The Purification and some Properties of the Polyphenol Oxidase from Tea (*Camellia sinensis* L.)

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1. Polyphenol oxidase (EC 1.10.3.–) from the shoots of the tea plant was purified about 5000-fold on a dry-weight basis. 2. At an intermediate stage of purification four soluble yellow fractions were obtained. They are believed to represent complexes of a basic enzyme protein with acidic phenolic oxidation products and nucleic acids. After removal of the complex-forming materials the fractions were blue and similar to each other. About 40% of the activity could not be extracted from the acetone-dried powder. 3. Each of the four blue fractions was resolved further into two species, A and B. The following results refer to species A. 4. The enzyme showed absorption maxima at 279 mμ ($E^{1\%}_{1cm.}$, 13·5) and 611 mμ ($E^{1\%}_{1cm.}$, 0·84) with a shoulder at 330 mμ. The enzyme was bleached by substrate under anaerobic conditions and the colour was restored by oxygen. 5. The molecular weight measured by sedimentation and diffusion was 144000 ± 16000. The copper content was 0·32% (w/w). 6. Kinetic constants are given for a number of substrates and inhibitors, including the natural substrates of the tea leaf. The specific activity towards pyrogallol was 373 units/mg. at 30°. 7. The best substrates were *o*-dihydric phenols. Quinol and *p*-phenylenediamine were slowly oxidized. Monohydric phenols and ascorbic acid were not oxidized. 8. The kinetics of oxidation of most substrates are consistent with a mechanism in which oxidized and reduced forms of the enzyme form binary complexes with phenol and oxygen respectively. A modified mechanism is postulated for the oxidation of chlorogenic acid. 9. The relation of the results to the mechanism of tea fermentation is discussed.

The controversy (Roberts, 1942) over the nature of the oxidase responsible for the browning of fermenting tea leaf was resolved by the partial purification of an active phenol oxidase from fresh leaf by Sreerangachar (1943), who also gave evidence for a copper prosthetic group. We have now confirmed the latter conclusion although Sreerangachar's (1943) evidence has been invalidated by our observation that a blue copper protein, devoid of oxidase activity, follows the tea polyphenol oxidase during the early stages of purification.

The properties of the enzyme as it occurs in washed acetone-dried powders of tea leaf were studied by Roberts & Wood (1950, 1951). The substrate specificity of the enzyme seemed to distinguish it from both the tyrosinase and the laccase enzyme groups (Bendall & Gregory, 1963). Catechol and pyrogallol, and many of their naturally occurring derivatives, were readily oxidized, but gallic acid and its depsides and flavonol glycosides were not oxidized, although myricetin did act as a substrate. In particular, *p*-cresol and tyrosine were not substrates, and although *p*-phenylenediamine was oxidized quite rapidly quinol and ascorbic acid were not.

Recently, the study of the prominent classes of phenol oxidase, tyrosinase (EC 1.10.3.1) and laccase (EC 1.10.3.2), has progressed considerably. In the present work an attempt has been made to purify the tea enzyme and to relate its properties to those of other phenol oxidases and to the mechanism of tea fermentation. Since the tea enzyme cannot be described unambiguously as either a tyrosinase or a laccase, we have preferred to retain the term 'polyphenol oxidase'.

METHODS AND MATERIALS

Analytical methods

Measurement of polyphenol-oxidase activity. The uptake of oxygen from a solution containing 0·1 M-pyrogallol in 0·05 M-sodium citrate buffer, pH 5·6, was followed with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) coupled to a Honeywell–Brown Electronik recorder. The reaction medium (3 ml.) was contained in a flat-bottomed cylindrical vessel (12 mm. internal diam.). The vessel was immersed in a water bath at

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30° and the solution stirred magnetically. A thin Perspex sheath with a narrow vertical groove was sealed to the electrode so that it formed a close fit with the reaction vessel.

Following the method of Dixon & Kleppe (1965*a*), the concentration of oxygen in the above buffer was found to be 7% less than that of water when both were saturated with air at standard pressure at 30°.

Assays were performed on 0·01–0·1 ml. of enzyme solution. One unit of oxidase activity is defined as the quantity of enzyme catalysing the uptake of 1 μmole of O_2/min. when the oxygen activity was that of air-saturated water and the other conditions were as above. The rate of oxygen uptake in the absence of added enzyme was subtracted from the rate obtained in the presence of the enzyme. The assay system gave a linear reponse in the range 0·02–0·2 unit and was sensitive to 0·001–0·002 unit.

Measurement of peroxidase activity. Peroxidase was estimated by the method of Gregory (1966).

Starch-gel electrophoresis. The vertical apparatus of Smithies (1959) was used with a gel consisting of 11% (w/v) starch either in 6 mM-sodium acetate buffer, pH 5·6, or in 10 mM-boric acid–6 mM-tris buffer, pH 8·6. In the latter case, the electrode vessels contained 1·5 mM-tris–citrate buffer, pH 9·1. Approx. 0·5–1 mg. of protein was applied to the gel, and a voltage gradient of 12 v/cm. was applied, cathode upward, for 6 hr. at 4°. The gel was then laid flat and divided horizontally into two. One portion was stained with 1% (w/v) Nigrosin WS dissolved in methanol–acetic acid–water (9:2:9, by vol.) for 1 hr. After washing with the same solvent mixture protein components were shown as blue–black zones. The other portion was irrigated with a reagent to reveal zones of polyphenol-oxidase activity. The reagent consisted of a fresh mixture of equal volumes of 4% (w/v) catechol in 0·1 M-sodium citrate buffer, pH 5·6, and 1% (w/v) ascorbic acid in the same buffer to which had been added sufficient saturated 2,6-dichlorophenol-indophenol solution to give a faint permanent blue colour. In the presence of polyphenol-oxidase zones the coupled oxidation of the reduced dye caused blue bands to appear.

Sedimentation and diffusion. Purified enzyme was examined in the Spinco model E analytical ultracentrifuge with the artificial-boundary cell. The boundary was observed by ultraviolet photography and the film analysed by a Joyce–Loebl microdensitometer. Sedimentation was peformed on an enzyme solution (0·733 mg./ml.) in 0·05 M-ammonium acetate buffer, pH 5·5, at a rotor speed of 44 770 rev./min. at 21°. Diffusion was carried out at 20° with a similar solution (1·1 mg./ml.) with a rotor speed of 2233 rev./min. to stabilize the boundary. Values of S and D were corrected to water at 20° but were not corrected for the concentration of protein, this being regarded as negligible.

Estimation of copper. The copper content of the enzyme was measured by adapting the methods of Stark & Dawson (1958) and Summers (1960). To a 2 ml. sample of the enzyme solution in a centrifuge tube calibrated to 3 ml. was added 0·2 ml. of conc. HCl. The mixture was heated at 100° for 10 min. and, while it was still hot, 0·8 ml. of 20% (w/v) trichloroacetic acid was added. The tube was left for 10 min. and then cooled to 25°. After adjustment of the volume to 3 ml. with water, the tube was centrifuged at 1000*g* for 10 min. A 2 ml. sample was taken from the supernatant fluid and the precipitate was retained for estimation of the dry weight. Saturated oxalyldihydrazide solution (1 ml.), 0·5 ml. of aq. NH_3 solution (sp.gr. 0·88) and 0·5 ml. of 40% (v/v) acetaldehyde were added in that order, with mixing after each addition. After 30 min. E_{540} was measured in a Unicam SP. 500 spectrophotometer. Blanks, standards and estimations were performed in duplicate. The treatment of blanks and standards differed only in that water or standard $CuSO_4$ (AnalaR) solution were used in place of the enzyme and that the centrifuging step was omitted.

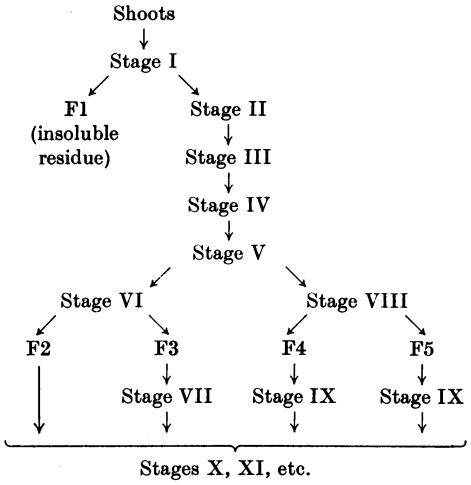
Determination of dry weight. The trichloroacetic acid precipitate from the above procedure was washed twice with 10% (w/v) trichloroacetic acid, three times with 70% (v/v) ethanol, and dried with ethanol and ether. It was dissolved in 100% formic acid and transferred to a small weighing bottle. The formic acid was evaporated and the residue dried to constant weight over P_2O_5 with the use of a mercury diffusion pump.

Spectrophotometry. The Unicam SP. 500 spectrophotometer was used for measurements at single wavelengths. The absorption spectra of the enzyme were obtained with the Beckman DK 2 recording spectrophotometer.

Chemicals

Preparation of catechins from tea. (+)-Gallocatechin, (−)-epigallocatechin, (−)-epigallocatechin gallate and (−)-epicatechin gallate were prepared from air-dried green shoots of tea by the method of Vuataz, Brandenberger & Egli (1959) modified by extracting the ethyl acetate-soluble fraction with wet ether (we are grateful to the late Dr E. A. H. Roberts for suggesting this). The freeze-dried ethyl acetate-soluble fraction (17 g.) was extracted repeatedly with wet diethyl ether (250 ml. portions). Occasional extractions with dry ether were included to prevent the residue from becoming too wet. The ether extracts were partially decolorized with activated charcoal, the ether was removed in a rotary evaporator and the aqueous residue was freeze-dried. The pale-yellow powder was further dried *in vacuo* over P_2O_5 (yield 8·5 g.) The preparation consisted essentially of the six catechins of the tea leaf, free from phenolic oxidation products but contaminated with small amounts of flavonol monoglucosides and traces of a few other substances showing up as faint fluorescent spots on two-dimensional paper chromatograms (Roberts, Wight & Wood, 1958) examined under ultraviolet light in the presence of NH_3 vapour. The catechins were separated by partition chromatography as described by Vuataz *et al.* (1959). The composition of fractions was checked by two-dimensional paper chromatography. Similar fractions were pooled, the organic solvent was removed on a rotary evaporator and the catechin was crystallized from water. The catechins were recrystallized once from water and dried *in vacuo* over P_2O_5.

Commercial chemicals. Pyrogallol and resorcinol (AnalaR) ascorbic acid (Roche Products Ltd., Welwyn Garden City, Herts.), 2,3-dihydroxynaphthalene, 3,4,5-trimethylphenol, putrescine hydrochloride, cadaverine hydrochloride, 2,3-dihydroxybenzoic acid, syringic acid, vanillic acid and protocatechuic aldehyde (L. Light and Co. Ltd., Colnbrook, Bucks.), chlorogenic acid (a gift from Dr T. Swain), adrenaline, toluene-3,4-dithiol and thioglycollic acid (Hopkin and Williams Ltd., Chadwell Heath, Essex), protocatechuic acid and catechol-3,5-disulphonic acid (British Drug Houses Ltd., Poole, Dorset) were used without further purification. Catechol, *o*-phenylenediamine, *p*-phenylenediamine, tetramethyl-*p*-phenylenediamine and quinol (Hopkin and



Scheme 1. Sequence of purification stages and the origins of the various fractions. See the text for further explanation.

Williams Ltd.), 4-methylcatechol and 3-methylcatechol (L. Light and Co. Ltd.) were recrystallized twice from toluene. *o*-Aminophenol, quercitin, *o*-nitrophenol, DL-β-dihydroxyphenylalamine and (−)-epicatechin (L. Light and Co. Ltd.), caffeic acid and ferulic acid (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) and (+)-catechin (Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.) were recrystallized from water. *p*-Cresol (Hopkin and Williams Ltd.), guaiacol (British Drug Houses Ltd.) and *o*-chlorophenol (L. Light and Co. Ltd.) were redistilled before use.

Preparation of the enzyme

The purification procedure described below is briefly summarized in Scheme 1. The starting material consisted of the young green shoots, including the bud and first two leaves, as used in the manufacture of black tea. For most of the work described here stage I was carried out in Malawi on freshly plucked shoots, but on some occasions the fresh material was sent by air to England.

Stage I: preparation of acetone-dried powder. Batches (about 150g.) of fresh leaf were blended with 650ml. of acetone in an MSE Ato-Mix blender at top speed for 1min. at room temperature. The suspension was filtered on a 1cm. Buchner funnel and the precipitate washed with small volumes of acetone. The solid was homogenized again with fresh acetone and filtered. After a third homogenization and filtration the powder was spread thinly on paper to dry at room temperature. The powder was mixed at intervals. As soon as the acetone had evaporated the powder was transferred to polyethylene bags and stored at −15°. It was stable for several months at −15°, but deteriorated rapidly at room temperature.

Stage II: extraction of acetone-dried powder. To prepare the extraction medium 35g. of glycine and 25g. of ascorbic acid were dissolved in 3l. of water and the pH was adjusted to 10·2 at room temperature with 5N-NaOH. Then NaCl (250g.), ice and water were added to give 5l. of medium at 0°.

Batches of powder (40–60g.) were extracted with 700–800ml. of medium in the blender run at top speed for 1min. The resulting suspension was centrifuged at 1000*g* for 25min. at 4°. The supernatant was strained through four layers of muslin and the solid was rehomogenized with additional medium. After the second centrifuging the residue (F1) contained about 40% of the original activity, and little more could be extracted. The supernatants were acidified to pH5 with 4N-citric acid and combined. Approx. 22l. of turbid fluid was obtained from 1kg. of powder; it was dialysed overnight against running tap water (10–12°).

Stage III: extraction of the dialysis residue. This consisted of coagulated protein, containing the polyphenol-oxidase activity, and an almost clear solution. The solid was centrifuged down as in stage II and the supernatant discarded. The precipitate was homogenized at room temperature with 0·1M-sodium citrate buffer, pH5·6 (300ml./kg. of acetone-dried powder), in the blender run at half speed for 1min. The suspension was centrifuged at 22000*g* in the Servall high-speed angle-head centrifuge at 4° for 20min. The supernatant was decanted and the precipitate re-extracted with buffer. The slightly turbid supernatants were combined and the residue was discarded.

Stage IV: fractionation with ammonium sulphate. The turbid brown solution from stage III was degassed by evacuation and treated with $(NH_4)_2SO_4$ (AnalaR) (390g./l. of solution). The solution was equilibrated at 4° for 2hr. and then centrifuged, at that temperature, in the MSE 13 centrifuge at 10000*g* for 10min. The precipitate was discarded. To the supernatant liquid was added, with stirring, 190g. of $(NH_4)_2SO_4$/l. and the suspension was equilibrated at 20°. The resulting precipitate was collected by centrifuging at 10000*g* for 10min. at 20° and the pale-yellow supernatant was discarded.

Stage V: precipitation at pH5·4. The precipitate from stage IV was dissolved in the minimum quantity of water and dialysed overnight against a large volume of 0·05M-sodium acetate buffer, pH5·4, at 4°. This treatment separated the oxidase activity into a soluble fraction and a fraction associated with a dark-brown precipitate. The dialysis residue was centrifuged and the supernatant set aside for treatment in stage VI. The precipitate was extracted with 0·1M-sodium citrate buffer, pH5·6. The extract was clarified by centrifuging and set aside for treatment in stage VIII.

Stage VI: ion-exchange chromatography on CM-cellulose. The soluble fraction from stage V was passed through a column (3cm. diam. × 20cm. long) of CM-cellulose (Whatman CM-70) equilibrated with 0·05M-sodium acetate buffer, pH5·4. Part of the activity and most of the brown colour were washed through with acetate buffer. This fraction of enzyme (F3) was passed to stage VII. The remainder of the activity adhered to the column in association with the lower of two blue bands. The column was developed with a linear concentration gradient of NaCl (0–1M) in acetate buffer over a volume of 300ml. Fractions from the column contained, in turn, a blue solution of the enzyme (F2), peroxidase, and a blue protein lacking polyphenol-oxidase activity. These substances were not completely separated and occurred in varying proportions in different preparations.

Stage VII: removal of coloured material by DEAE-cellulose. The brown solution of fraction F3 from stage VI was passed through a column (5cm. diam. × 20cm. long) packed with

DEAE-cellulose (Whatman DE-50). Most of the enzymic activity was obtained as a relatively pale solution after being washed through with 0·05 M-acetate buffer, pH 5·4. A dense-brown band remained at the top of the column and small amounts of activity were associated with faint bands that moved slowly down. The unretarded fraction was reapplied to a CM-cellulose column, the procedure of stage VI being followed. The resulting enzyme preparation resembled fraction F2.

Stage VIII: precipitation at pH 8·4. The citrate-buffer extract from stage V was dialysed overnight at 4° against 0·05 M-tris–chloride buffer, pH 8·4. A brown precipitate was collected by centrifuging. The brown supernatant (F4) was treated in stage IX. Extraction of the precipitate with 0·1 M-citrate buffer, pH 5·6, gave a dark-brown residue that was discarded and a solution that was dialysed again against the tris buffer at pH 8·4. A precipitate formed containing nearly all the activity. The precipitate was extracted with 0·1 M-sodium citrate buffer, pH 5·6, to yield, after clarification, a light-brown solution of enzyme (F5).

Stage IX: removal of nucleic acid from fractions F4 and F5. A solution of clupeine sulphate (5%, w/v) was added to fractions F4 and F5 until maximum precipitation was obtained. The suspensions were separately dialysed at 4° against 0·05 M-sodium acetate buffer, pH 5·4. The precipitates that formed were removed by centrifuging. The supernatants, which contained the enzymic activity, were then treated as in stage VI and yielded blue or green fractions similar to F2.

Stage X: chromatography on hydroxyapatite. Hydroxyapatite (Levin, 1962) was suspended in 0·01 M-sodium phosphate buffer, pH 6·8, at 4° and packed in a column (1·5 cm. diam. × 15 cm. long). The enzyme solution, dialysed against the same buffer, was passed through the column. The activity was adsorbed in association with a blue band. The column was washed with 0·01 M-phosphate buffer and a linear gradient of 0·01–0·3 M-sodium phosphate buffer, pH 6·8, was then applied over a volume of 300 ml. Two blue bands were eluted with slight overlap. Both showed polyphenol-oxidase activity and are referred to as A and B species in order of elution. This resolution was achieved with F2 and F3 preparations.

Stage XI: ion-exchange chromatography on Amberlite CG-50. The resin (grade II: British Drug Houses Ltd.) was sedimented through a large volume of 0·05 M-tris–chloride buffer, pH 8·0, to remove fine particles. A column (1 cm. diam. × 10 cm. long) was packed with resin at 4° and the enzyme solution in the same buffer was applied to the column and washed through; the activity was retained as a blue band. It was eluted by a linear concentration gradient of NaCl (0–1 M) in tris buffer over a volume of 80 ml. The pH of the effluent did not vary by more than 0·2 units.

In stages X and XI and the repetition of stage VI 5 ml. fractions were taken and assayed for E_{279} and polyphenol-oxidase and peroxidase activities.

RESULTS

General properties

Purification. The progress of the purification is shown in Table 1. The catalytic behaviour of the enzyme was studied with preparation F2A and other studies were made with preparation F3A.

The relative proportions of fractions 2–5 varied widely, and usually one fraction predominated in any one preparation. After stages VII and IX (Scheme I) all the fractions behaved in a similar manner on chromatography, were basic, and could be purified to preparations having similar spectra and catalytic properties not markedly different with regard to specific activity towards pyrogallol, K_m towards oxygen and ratios of activities towards different substrates. The similarities mentioned above, except for the behaviour on chromatography, also apply to the A and B species. The fractionation into A and B species, which is relatively clear-cut in stage X, could also be seen in stage XI, although the larger overlap made separation of the two species impracticable. A second treatment with ammonium sulphate at 4° effected considerable separation. When the enzyme concentration was 200 units/ml. in 0·1 M-sodium phosphate buffer, pH 7·0, 3·05 M-ammonium sulphate precipitated mainly species B and 3·50 M-ammonium sulphate precipitated species A. The separation was observed with all four soluble fractions, although fractions F4 and F5 were not analysed on hydroxyapatite. Species A is the easier to purify, and so far no preparation of species B has been obtained sufficiently pure for a comparison to be made with species A.

Starch-gel electrophoresis. Samples (0·3, 0·6 and 1·0 mg.) of preparation F3A were applied to the gel at pH 8·6 and also at pH 5·6, and in each case they moved very slowly towards the cathode. A single coincident band was observed with the stains for both protein and the polyphenol oxidase. It was concluded that the enzyme penetrated the gel with great difficulty, even though the gel was more dilute than usual. Had faster-moving proteins been present, they would have been detected by the stain at a concentration less than 5% of the oxidase band. These results, taken with the single boundary detected in the ultracentrifuge, provide some evidence for homogeneity. It may be added that the best preparations seemed to approach a limiting specific activity.

In another experiment with horizontal gel (Smithies, 1955) with 0·01 M-sodium acetate buffer, pH 5·6, the following samples of enzyme were placed in the starting slots: F3 at stage VI, and F2A, F3A and F5 each after stage IX. A gradient of 12 v/cm. was applied for 17 hr. at 4°. The zone of oxidase activity of preparation F3 migrated approx. 1·5 cm. towards the anode. All the other samples moved a similar distance towards the cathode. A considerable degree of streaking rendered further comparisons valueless. It is concluded that in preparation F3, as obtained in stage VI, the basic enzyme protein is present as a complex with acidic material, which is subsequently removed by passage through DEAE-cellulose.

Table 1. *Progress of the purification of polyphenol oxidase*

The yields quoted are representative of several preparations up to stage VII; those given for stages X and XI refer to the two particular preparations used for studying the properties of the enzyme.

	Stage	Specific activity (units/ml. when $E^{1cm.}_{279m\mu}=1$)	Specific activity (units/mg. dry wt.)	Amount recovered from previous stage (%)
	Fresh tea shoots		0·01–0·015*	–
I	Acetone-dried powder		0·07–0·10	80
III	Soluble extract		5	20–60
IV	60–80%-saturated $(NH_4)_2SO_4$ fraction		10	90
VI	Fraction F2 after CM-cellulose	10–12	30	80
X	Fraction F2A after hydroxyapatite	60–200		10–40
	Fraction F2B after hydroxyapatite	110		30
XI	Fraction F2A after Amberlite CG-50	100–210		10–60
	Fraction F2A from stage XI after repeat chromatography on CM-cellulose†	265	342	60
VII	Fraction F3 after DEAE-cellulose	5		85
	followed by CM-cellulose	90		80
XI	After Amberlite CG-50	180		50
X	After hydroxyapatite:			
	Fraction F3A (best fraction)‡	276	373	20
	Fraction F3A (remainder)	130		36
	Fraction F3B	190		40

* Fresh-weight basis.

† This preparation was used for the study of the catalytic properties of the enzyme.

‡ This preparation was used for the study of the general properties of the enzyme.

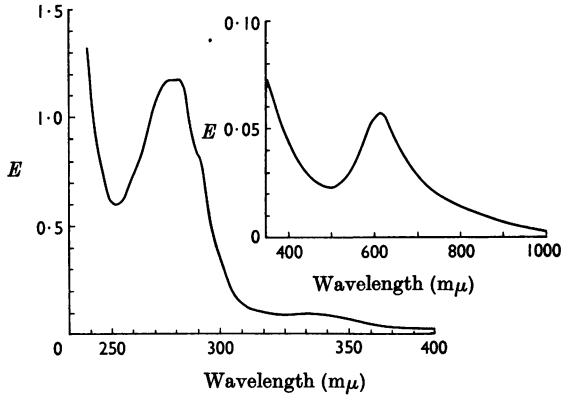


Fig. 1. Absorption spectrum of the purified enzyme (F2A) under aerobic conditions. The enzyme was at a concentration of 1·1 mg./ml. (335 units/ml.) in 0·3 M-sodium phosphate buffer, pH 6·8. The light-path was 1 cm.

Molecular weight and copper content. The sedimentation constant corrected to water at 20° was 5·25 s and $D_{20,w}$ was $3·33 \times 10^{-7}$ cm.^{2}/sec. Therefore if $\bar{v}$ lies between 0·70 and 0·76, the molecular weight lies between 128000 and 160000. The copper content was 0·32% (w/w), giving a weight of 19900 g./g.atom copper, or 6·4–8·0 copper atoms/enzyme molecule. The specific activity was 373 units/mg. when measured under standard conditions with pyrogallol as substrate.

Absorption spectrum. The absorption spectrum of preparation F2A in the aerobic state is given in Fig. 1. $E^{1\%}_{1cm.}$ was 13·5 at 279 mμ and 0·84 at 611 mμ, and the molar extinction coefficient, in terms of copper, is 1680 at 611 mμ. Thorough evacuation of a solution of the enzyme in a Thunberg spectrophotometer cell caused no change in the height of the

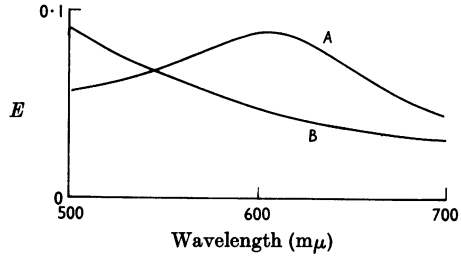


Fig. 2. Effect of (−)-epigallocatechin gallate on the 611 mμ absorption band. *A*, Spectrum of 3 ml. of enzyme solution (1·3 mg. of fraction F3A/ml. in 0·1 M-sodium citrate buffer, pH 5·6) in a Thunberg spectrophotometer cell after thorough evacuation; the light-path was 1 cm. *B*, The same after tipping in a solution of epigallocatechin gallate (0·5 mg.) in 0·5 ml. of water.

absorption band at 611 mμ. Therefore the blue colour of the enzyme is probably due to an oxidized state rather than an oxygenated complex. When (−)-epigallocatechin gallate was tipped into the evacuated solution from the hollow stopper the 611 mμ band disappeared at once, leaving a yellow solution (Fig. 2). The contents of the tube were passed through a column of Sephadex G-75 to remove phenols and concentrated on a small column of hydroxyapatite. A blue solution of the enzyme was recovered which in colour and activity accounted for some 80% of the original.

Catalytic properties

Substrates. The rates of enzymic oxidation of different concentrations of phenols at different concentrations of oxygen were estimated by drawing tangents to the recorded curves. In most cases it was shown that reoxygenating the reaction medium gave a second series of results similar to the first, and therefore no inactivation of the enzyme was taking place during the time taken to consume 1 μmole of oxygen, nor was there any significant product inhibition. Exceptions to this rule were *o*-aminophenol, which showed 7% inactivation during the consumption of 1 μmole of oxygen (over a period of 3·5 min.), and (−)-epicatechin gallate, which showed marked product inhibition. Secondary non-enzymic reactions involving an uptake of oxygen are known to occur in several cases, and no allowance has been made for these. The amenability of the results to kinetic analysis suggests that any such secondary steps were not rate-limiting.

Table 2. *Kinetic constants for tea polyphenol oxidase (fraction F2A)*

Meanings of symbols: $\overline{\overline{V}}$, maximum velocity at infinite concentrations of both hydrogen donor and oxygen, except for substrates which are inhibitory at high concentrations, when $\overline{\overline{V}}$ was obtained by extrapolation from rates at low substrate concentrations; $\overline{V}$ (air), velocity at infinite concentration of hydrogen donor and oxygen concentration of air-saturated water; $K_m^{O_2}$, Michaelis constant for oxygen at infinite concentration of hydrogen donor; K_m^S, Michaelis constant for hydrogen donor at infinite concentration of oxygen; k_m^S, (air), Michaelis constant towards hydrogen donor at oxygen concentration of air-saturated water; $[S]_{opt.}$, optimum concentration of hydrogen donor; N.D., not determined. Further details are in the text.

Substrate	$\overline{\overline{V}}$ (μmoles/min./unit)	$K_m^{O_2}$ (M)	K_m^S (M)	k_m^S (air) (M)	Form	Notes
Catechol	0·49	$2·1\times10^{-5}$	$2·9\times10^{-2}$	$2·6\times10^{-2}$	1b	
3-Methylcatechol	0·25	$3·9\times10^{-5}$	$6·8\times10^{-2}$	$5·83\times10^{-2}$	1b	
4-Methylcatechol	1·94	$1·3\times10^{-4}$	$2·0\times10^{-2}$	$1·5\times10^{-2}$	1	
Caffeic acid	0·091	$3·5\times10^{-6}$	$8·1\times10^{-3}$	$7·98\times10^{-3}$	1	
Chlorogenic acid	very high	$1·1\times10^{-5}$	0	0	2	$K_m^S/\overline{\overline{V}}=2·68\times10^{-2}$ mole/min./unit/μmole
Adrenaline	N.D.	N.D.	N.D.	6×10^{-4}	—	$\overline{V}$ (air) = 0·0034 μmole/min./unit
DL-β-Dihydroxyphenylalanine	N.D.	N.D.	N.D.	$2·8\times10^{-2}$	—	$\overline{V}$ (air) = 0·022 μmole/min./unit
Pyrogallol	2·00	$1·1\times10^{-4}$	$5·3\times10^{-2}$	$3·6\times10^{-2}$	1	
Gallic acid	0·04	$1·0\times10^{-5}$	$8·3\times10^{-2}$	$7·96\times10^{-2}$	1	
Ethyl gallate	0·13	$1·6\times10^{-5}$	$2·6\times10^{-2}$	$2·4\times10^{-2}$	1	
Quercitin	0·8	N.D.	N.D.	5×10^{-4}	—	When [quercitin] = 0·13 mM and $[O_2]$ = 0·23 mM, v = 0·18 μmole/min./unit
(+)-Catechin	1·0	$4·7\times10^{-5}$	$1·8\times10^{-3}$	$1·5\times10^{-3}$	1a	$[S]_{opt.}$ = 10 mM
(−)-Epicatechin	1·8	$1·3\times10^{-4}$	$1·5\times10^{-3}$	$9·8\times10^{-4}$	1a	$[S]_{opt.}$ = 6 mM
(−)-Epicatechin gallate	High	N.D.	N.D.	N.D.	—	$[S]_{opt.}$ = 1 mM, when v (air) = 0·4 μmole/min./unit
(+)-Gallocatechin	1·07	$7·9\times10^{-5}$	$4·8\times10^{-3}$	$3·6\times10^{-3}$	1	
(−)-Epigallocatechin	1·7	$1·7\times10^{-4}$	$5·7\times10^{-3}$	$3·3\times10^{-3}$	1	
(−)-Epigallocatechin gallate	0·82	—	—	$2·4\times10^{-4}$	3	$[S]_{opt.}$ = 3·3 mM, when K_m (oxygen) = $2·4\times10^{-5}$ M
o-Aminophenol	0·31	$1·0\times10^{-5}$	0·2	0·2	—	Qualification in text
o-Phenylenediamine	0·24	2×10^{-5}	0·53	0·49	1	
Quinol	0·03	$1·5\times10^{-5}$	$1·4\times10^{-2}$	$1·3\times10^{-2}$	1	
p-Phenylenediamine	0·05	8×10^{-6}	2×10^{-2}	2×10^{-6}	1	
NNN'N'-Tetramethyl-*p*-phenylenediamine	0·05	8×10^{-6}	2×10^{-2}	2×10^{-6}	1	

When reciprocals of the rates were plotted against reciprocals of the concentrations of oxygen or hydrogen donor (Lineweaver & Burk, 1934) straight lines were obtained in most cases. In other cases the plots became linear at low concentrations. These lines were extrapolated to infinite concentration of one reactant, and the maximum velocities so obtained were plotted on the other graph in terms of the other reactant. In this way values of $\bar{\bar{V}}$ (the rate/unit of enzyme at infinite concentrations of oxygen and hydrogen donor), and the K_m values for each reactant at infinite concentration of the other, were obtained. Table 2 gives these parameters. Also given are K_m values for the hydrogen donors at the oxygen concentration (0·24 mM) of air-saturated water, and the optimum substrate concentration is quoted for substrates that in high concentration inhibited the enzyme. The results are described below in groups according to the forms of the families of lines on the Lineweaver–Burk plots.

Form 1. The families of lines were parallel in both plots (Fig. 3 and 4) and the line representing infinite concentration did not pass through the origin. Most substrates fell under this heading: pyrogallol, 4-methylcatechol, gallic acid, ethyl gallate, caffeic acid, (+)-gallocatechin, (−)-epigallocatechin, *o*-phenylenediamine, *p*-phenylenediamine, *NNN'N'*-tetramethyl-*p*-phenylenediamine and quinol.

Form 1a. (+)-Catechin and (−)-epicatechin showed inhibition at high concentration (Fig. 5), but at lower concentrations form 1 kinetics were obtained. The plots with respect to oxygen were linear and parallel.

Form 1b. The progress curve for the oxidation of catechol showed a lag phase, as was found by

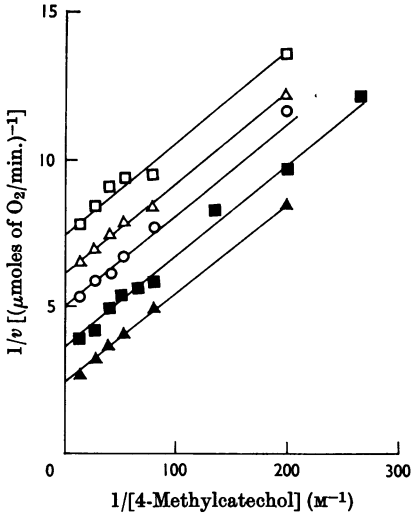


Fig. 3. Form 1 kinetics. Oxidation of 4-methylcatechol: plot of $1/v$ against 1/[4-methylcatechol], obtained with 0·23 unit of enzyme and the following O_2 concentrations: □, 0·05 mM; △, 0·067 mM; ○, 0·10 mM; ■, 0·20 mM; ▲, infinite, extrapolated from Fig. 4.

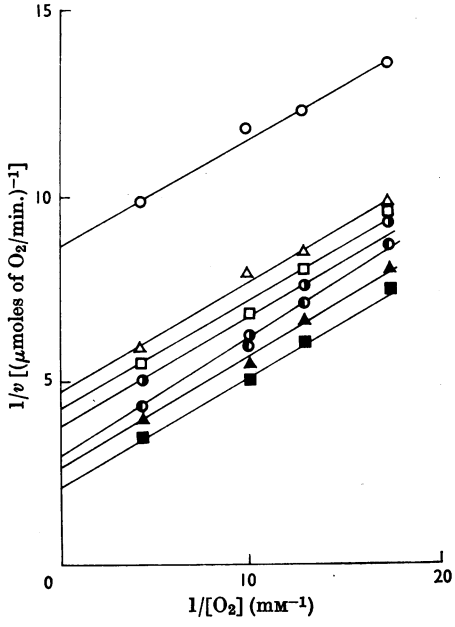


Fig. 4. Form 1 kinetics. Oxidation of 4-methylcatechol: plot $1/v$ against $1/[O_2]$ from the same data as Fig. 3, with the following 4-methylcatechol concentrations: ○, 6·7 mM; △, 16·7 mM; □, 25 mM; ◑, 33 mM; ◐, 50 mM; ▲, 0·1 M; ■, infinite, extrapolated from Fig. 3.

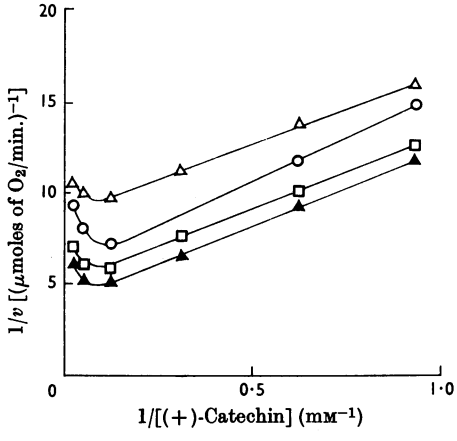


Fig. 5. Form 1a kinetics. Oxidation of (+)-catechin: plot of $1/v$ against 1/[(+)-catechin], obtained with 0·23 unit of enzyme and the following O_2 concentrations: △, 0·05 mM; ○, 0·067 mM; □, 0·2 mM; ▲, infinite.

Roberts & Wood (1950), and 3-methylcatechol behaved similarly. In the presence of ascorbic acid (0·1–0·03 M) a steady rate of oxygen uptake was attained immediately. After the steady state had been reached (without ascorbate) measurements yielded plots of form 1.

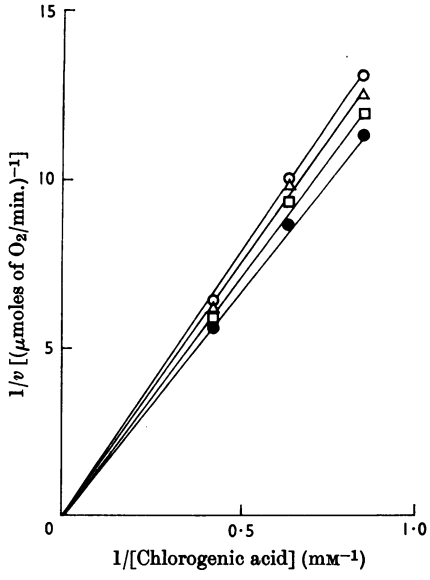


Fig. 6. Form 2 kinetics. Oxidation of chlorogenic acid: plot of $1/v$ against 1/[chlorogenic acid], obtained with 2·2 units of enzyme and the following O_2 concentrations: ○, 0·05 mM; △, 0·067 mM; □, 0·1 mM; ●, 0·2 mM.

Form 2. The K_m for the phenolic substrate is infinite, the lines diverging from the origin, whereas that for oxygen is constant (chlorogenic acid; Fig. 6).

Form 3. (−)-Epigallocatechin gallate showed very marked inhibition at high substrate concentrations (Fig. 7). With respect to oxygen, straight lines were obtained, K_m passing through a minimum value (Fig. 8).

o-Aminophenol was studied in the same way, but the Lineweaver–Burk plots were not linear, particularly at low concentrations of oxygen. There was slight enzyme inactivation (7% in 3 min.), and the progress curves were the same when the observations were repeated after more oxygen had been added to the system. The results in Table 2 were obtained by extrapolation of the approximately linear part of the Lineweaver–Burk plots at the higher oxygen concentrations (0·05–0·24 mM).

Some other substrates were only studied at one concentration of oxygen (0·24 mM). With quercitin, adrenaline and DL-β-dihydroxyphenylalanine linear Lineweaver–Burk plots were obtained. With (−)-epicatechin gallate severe product inhibition was observed. The initial rates of oxygen uptake show some inhibition at substrate concentrations approaching the maximum solubility (Fig. 9).

The oxidation of toluene-3,4-dithiol was examined in Warburg manometers containing potassium hydroxide in the centre wells of the vessels. The main compartments contained 1·8 ml. of 0·1 M-sodium citrate buffer, pH 5·6, and 1·0 ml. of dimethylformamide containing toluenedithiol (15 μmoles) or pyrogallol (0·3 m-mole), and the side arms contained 4 units of enzyme in 0·2 ml. of

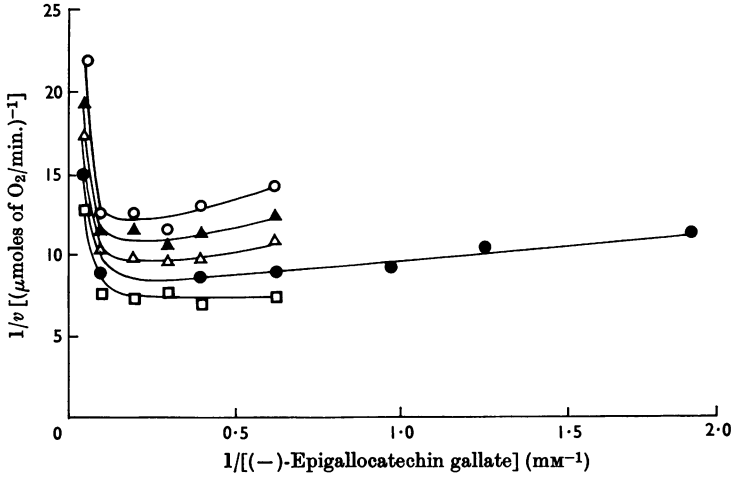


Fig. 7. Form 3 kinetics. Oxidation of (−)-epigallocatechin gallate: plot of $1/v$ against 1/[(−)-epigallocatechin gallate], obtained with 0·23 unit of enzyme and the following O_2 concentrations: ○, 0·05 mM; ▲, 0·067 mM; △, 0·1 mM; ●, 0·2 mM; □, infinite.

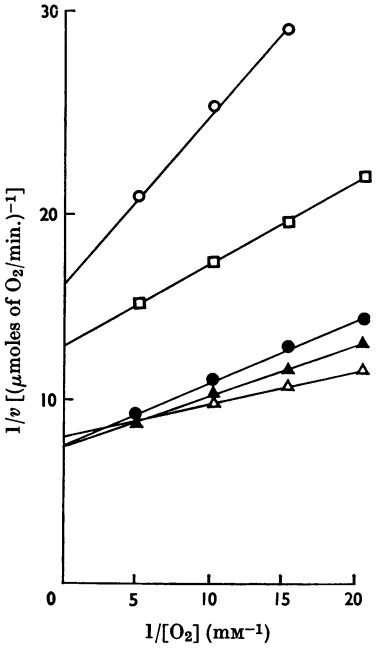


Fig. 8. Form 3 kinetics. Oxidation of (−)-epigallocatechin gallate: plot of $1/v$ against $1/[O_2]$, from the same data as Fig. 7, with the following (−)-epigallocatechin gallate concentrations: ○, 40 mM; □, 20 mM; ▲, 5 mM; △, 3·3 mM; ●, 1·6 mM.

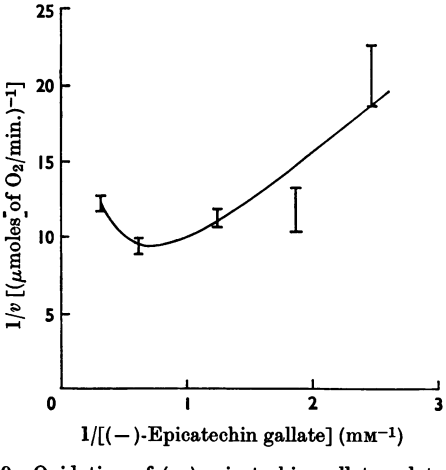


Fig. 9. Oxidation of (−)-epicatechin gallate: plot of $1/v$ against $1/[(-)$-epicatechin gallate], with 0·22 unit of enzyme and an O_2 concentration of 0·24 mM.

buffer. Under these conditions the initial rate of oxygen uptake with pyrogallol was 0·44 μmole/min., whereas that with toluenedithiol was 0·15 μmole/min. There was rapid inactivation of the enzyme by the solvent.

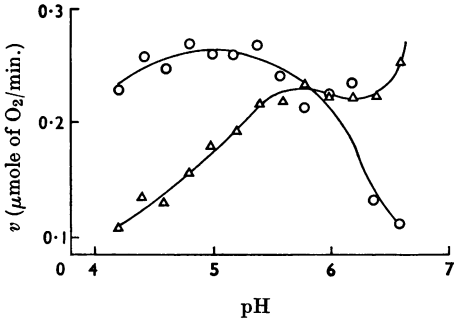


Fig. 10. Effect of pH on the oxidation of 0·1 M-pyrogallol (△) and 0·1 M-methylcatechol (○). The buffers were prepared by titrating 0·1 M-Na_2HPO_4 with 0·1 M-citric acid. In each case the non-enzymic rate of oxygen uptake was subtracted from that observed in the presence of the enzyme. The blank rate increases sharply above pH 6 in each case, and is prohibitively high above pH 6·6.

Effects of pH. The effects of pH on the activity of the enzyme, with pyrogallol and 4-methylcatechol respectively as substrate, are shown in Fig. 10. The pH optima were 5·7 with pyrogallol and 5·0 with 4-methylcatechol.

Inhibitors. The effects of inhibitors were examined in the presence of 0·1 M-pyrogallol and 0·1–1 unit of enzyme. With potassium cyanide inhibition was complete at concentrations above 1 mM. Diethylammonium diethyldithiocarbamate caused complete inhibition above 5 mM, but both these inhibitors showed a time-dependent effect. With 10 mM-thiourea 80% inhibition was observed. Protocatechuic acid, protocatechuic aldehyde, ferulic acid, tetramethylenediamine, pentamethylenediamine and resorcinol had inhibitory effects on the oxidation of pyrogallol that gave linear Lineweaver–Burk plots. The results are described in Table 3. Sodium EDTA (10 mM), saturated 8-hydroxyquinoline and 10 mM-chloroacetophenone had no inhibitory effects.

Inactive phenols. The rates of autoxidation of the following compounds were not increased during a 10 min. period by the addition of 2–3 units of enzyme nor did these compounds have any inhibitory effect on the rate of oxidation of 0·1 M-pyrogallol by 0·1 unit of enzyme: naphthalene-2,3-diol, 1-amino-2-naphthol-4-sulphonic acid, catechol-3,5-disulphonic acid, 2,3-dihydroxybenzoic acid, cyclohexane-1,2-diol, *p*-cresol, ascorbic acid, a mixture of *p*-cresol and ascorbic acid, *o*-nitrophenol, *o*-chlorophenol, *o*-methoxyphenol, 3,4,5-trimethylphenol, syringic acid and vanillic acid.

Table 3. *Effects of inhibitors on tea polyphenol oxidase*

The standard assay procedure was used with 0·1 M-pyrogallol as substrate and enzyme preparation F2A. The effects on the Lineweaver–Burk plots are described.

Inhibitor	Effect with respect to pyrogallol				Effect with respect to O_2			
	Concn. (M)	Effect	Relative change in		Concn. (M)	Effect	Relative change in	
			Slope	Intercept			Slope	Intercept
Protocatechuic acid	0·09	Competitive	2·5	1·0	0·18	Upward displacement and increased slope	1·23	1·57
Protocatechuic aldehyde	0·044	Competitive	5·0	1·0		Not determined		
Ferulic acid	0·033	Non-competitive	2·0	2·0	0·047 0·021	Non-competitive	3·7 2·0	3·7 2·0
Tetramethylene-diamine	0·10	Parallel upward displacement	1·0	2·22	0·10	Non-competitive	3·7	3·7
Pentamethylene-diamine	0·10	Parallel upward displacement	1·0	2·22	0·10	Non-competitive	3·7	3·7
Resorcinol	0·090	Mixed type	2·74	1·34	0·090	Upward displacement and increased slope	1·25	1·63

DISCUSSION

At least 98% of the enzyme in the living leaf is bound to insoluble material. The enzyme is partly released during the preparation of the acetone-dried powder, but it is not clear whether preparation F1 is a bound (or tanned) form of the soluble enzyme or is a distinct species. The insoluble fraction is relatively greater if much oxidation of polyphenols is allowed to occur during preparation or extraction of the powder, and in powders prepared from fermented leaf. The fact that fractions 3, 4 and 5 can be resolved into material similar to preparation F2 suggests that these fractions are artifacts that arise by combination of the same basic protein with acidic material, phenol oxidation products or nucleic acid or both. The difference between the A and B species of the enzyme seems likely to reside in the protein moiety, but the nature and origin of this difference remain obscure at present.

Evidence for the mechanism of action of the enzyme comes from the observation of a reversible bleaching, which suggests cupric and cuprous states, and from the kinetics. Dalziel (1957) has shown that form 1 kinetics, which are followed by most of the substrates studied here, are given by a type of mechanism in which reaction of the enzyme with S_1 gives a modified form of the enzyme, which is then returned to the original state by reaction with S_2 [Dalziel's type IV (i)]. With polyphenol oxidase, reaction with oxygen would yield E″ at an oxidation level of +4 relative to E. E would be re-formed by reaction of E″ with an *o*-dihydric phenol in either two successive 2-equivalent changes, or four 1-equivalent steps as has been shown for the lac-latex laccase by Nakamura (1960). In mechanism Ia the former is assumed (Scheme 2). The steady state equation for this mechanism is:

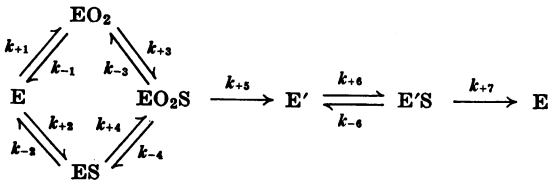
$$\mathrm{E}+\mathrm{O_2} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{EO_2} \xrightarrow{k'} \mathrm{E''} \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} \mathrm{E''S} \xrightarrow{k_{+9}} \mathrm{E'} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{E'S} \xrightarrow{k_{+7}} \mathrm{E}$$

Scheme 2. Mechanism Ia, where E′ has an oxidation level of +2 relative to E.

$$\frac{e}{v} = \frac{1}{k'} + \frac{1}{k_{+7}} + \frac{1}{k_{+9}} + \left[\frac{k_{-6}+k_{+7}}{k_{+6}k_{+7}} + \frac{k_{-8}+k_{+9}}{k_{+8}k_{+9}}\right]\frac{1}{[\mathrm{S}]} + \frac{k_{-1}+k'}{k_{+1}k'}\cdot\frac{1}{[\mathrm{O_2}]} \qquad (1)$$

However, mechanism Ia is not a unique explanation of form 1. In special circumstances mechanisms involving the ternary complex EO_2S may also give families of Lineweaver–Burk plots with constant slope. If the rate equation for a two-substrate reaction is written in the general form given by Dalziel:

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{[\mathrm{O_2}]} + \frac{\phi_2}{[\mathrm{S}]} + \frac{\phi_{12}}{[\mathrm{O_2}][\mathrm{S}]}$$



Scheme 3. Mechanism Ib.

where ϕ stands for a constant derived from rate constants only, we require the individual rate constants to be such as to render ϕ_{12} negligible. This may be achieved with the types I (a), II (i), II (ii, a), II (ii, b) and II (iii) of Dalziel. For the type of reaction catalysed by polyphenol oxidase, the ternary complex may be approached through each binary complex, as in mechanism Ib, or by a compulsory sequence of addition of the two substrates. EO_2S is assumed to decompose into the quinone and the oxidized form E′, which is then reduced back to E by reaction with a second molecule of hydrogen donor. For substrate addition in random order the mechanism may be written as shown in Scheme 3. It may be noted that linear Lineweaver–Burk plots indicate that the enzyme cannot form a single complex with the two molecules of hydrogen donor required to reduce one molecule of oxygen.

Application of the equilibrium treatment of Michaelis & Menten (1913) to mechanism Ib gives the rate equation:

$$\frac{e}{v} = \frac{1}{k_{+5}} + \frac{1}{k_{+7}} + \frac{k_{-4}}{k_{+4}k_{+5}[O_2]} + \left[\frac{1}{k_{+6}} + \frac{k_{-3}}{k_{+3}k_{+5}} + \frac{k_{-6}}{k_{+6}k_{+7}}\right]\frac{1}{[S]} + \frac{k_{-1}k_{-3}}{k_{+1}k_{+3}k_{+5}[O_2][S]} \quad (2)$$

The steady-state treatment does not yield a linear form of equation. Form 1 is satisfied when ϕ_{12} is small, and thus $k_{+1} \gg k_{-1}$. The relation imposed by the equilibrium conditions on the dissociation constants of the various enzyme complexes is:

$$\frac{k_{-1}}{k_{+1}} \cdot \frac{k_{-3}}{k_{+3}} = \frac{k_{-2}}{k_{+2}} \cdot \frac{k_{-4}}{k_{+4}}$$

but equality of k_{-1}/k_{+1} and k_{-4}/k_{+4} is excluded. Thus combination of the enzyme with one substrate influences its affinity for the other.

The formation of the ternary complex by a compulsory sequence of addition of oxygen and S gives a rate equation consistent with form 1

$$E + O_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EO_2 \xrightarrow{k(S)} E' \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} E'S \xrightarrow{k_{+7}} E$$

Scheme 4. Mechanism II.

provided that k_{-1} is small and the rate of decomposition of the ternary complex is large enough to destroy equilibrium conditions. The addition of oxygen before S we may refer to as mechanism Ic, and the reverse order as mechanism Id. The rate equation for mechanism Ic, with the same system of enumeration of constants as in mechanism Ib, is:

$$\frac{e}{v} = \frac{1}{k_{+5}} + \frac{1}{k_{+7}} + \frac{1}{k_{+1}[O_2]} + \left[\frac{1}{k_{+3}} + \frac{k_{-3}}{k_{+3}k_{+5}} + \frac{1}{k_{+6}} + \frac{k_{-6}}{k_{+5}k_{+7}}\right]\frac{1}{[S]} + \left[\frac{k_{-1}}{k_{+1}k_{+3}} + \frac{k_{-1}k_{-3}}{k_{+1}k_{+3}k_{+5}}\right]\cdot\frac{1}{[O_2][S]} \quad (3)$$

Three lines of argument favour mechanism Ib.

(1) Form 1 needs to be reconciled with the kinetics of oxidation of chlorogenic acid (form 2). Form 2 imposes the stringent conditions that $1/K_m^S = 0$, that $1/K_m^{O_2}$ is independent of [S] and that the slopes of the lines obtained by plotting $1/v$ against $1/[O_2]$ become zero when $[S] = \infty$. The mechanism shown in Scheme 4 can account for these observations provided that k is small and k_{+7} large, so that the equilibrium treatment is valid. The rate equation under these restricted conditions reduces to:

$$\frac{e}{v} = \frac{1}{k[S]} + \frac{k_{-1}}{k\,k_{+1}[S][O_2]}$$

No other type of mechanism has been found to satisfy form 2.

Mechanism II may be seen as a limiting case of mechanism Ib or Ic. It is difficult to reconcile with mechanism Ia because chlorogenic acid could not be expected to prevent the spontaneous decomposition of EO_2 into E″ that occurs in mechanism Ia. Mechanism II is more readily compatible with mechanism Ib than with mechanism Ic because equilibrium conditions exist in mechanisms II and Ib but not in mechanism Ic. Mechanism Id, which requires that S adds on to the enzyme before oxygen, is clearly incompatible with mechanism II.

(2) The values of the slopes of the Lineweaver–Burk plots for oxygen (i.e. $K_m^{O_2}/\overline{\overline{V}}$) for the variety of hydrogen donors provide a useful method of distinguishing mechanisms (Dixon & Kleppe,

1965b). The kinetic interpretations of these slopes, as may be seen from eqns. (1), (2) and (3) respectively, are:

Mechanism Ia: $(k_{-1}+k')/k_{+1}k'$

Mechanism Ib: $k_{-4}/k_{+4}k_{+5}$

Mechanism Ic: $1/k_{+1}$

Mechanisms Ia and Ic, but not mechanism Ib, predict that the slope is independent of the nature of the hydrogen donor. The values given in Table 2 show that the observed slopes varied from $3{\cdot}8 \times 10^{-5}$ with caffeic acid to 5×10^{-4} with quinol, a difference that is beyond experimental error.

(3) The mechanism must be consistent with the effects of inhibitors (Table 3). Although a complete kinetic interpretation of the observed effects has not been attempted, it can be shown that the non-competitive effects observed with ferulic acid, protocatechuic acid and tetramethylenediamine are incompatible with mechanism Ia. Dixon & Webb (1964) pointed out that strictly non-competitive inhibition cannot occur with an enzyme obeying Briggs–Haldane kinetics. If, in mechanism Ia, k' were so small as to allow an effective equilibrium between enzyme and oxygen, non-competitive inhibition with respect to oxygen would be possible by an effect on k'. Nevertheless, simultaneous non-competitive inhibition towards hydrogen donor would be impossible; an effect on k' would increase K_m^s. Thus the doubly non-competitive inhibition observed with ferulic acid is inconsistent with mechanism Ia. However, the deficiency of mechanism Ia runs deeper. An inhibition that is competitive towards hydrogen donor may be due to an increase in ϕ_2, in ϕ_{12} or in both. However, if the effect is on ϕ_2 alone, as is required by mechanism Ia since no ϕ_{12} exists in this case, the inhibitor would cause a parallel upward displacement of the plots of $1/v$ against $1/[O_2]$, i.e. the inhibitor would be 'uncompetitive' with respect to oxygen. Thus the non-competitive effect of protocatechuic acid with respect to oxygen cannot be reconciled with mechanism Ia, but becomes mathematically feasible with mechanisms Ib and Ic, which contain a ϕ_{12} term, albeit one that is quantitatively insignificant in the absence of inhibitor.

The tea enzyme is distinguished from tyrosinases and laccases by its inability to oxidize monohydric phenols, either by hydroxylation or by dehydrogenation, a deficiency also shown by the oxidases from sweet potatoes (Eiger & Dawson, 1949) and tobacco leaves (Clayton, 1959). However, the tea enzyme does possess the classical distinctive attribute of the laccases, the ability to oxidize quinol and *p*-phenylenediamine, although the activities are low. As with other phenol oxidases, the best substrates are *o*-dihydric phenols. The effect of substituting a carboxyl group in the ring is striking. The maximum velocity at infinite concentrations of phenol and oxygen for gallic acid is only 2% of the value for pyrogallol (Table 2). Catechol is oxidized at a quarter of the rate of pyrogallol, but protocatechuic acid is an inhibitor. It is noteworthy that maximum velocities are related inversely to the oxidation–reduction potentials of the phenols. Values of E_0 determined by Ball & Chen (1933) are 0·713 and 0·799 v for pyrogallol and gallic acid respectively, and 0·792 and 0·883 v for catechol and protocatechuic acid. Substitution of a methyl group at position 4 of catechol lowers the potential by 37 mv (Kvalnes, 1934), and also increases the maximum velocity. It therefore seems that the catalytic properties of the tea oxidase are partly limited by its oxidation–reduction potential. This may afford an explanation of the failure to oxidize monohydric phenols, but it should be noted that the lack of any inhibitory effect of monohydric phenols on pyrogallol oxidation suggests that they are not bound to the enzyme.

The relatively feeble rates of oxidation of gallic acid and ethyl gallate have an important bearing on the mechanism of tea fermentation, for Roberts (1962) has made the suggestion, which has been confirmed by the work of Takino & Imagawa (1963, 1964), that an early stage in the formation of theaflavins and thearubigins is the condensation of two catechin quinones through the B rings of their flavonoid nuclei to form dimers. The gallate residues do not appear to participate in this oxidative condensation. The inhibitory effect of high concentrations of epigallocatechin gallate is also significant, because the concentration of this catechin in fresh leaf (46 mM, calculated from Vuataz *et al.* 1959) is well above the optimum. The low rate of fermentation compared with the measured activity of the oxidase (Bendall & Gregory, 1961) and the stimulation of fermentation by dilution of the tissue suspension with water (Roberts, 1940) can be mainly explained by this. The powerful inhibition by the product of oxidation of epicatechin gallate may also be of consequence, but this is rendered less likely by the difference in the pathway of oxidation in the presence of other catechins; epicatechin gallate alone yields mainly 'substance Q' (Roberts, 1962), but in the presence of gallocatechins it gives rise to theaflavins (Takino & Imagawa, 1963, 1964).

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