe-porphyrin protein enzymes, show no specific absorption bands in the visible region of the spectrum, the absorption, if any, being only diffuse. A spectroscopic examination of tea oxidase has failed to reveal any characteristic absorption bands [Sreerangachar, 1943], and the enzyme is, therefore, analogous in this behaviour to Cu-proteins.

Consequent on the finding that tea oxidase was a polyphenol oxidase with respect to its substrate specificity, further investigations of the material

and chemical nature of this enzyme became desirable, and it was thought that a study of the prosthetic group was a fruitful line to follow in the first instance. In particular, it was intended to see whether, like the other known examples of polyphenol oxidases, tea-polyphenol oxidase was also a Cu-protein compound.

Cu is easily detected in tea-oxidase preparations, but it was found that the crude preparations also contained Mn and Fe. As these two metals are also frequently associated with certain oxidizing enzymes, the possibility of their forming the active group of tea oxidase had to be considered in the early stages. According to Bertrand & Mallèvre [1895], there exists a close connexion between Mn and laccase, and Shaw & Jones [1935] have emphasized the importance of Mn in the study of the oxidative enzyme in tea. That the fresh tea leaf contains appreciable amounts of Mn can be seen by the fact that the ash is generally green because of the presence of manganous salts, which readily change over to the characteristic permanganate colour when wetted with oxidizing solutions. But in spite of this, the acetone-prepared enzyme contains only a fraction of the original amount (200–400 µg./g.). Further, Mn is almost completely absent even in partially purified preparations of enzyme and there-

fore it clearly cannot be associated with the active group of the enzyme.

The importance of Fe lies in the fact that, should it be possible to establish its constant association with tea oxidase, the latter would have to be classified along with Fe-containing enzymes or haemochromogens. This would not, however, be quite consistent with the chemical constitution of a polyphenol oxidase as understood at present. Tea-enzyme preparations do contain Fe, which not only persists in most of the purification stages, but also increases in proportion as purification proceeds. In this case, therefore, a more detailed examination was necessary, in order to ascertain if Fe was an essential component of the enzyme. It will be shown later that adsorption on tricalcium phosphate gel, and subsequent elution, completely eliminates all Fe from the enzyme, the activity of which is thereby enhanced. Fe is thus a non-essential constituent so far as enzyme activity is concerned.

Cu, on the other hand, is consistently present in all the purified enzyme preparations, which become richer in this element with each step in purification. A study, therefore, of the relation between the Cu content and the activity of purified tea-oxidase preparations would throw some light on the active group of the enzyme. The experiments described in the present communication provide evidence for the postulate that tea oxidase is a Cu-protein complex.

EXPERIMENTAL

As far as possible, particular care was taken to avoid any metallic contamination during preparation of enzyme and in the subsequent experiments. The use of sand in grinding leaf under acetone, as practised previously, was dispensed with in the present investigation. Cu-free distilled water was prepared by distilling ordinary distilled water successively with KMnO_4 and metaphosphoric acid in an all-glass apparatus. In the estimation of Cu and Fe, necessary corrections were applied for the small respective metallic contents of reagents.

Estimation of Fe and Cu

Fe was estimated by the α - α' -dipyridyl method of Shorland & Wall [1936]. Digestions were carried out in 6 in. $\times$ 1 in. pyrex test-tubes with 1 ml. of conc. H_2SO_4 (Analar) and a few drops of Analar HNO_3 . The digests were made up to 10 ml., 1 ml. of which was mixed in a $\frac{1}{4}$ in. Lovibond cell with 1 ml. of dipyridyl reagent and a trace of solid $\text{Na}_2\text{S}_2\text{O}_4$. The colorimetric determinations were carried out in a Lovibond tintometer, the red units being found proportional to the Fe content of the test material. With Fe solutions of known strengths, a standardization curve can be drawn (Fig. 1), and the Fe content of any unknown material can be calculated from its Lovibond readings by reference to the standard curve.

The Cu content of the preparations was determined by the colorimetric method of Keilin & Mann [1938], with sodium diethyl-dithiocarbamate reagent. Digestion of the material was carried out as above and 5 ml. digests were employed for the test. The CCl_4 solution was made up to 10 ml. and dehydrated by shaking with a small quantity

Fig. 1.

of anhydrous sodium sulphate. Colorimetric determination was again carried out in a Lovibond tintometer, with $\frac{1}{2}$ in. cells for all tests. The yellow readings are found to be proportional to the Cu content of the material as shown by Fig. 2, which was obtained by employing solutions of known Cu content. Measurements could be made correct to 0.1 yellow Lovibond units, corresponding to about 0.3 µg. of Cu in 10 ml. of CCl_4 test solution.

Fig. 2.

Total blanks were run for both the Fe and Cu contents of all reagents except the test solution, the procedure being identical with that of an experiment.

Estimation of enzyme activity

The activities of enzyme preparations were estimated by the method described in Part 4 of this series. Activities are expressed in terms of mg. ascorbic acid oxidized in 1 hr. at room temperature under the conditions described. Where necessary, activities are calculated/g. dry weight of the material.

Purification of enzyme

(a) *Extraction of enzyme from acetone-prepared leaf powder.* The acetone preparation of enzyme was obtained as described previously [Lamb & Sreerangachar, 1940] but without using sand for grinding leaf. It was observed previously that extraction of soluble enzyme from this acetone preparation varied with the reaction of the extracting medium up to pH 7.0. Alkaline reactions were not then tried. In the present experiment it has been found that the amount of enzyme extracted increases up to pH 10.0, beyond which the enzyme undergoes considerable inactivation. Sørensen's glycine buffer and Ringer's phosphate buffer were employed to obtain these alkaline reactions, and Table 1 gives results of extraction of 20 mg. acetone-prepared enzyme. After extraction the enzyme solution was adjusted to pH 5.0 with dilute acetic acid, and a fraction equivalent to 20 mg. of original powder was employed for activity determinations. All reactions were carried out in presence of McIlvaine's buffer at pH 5.0 and activity expressed in mg. ascorbic acid oxidized in 1 hr.

Table 1. *Enzyme extraction in alkaline solution*

pH ...	5.0	8.0	9.0	10.0	11.0	12.0
Insoluble enzyme activity	1.51	1.31	1.35	1.05	0.62	0.53
Soluble enzyme activity	0.98	1.02	1.38	1.56	0.32	0.05
Total activity	2.49	2.33	2.73	2.61	0.94	0.58

The unextracted powder gave an average activity of 2.60, which shows that up to pH 10 there is no enzyme inactivation. The amount of enzyme extracted is maximum (60% of total) at pH 10.0, beyond which both the soluble and insoluble enzymes are considerably inactivated. It appears, therefore, that the most suitable reaction for extraction of soluble enzyme is pH 10.0, which was accordingly employed in the present investigation.

About 10 g. of the acetone-prepared leaf powder were extracted with 400 ml. buffer at pH 10.0 for 2 hr., filtered through muslin and squeezed by hand. The residue was extracted twice with 200 ml. buffer, the extract being squeezed out each time through muslin. The extracts were all collected, filtered through paper to give a clear reddish brown extract and adjusted to pH 6.0-6.5 by addition of dilute acetic acid. This acetone preparation had an activity of 0.21/mg. dry weight, while the activity of its extract was 0.43. If we define the amount of enzyme that oxidizes 1 mg. ascorbic acid as equivalent to a unit of enzyme, the crude extract was found to contain 966 units out of 2110 units of enzyme in the powder, i.e. about 46% extraction.

(b) *Fractional precipitation with ammonium sulphate.* Solid ammonium sulphate was gradually added to the crude extract so as to give, as shown below, increasing concentrations of the salt. The precipitate appearing at each stage was filtered off before the next addition of $(\text{NH}_4)_2\text{SO}_4$.

Concentration of $(\text{NH}_4)_2\text{SO}_4$ (% saturation)	Resulting precipitate
5	No ppt.
10	No ppt.
25	Turbidity but no ppt.
33.3	Slight brown ppt. (A)
50.0	Thin dark ppt. (B)
75.0	Dark ppt. (C)
100.0	Dark ppt. (D)

Precipitates A-D were collected separately, suspended in water and dialyzed against distilled water until free of $(\text{NH}_4)_2\text{SO}_4$. They were all found to be completely tannin-free when tested with FeCl_3 . The precipitates A and B, on dialysis, gave cloudy suspensions containing some insoluble matter, while C gave a brownish solution with very little insoluble substance. Precipitate D, on the other hand, yielded on dialysis a dark brown solution. Table 2 shows the activities and other particulars of these four fractions.

Table 2. *Properties of fractions obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation*

Fraction	Vol. of suspension (ml.)	Solid content (mg./ml.)	Activity (units/mg. dry wt.)	Enzyme content (units)
A	50	1.80	1.20	105
B	100	1.76	1.31	231
C	30	0.53	5.70	91
D	100	0.88	4.48	394

It will be seen that from an initial quantity of about 966 units a total of about 821 units of enzyme are recovered in the process. The fractions A and B seem to be similar in their activities and also in their solid-matter content. Most of the activity in these fractions appears to be associated with the insoluble matter; for the filtrates from A and B gave activities of only 0.42 and 0.41 unit/ml. respectively, while the original suspensions had activities of 2.17 and 2.31 units/ml. respectively. The dialysates resulting from fractions C and D had much higher activities than A and B, and they were, therefore, mixed and employed for further purification.

(c) *Purification with lead acetate.* In accordance with the procedure of Keilin & Mann [1938] for the purification of mushroom polyphenol oxidase, further purification of tea oxidase by means of lead acetate was attempted. In order to determine the amount of lead acetate required to remove impurities without affecting the enzyme, a trial experiment was conducted. 5 ml. portions of the dialysates (§ (b) above) were treated separately with 0.1, 0.2, 0.3 and 0.4 ml. 1/100 saturated lead acetate and the resulting

brownish gelatinous precipitate was immediately centrifuged off. The colour of the liquid diminished proportionately with the amount of lead acetate added, and in the last instance the centrifugate was almost colourless. The activities of the various solutions resulting from the above treatment are shown in Table 3.

Table 3. *Activity of material obtained by lead acetate fractionation*

Lead acetate solution added (ml.)	Activity of resulting solution (units/ml.)	Percentage of the original activity recovered
0	2.90	100.0
0.1	2.80	96.5
0.2	2.28	78.6
0.3	0.64	22.0
0.4	0.36	12.4

This preliminary test shows that lead acetate, even in small quantities, brings about a rapid inactivation of enzyme. On the dry weight basis, the activity of the solution resulting from the addition of 0.2 ml. lead acetate solution was 2.85 units/mg., which was only 70% of original activity. The method was, in consequence, useless for the purification of tea oxidase and was abandoned.

(d) *Fractional precipitation with acetone in the cold.* 40 ml. enzyme solution (§ (b) above) were cooled to -2 to -3° and mixed with cold acetone to give successively the following concentrations of acetone: (1) 5% acetone as employed by Keilin & Mann [1938] to remove readily precipitable impurities; (2) 40% acetone similar to Kubowitz's method [1938] for precipitating the potato oxidase, and (3) >80% acetone which effected more or less total precipitation. After good stirring the brown precipitates were centrifuged off, dissolved in a small quantity of McIlvaine's buffer at pH 7.0 and dialyzed against water.

Table 4. *Properties of the material obtained by fractional precipitation with cold acetone*

Conc. of acetone (%)	Vol. of fraction on dialysis (ml.)	Activity (units/ml.)	Dry matter (mg./ml.)	Activity (units/mg. dry wt.)
5	No ppt.	—	—	—
40	11	13.2	2.8	4.7
>80	9	6.4	2.3	2.8

The activity of the original solution is 5.0 units/mg. dry weight, and this value is not reached by any of the fractions obtained, although the 40% acetone fraction is very near to it. At 40% acetone concentration, therefore, the enzyme is concentrated into a smaller volume, but on the dry-weight basis no purification of enzyme has been achieved. Concentrations of acetone above 40% appear to bring about inactivation of enzyme. Thus acetone precipitation gave no more successful results in the purification of tea oxidase than the precipitation with lead acetate. (Table 4.)

(e) *Adsorption on calcium orthophosphate.* Calcium orthophosphate gel has been successfully employed in the purification of several oxidizing enzymes, e.g. mushroom polyphenol oxidase [Keilin & Mann, 1938], laccase [Keilin & Mann, 1938], and tyrosinase [Venkateswaran & Sreenivasaya, 1940].

Preliminary experiments on the use of this material for purification of tea oxidase gave promising results, and it has, therefore, since been adopted. In a typical adsorption experiment 150 ml. of $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme, containing 842 units, were acidified to pH 6.5 and mixed with 1.8 g. of freshly prepared gelatinous tricalcium phosphate. After standing for 30 min. the mixture was centrifuged and the colourless centrifugate, which contained only 42 units, was discarded. The phosphate cake was extracted three times with 10 ml. M/2 K_2HPO_4 solution, elution being carried out for $\frac{1}{2}$, 2 and 4 hr. respectively. The mixture was centrifuged once again, and the eluates all combined and dialyzed against distilled water. 120 ml. of pale yellow solution resulted, with 0.53 mg. dry weight/ml. and an activity of 5.0 units/mg. dry weight.

A second adsorption as above resulted in 30 ml. of an almost colourless dialyzed solution: dry weight, 0.20 mg./ml. and activity 10.1/mg. dry weight. A 60–70-fold purification of enzyme in the acetone preparation (activity 0.15 unit/mg. dry weight) has thus been effected by $(\text{NH}_4)_2\text{SO}_4$ fractional saturation and the two adsorptions just described. This, taken in conjunction with the fact that the soluble enzyme forms, under the most favourable conditions, only 60% of the total quantity of enzyme present in the acetone preparation, shows that the actual concentration of soluble enzyme effected is about 100-fold. Further, on an average the acetone-prepared enzyme forms about 50% of the dry weight of leaf and the latter roughly amounts to a fourth of the fresh weight. These data, therefore, indicate that the activity of the purest preparation obtained is at least 800 times greater than the activity of fresh tea leaf.

There are indications that the stability of the enzyme suffers considerably on purification by adsorption, elution and subsequent dialysis. Most of the inactivation occurs during dialysis which, however, is necessary for the dry-weight determinations and therefore cannot be excluded. It is, therefore, advisable to neutralize the eluate and determine its activity before dialysis. Dry-weight determinations could be done on the dialyzed preparations, with proper allowance for the increase in volume during dialysis. Inactivation can be much reduced if the alkaline eluate is neutralized before dialysis. Thus it is possible that the protein carrier of the enzyme in a purer state cannot stand continuous contact with the alkaline medium, which may rapidly denature the protein. The possibility also of the existence of a protecting substance or an additional prosthetic group which is removed during adsorption cannot be excluded. The work of Keilin & Mann [1940] on the blue pigment of laccase lends support to the latter hypothesis.

*Relationship between iron and copper contents
and enzyme activity*

In order to elucidate any possible relationship between Cu and Fe on the one hand and the activity of the enzyme on the other, quantitative data were obtained on the contents of these metals in the several purified preparations of enzyme as described above. Table 5 contains some typical results of

tion to all the rest of the adsorption experiments, where enzyme activity was determined immediately after elution and neutralization. § 3 (e) above gives reasons for suspecting that the continued contact with alkaline phosphate inactivates the enzyme in its purer state, and for this reason the low value of enzyme activity here shown is also suspect. No. 13 refers to a fractional precipitation of the enzyme solution with 40 % acetone. It is not unreasonable

Table 5. *Relationship between Fe and Cu contents and enzyme activity*

No.	Stage of purification	Activity (units/g. dry enzyme)	Cu content		Fe content	
			($\mu\text{g./g.}$)	($\mu\text{g./unit}$ activity)	($\mu\text{g./g.}$)	($\mu\text{g./unit}$ activity)
1	Acetone-prepared enzyme	151	32	0.21	150	1.00
2	Crude extract	330	60	0.18	119	0.36
3	$(\text{NH}_4)_2\text{SO}_4$ $\frac{1}{2}$ sat. ppt.	890	145	0.16	1364	1.53
4	$(\text{NH}_4)_2\text{SO}_4$ full sat. ppt. I	3,100	267	0.09	2400	0.77
5	After adsorption on and elution from $\text{Ca}_3(\text{PO}_4)_2$ gel. I	5,060	625	0.12	0	0
6	$(\text{NH}_4)_2\text{SO}_4$ full sat. ppt. II	6,600	520	0.08	1558	0.24
7	$\text{Ca}_3(\text{PO}_4)_2$ adsorbed and eluted enzyme II	4,000	364	0.09	0	0
8	$(\text{NH}_4)_2\text{SO}_4$ full sat. ppt. III	3,546	335	0.09	789	0.22
9	$\text{Ca}_3(\text{PO}_4)_2$ adsorbed and eluted enzyme III, first adsorption	5,000	357	0.07	0	0
10	$\text{Ca}_3(\text{PO}_4)_2$ adsorbed and eluted enzyme III, second adsorption	10,100	800	0.08	0	0
11	Adsorbed cake residue from no. 9 eluted again with alkaline phosphate	2,830	313	0.11	0	0
12	Cake from no. 11 eluted with $\frac{1}{2}$ sat. $(\text{NH}_4)_2\text{SO}_4$	2,440	244	0.10	610	0.25
13	$(\text{NH}_4)_2\text{SO}_4$ full sat. enzyme precipitated at 40 % acetone concentration	4,700	714	0.15	1786	0.38

these determinations and also the activities of the preparations/g. dry enzyme. To facilitate comparison, contents of Fe and Cu/unit activity of enzyme are also given.

It will be noticed that Fe is present in the enzyme preparations up to a certain stage in purification but disappears as purification proceeds further. It actually increases during the initial stages, although this increase is not in proportion to the increase in activity. Absence of Fe in the adsorption-purified enzyme makes its association with the active molecule of the enzyme impossible.

On the other hand, there is a steady increase in the Cu content of the preparations, with increasing activity/g. dry weight of material. In the more active preparations, however, there exists a fair proportionality between enzyme activity and Cu content. The Cu content/unit activity approaches a constant value on progressive purification. When the activities of the purified enzyme preparations are plotted against their corresponding copper contents an approximate straight-line relationship becomes evident (Fig. 3).

There are two points which diverge very appreciably from the linear relationship between activity and Cu content shown by the rest of the data. Against these points the number of the preparation to which they refer is shown. No. 5 is a preparation in which the activity was determined after dialysis following adsorption and elution, in contradistinction

to suggest that the acetone effected both a concentration of the enzyme (which would give a value much higher than the original 5.0) and also inactivated a portion of it. In this case the apparent

Fig. 3. Relationship between the Cu content and the activity of tea oxidase.

drop to 4.7 would not represent the full extent of inactivation, and again, this result is rendered suspect. In both these cases, therefore, there are

reasonable criteria for rejection of these two data when the activity-Cu relationship is considered.

The proportional relationship between the Cu content and the activity of the preparations is apparent even amongst the batches of acetone-prepared enzyme when such preparations are obtained from individual bushes whose leaf showed a fairly wide range of activity. For instance, the bushes whose acetone-prepared enzyme preparations had activities of 224, 193, 185 and 80 respectively/g. of material contained 20, 16, 16 and 4 μ g. of Cu/g. respectively. Since even in these preparations, which can by no means be considered pure, the ratio between the enzyme activities and Cu appears to be of the same order as that obtained with further purified preparations, it is possible that the Cu found in these preparations is mostly enzymic. This idea receives support from the fact that some of the fractions exhibiting only slight activity do also contain some Cu, but it is not in any great excess over the amount that corresponds to their activity. However, not all the Cu present in the fresh leaf belongs to the enzyme. Part of the Cu present in fresh leaf but not essential to the enzyme is indeed removed during extraction with acetone.

Dialysis against KCN

While ordinary dialysis removes all 'free' or ionic Cu from the enzyme preparations, dialysis against cyanide results also in loss of bound Cu from the enzyme. This method of removal of bound Cu was originally devised by Kubowitz [1937; 1938] in his study of potato oxidase and later employed by others [Meiklejohn & Stewart, 1941; Allen & Bodine, 1941]. In the Cu-protein systems studied, complete removal of Cu resulted in total inactivation of enzyme, which showed that Cu formed part of the enzyme molecule.

In experiments with tea-oxidase preparations, dialysis against KCN has also been carried out, with a similar object. To 10 ml. samples of (a) $(\text{NH}_4)_2\text{SO}_4$ -purified enzyme and (b) $\text{Ca}_3(\text{PO}_4)_2$ -adsorbed enzyme, solid KCN was added to give a concentration of M/100 KCN, and the mixtures dialyzed against Cu-free water until all KCN was removed. In Exps. 2 and 3, the enzyme solutions were first dialyzed for about 4 hr. against M/1000 KCN solution and then against Cu-free water. Table 6 gives the activities and iron and copper contents before and after KCN treatment.

In the case of $(\text{NH}_4)_2\text{SO}_4$ -purified enzyme there is apparently a tendency to retain part of the original Cu after treatment with KCN. By continued dialysis against KCN, as in Exp. 2, most of the Cu can, however, be removed from the enzyme solution. The enzyme preparation obtained after adsorption loses its Cu more easily when subjected

Table 6. *Dialysis of tea oxidase against KCN*

	Original enzyme	KCN-dialyzed enzyme	Percentage remaining after KCN treatment
Exp. 1. $(\text{NH}_4)_2\text{SO}_4$ -purified enzyme			
Dialyzed against water			
Activity/g.	6000	2800	42
Cu, μ g./g.	520	206	40
Fe, μ g./g.	1558	1647	106
Exp. 2. $(\text{NH}_4)_2\text{SO}_4$ -purified enzyme			
Dialyzed against KCN + water			
Activity/g.	2872	206	7
Cu, μ g./g.	335	35	10
Fe, μ g./g.	789	701	89
Exp. 3. $\text{Ca}_3(\text{PO}_4)_2$ -adsorbed enzyme			
Dialyzed against KCN + water			
Activity/g.	3562	0	0
Cu, μ g./g.	313	0	0
Fe, μ g./g.	0	0	0

to KCN dialysis, which renders this preparation completely free from Cu. It is significant that in every case the activity of the KCN-treated preparation is proportional to the amount of Cu retained in the solution; when the entire Cu is removed, the preparation becomes completely inactive in catalyzing the oxidation of polyphenols.

The Fe content is not significantly affected by the KCN treatment, and its presence seems to be immaterial for the activity of the preparations.

Previous workers, already cited, have found it possible, by addition of inorganic Cu, to restore the activity of preparations inactivated by dialysis against KCN. Kubowitz [1937; 1938] reports a restoration of 93 %, Meiklejohn & Stewart 67 %. The behaviour of tea oxidase, however, seems to be different in this respect from the above instances. For, when Cu is removed from tea oxidase by KCN dialysis, it has not been possible to restore activity even after addition of six to ten times the original amount of Cu. For this purpose the inactive dialyzed preparations were allowed, after addition of CuSO_4 , to stand for a few hours and were then dialyzed against water to remove excess Cu. The preparations remained inactive.

The failure of inorganic Cu to restore the enzyme activity may perhaps be due to its inability, after once being split off, to recombine with the protein residue of the enzyme molecule. The possibly labile nature of the protein part has already been mentioned, and during KCN dialysis it is likely that the protein may have been altered in a manner which makes reconstruction of enzyme impossible. Even in Meiklejohn & Stewart's [1941] experiments, the necessity for adding large excesses of inorganic Cu to restore only part of the original activity may be considered as indicative of the unstable nature of

the protein residue of the enzyme. Although restoration of inactive tea-oxidase preparations has not been successful, the complete loss of activity that attends a total removal of Cu must be regarded as providing evidence for Cu forming the active group of the enzyme.

N content of active preparations

Since the purest enzyme is considered to be a Cu protein, its N content may be expected to be about 15–16% of dry weight, as in the case of other proteins. N determinations of partially purified preparations may, therefore, be taken to indicate the extent of purification effected, provided the preparations do not contain any nitrogenous impurities. It seems unlikely that, after $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis and the operations preceding it, the tea-oxidase preparations contain any unknown nitrogenous impurities. Table 7 gives the results of micro-determinations of N on some of the more active preparations of enzyme, together with their activities.

Table 7. N contents of enzyme preparations

Preparations	Activity (units/g. dry wt.)	N (%)
1. $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme I	4,700	3.96
2. $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme II	3,546	3.47
3. No. 2 after adsorption on $\text{Ca}_3(\text{PO}_4)_2$	5,000	4.97
4. No. 3 after a second adsorption	10,100	6.60
5. No. 3 elution with $\frac{1}{2}$ sat. $(\text{NH}_4)_2\text{SO}_4$	2,882	2.73

It will be noticed that the N content of the preparations varies with the degree of purification obtained. If the N content of the pure substance is about 15%, our best preparation is only about 40% pure.

DISCUSSION

In plant extracts, a certain amount of catalytic oxidation of ascorbic acid is probably due to the presence of ionic Cu, as distinct from the action of enzymic Cu, which is no doubt very much greater than the former. Since, in the present investigation, estimations of polyphenol oxidase activity have been carried out by means of ascorbic acid, it is relevant to consider if there has been any interference in the results from this source. Such interference, if any, is possible only in the case of acetone-prepared enzyme powder and the crude extract. All other preparations clearly cannot contain any ionic Cu, as they have all been subjected to a prolonged dialysis during the course of purification. The more important conclusions reached in these experiments have been based on the dialyzed preparations, and as such they are unaffected by any possible presence of Cu ions in the fresh leaf.

No correlation has been perceived between the Fe content and the activity of preparations studied. On the contrary, the most active preparations are characterized by a complete absence of Fe, while the Fe contents in other preparations rendered inactive by dialysis against KCN remained unaffected. The possibility, therefore, of the enzyme being of the nature of a haemochromogen is excluded. This is in support of our previous conclusions based on spectroscopic and other evidence.

On the other hand, the evidence presented in this investigation shows a close relationship between the Cu content and tea-oxidase activity. In the more concentrated preparations the catalytic activity runs fairly parallel to the Cu content. Removal of Cu from the enzyme complex by dialysis against KCN results in its inactivation. These results point to the conclusion that, like other known polyphenol oxidases, tea oxidase also is a metalloprotein with Cu as its prosthetic group.

To obtain an idea of the degree of success in the purification of the tea enzyme in our best preparation, a comparison may be made between the activities of our enzyme and that of Kubowitz. Calculated in terms of O_2 uptake, the purest preparation of tea oxidase obtained in these experiments has an activity of $27 \mu\text{l. O}_2/\text{min.}/\mu\text{g.}$ of enzymic Cu ($Q_{10}/\text{Cu} = 1.6 \times 10^6$), which is roughly one-tenth the figure obtained by Kubowitz for the potato oxidase. With the limited equipment available here, attempts to purify the enzyme further have not as yet proved successful.

SUMMARY

1. Tea oxidase has been purified to a concentration of at least 800 times that present in fresh leaf.

2. The purest preparation obtained had an activity of 10,100 units/hr./g. dry weight of enzyme, 1 unit of enzyme being defined as the amount which brings about the oxidation of 1 mg. ascorbic acid. The Cu content of this preparation is 0.08% and nitrogen 6.60%. It has been calculated that $1 \mu\text{g.}$ of enzymic Cu catalyzes an O_2 uptake of $27 \mu\text{l. O}_2/\text{min.}$

3. The enzyme preparations purified by adsorption on $\text{Ca}_3(\text{PO}_4)_2$ gel were completely free from Fe.

4. In the more active preparations the polyphenol oxidase activity is proportional to the amount of Cu present. Complete removal of Cu inactivates the enzyme; resynthesis of enzyme by addition of CuSO_4 has not been possible.

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Oxidation of Acetic Acid in Animal Tissue

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Although it is known that acetic acid is readily oxidized in the animal, little is known of the intermediate stages on its oxidative path in the animal body, and numerous schemes have been suggested for its intermediate metabolism. In the present paper the oxidation of acetic acid and related 2-carbon compounds in animal tissue was examined. The results suggest that neither glycollic acid, glyoxylic acid, nor glycine are intermediates in the oxidation of acetic acid.

In the course of the investigation it was found that glyoxylic acid is a strong inhibitor of tissue respiration and of carboxylase. This observation may be useful in the study of enzymic reactions.

METHODS

Preparation of glyoxylic acid [Mohrschulz, 1926]. The Ca-salt was isolated and recrystallized three times (analysis: Ca, found 17.9%; calc. 17.9%). The Na-salt used was prepared from the Ca-salt by an equimolecular quantity of Na_2CO_3 .

Manometric experiments were carried out at 40° in Warburg's conical flasks. Fresh tissue was either sliced or minced. In every experiment tissue from the same normally fed animal was used, and the results are therefore directly comparable.

The rate of disappearance of carboxylic acids was studied by a method first described by Meyerhof & Lohmann [1926] [see also Elliott & Schroeder, 1934]. The method is based on the fact that the oxidation of a carboxylic acid in a neutral buffered medium yields, under certain conditions, bicarbonate. The increase of bicarbonate in the solution is therefore a measure of the oxidation of the organic acid. Tissue slices (dry weight 7–30 mg. according to species and tissue) were suspended in 3 ml. physiological saline [Krebs & Henseleit, 1932] containing 80 μl . bicarbonate/ml. The substrates were added as Na-salts to the main compartment of the flask. 0.2 ml. 2N NaOH was placed into the centre well and, unless otherwise stated, the gas space of the cup

was filled with O_2 . The manometers were put into the thermostat and the O_2 uptake was measured after an equilibration period of 10 min. The total O_2 uptake was calculated by extrapolating for the equilibration period. After the incubation period (90–120 min.) the cups were cooled with ice, and the slices removed, washed, dried, and weighed. The NaOH in the centre well was replaced by 0.1 ml. 10% H_2SO_4 , and 0.2 ml. 10% H_2SO_4 was measured into the side-arm. The gas space was filled with 5% CO_2 in O_2 , and, after equilibration, the amount of NaHCO_3 in the solutions was estimated by acidification. The values found were corrected for the amount of NaHCO_3 carried by the tissue slices, on the assumption that a volume of liquid equalling 10 times the dry weight of tissue is introduced or removed with the tissue. From the values obtained, Q_{O_2} and $Q_{\text{bicarbonate}}$ ($Q_{\text{bic.}}$) were calculated. $Q_{\text{bic.}}$ is equal to the negative metabolism quotient of the substrate investigated ($-Q_{\text{substrate}}$), provided that no acid groups are formed during the experiment, and the carboxylic group is oxidized. When the carboxylic group is not altered as a result of the metabolism, the changes of bicarbonate found in the experiment are not related to the utilization of the substrate.

In experiments with minced tissue, 3 ml. of a suspension of tissue in phosphate saline [Krebs, 1933] were measured into the main compartment; 0.2 ml. 2N NaOH was placed into the centre well, and the substrate was measured into the side-arm and made up to 1 ml. with the saline medium. The gas space was filled with O_2 . After an equilibration period of 10 min. the contents of the cup were mixed and the O_2 uptake was measured.

In anaerobic experiments, yellow phosphorus was placed in the centre well, the gas space filled with 5% CO_2 in N_2 , and the CO_2 output measured.

In some of the experiments lactic acid was estimated according to Friedemann, Cotonio & Shaffer [1927].

RESULTS

Oxidation of acetate. As measured by the formation of bicarbonate, this is more rapid in guinea-pig kidney cortex than in any other tissue examined