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RING CLEAVAGE OF PLANT CATECHOLS BY CRYSTALLINE OXYGENASES*

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Abstract—The aromatic ring of dihydroxyphenylpropanoids was found to be cleaved by two types of highly characterized crystalline oxygenases derived from bacteria. Oxidation of caffeic acid, chlorogenic acid, dihydroxyphenylalanine, and dihydroxyphenylethylamine by the intradiol and extradiol-cleaving enzymes, protocatechuate 3,4-dioxygenase and metapyrocatechase, respectively, permits description of rates and characteristics of the cleavage reactions and spectral properties of the products. The described reactions and oxidation products are pertinent to the study of plant aromatic metabolism and the degradation of plant constituents by soil bacteria.

INTRODUCTION

THE METABOLIC cleavage of dihydroxyaromatic ring compounds by plants has been suggested by experimental findings,[1,2] but the recognition and identification of cleavage products in plant extracts could be more satisfactorily accomplished if major properties of such products were known. Oxygenases that cleave simple hydroxyaromatic compounds have been reported in bacteria and present a means of obtaining and studying directly the properties of oxidative cleavage products. Since most of the substrates that have been studied are not pertinent to plant metabolism, experiments were undertaken to (A) test whether some representative higher plant catechols can be directly attacked by oxygenase type ring-cleaving enzymes and (B) ascertain some properties of the oxidized cleavage products and their modes of formation. We studied reactions with caffeic acid (CF), chlorogenic acid (CG), dihydroxyphenylalanine (Dopa), dihydroxyphenylethylamine (Dopamine), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and isoferulic acid (3-hydroxy-4-methoxy-cinnamic acid).

Reactions of the test compounds with two crystalline oxygenase enzymes from soil pseudomonads were investigated, these enzymes being used as models of two distinctive

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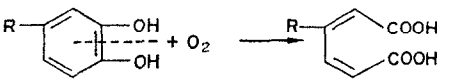
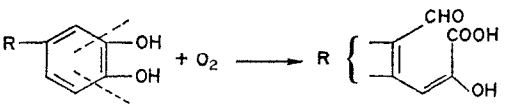
[1] S. SENOH and T. SAKAN, in *Biological and Chemical Aspects of Oxygenases* (edited by K. Bloch and O. HAYAISHI), p. 93, Maruzen, Tokyo (1966).

[2] H. E. MILLER, H. ROSLER, A. WOHLPART, H. WYLER, M. E. WILCOX, H. FROHOFER, T. J. MABRY and A. S. DREIDING, *Helv. Chim. Acta.* **51**, 1470 (1968).

types of reactions. Protocatechuate 3,4-dioxygenase (protocatechuate:oxygen 3,4-oxidoreductase, E.C. 1.13.1.3) (PCase) is known to attack its substrates by cleaving the aromatic ring between the hydroxyl groups (referred to as 'ortho' or 'intradiol' cleavage), producing two carboxyl groups at the site of ring opening:[3]



Metapyrocatechase (catechol:oxygen 2,3-oxidoreductase, E.C. 1.13.1.2) (MPase), on the other hand, cleaves its substrates adjacent to one of the ring hydroxyl groups ('meta' or 'extradiol' cleavage), producing an α-hydroxy acid semialdehyde:[4]



Crystalline preparations of these highly specific enzymes were employed against recrystallized substrates to minimize ambiguity about the nature of the cleavage reactions occurring and the products formed. Cleavage of the substrates by both types of enzymes was observed and the products obtained and some of their characteristics are reported.

RESULTS

Both types of enzymes reacted with CF and CG and also with Dopamine or Dopa as substrates, with accompanying changes in their adsorption spectra.

Protocatechuate 3,4-*Dioxygenase*

The colorless solution of product formed from reaction with CF showed an absorption peak at 273 nm (ϵ 1·9 × 10^4) in 0·05 M Tris-acetate buffer at pH 7·5; isosbestic points were observed at 236 and 285 nm during the reaction (Fig. 1). Similar results occurred even with a large variation of enzyme concentration or reaction time. In the absence of added enzyme no change in spectrum of the substrate was observed. Likewise, the spectrum of the reaction product was stable for 30 min at room temp. When NaOH was added (approximately 0·2 M in NaOH, pH 11–11·5, uncorrected) there was no change in spectral peak or absorptivity, nor did the reaction solution turn pinkish as occurs when solutions of substrate are made alkaline. When HCl was added (approximately 0·2 M in HCl, pH 1–1·4, trace of protein turbity) the spectral peak was but slightly shifted reversibly to 271 nm with a 15 per cent increase in absorptivity, but with a 1 per cent/min loss of absorbance on standing at room temp.

The product formed from CG has an absorption peak at 274 nm (ϵ 1·6 × 10^4) and the spectral curves displayed an isosbestic point at 294 nm during the reaction (Fig. 2). This product too was stable and colorless at pH 7·5 and was also colorless at pH 11, unlike substrate. When thus made alkaline, the product solution changed but little in peak position and there was only a slow loss of absorption. At pH 2, a slight shift of absorption peak occurred accompanied by a slow loss of absorption, as with the product from CF.

[3] H. FUJISAWA and O. HAYAISHI, *J. Biol. Chem.* **243,** 2673 (1968).
[4] M. NOZAKI, K. ONO, T. NAKAZAWA, S. KOTANI and O. HAYAISHI, *J. Biol. Chem.* **243,** 2682 (1968).

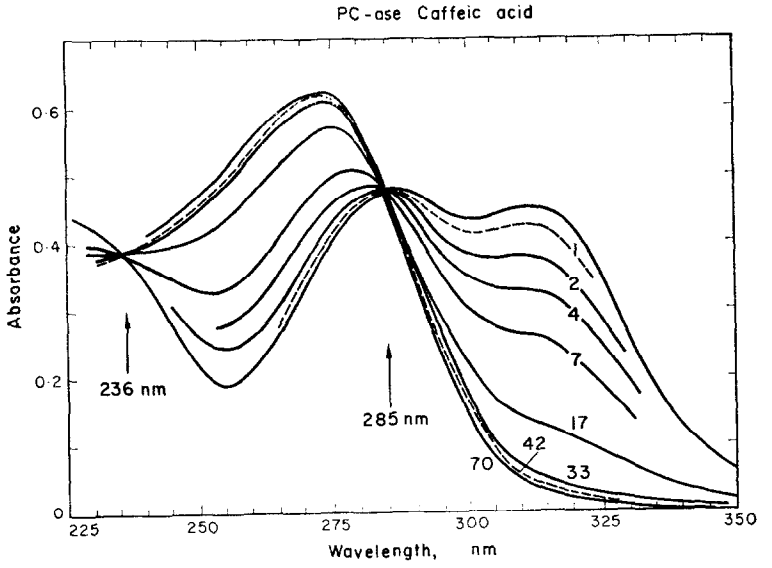


FIG. 1. SPECTRAL CHANGES DURING PCASE REACTION WITH CAFFEIC AND ENZYME, FINAL CONC. 0·02 mg/ml, ADDED FIRST TO BLANK CUVETTE, THEN TO SAMPLE CUVETTE AT ZERO TIME.

Numerical labels on the curves indicate min after zero time. Substrate concentration, 3·33 × 10^{-5} M.

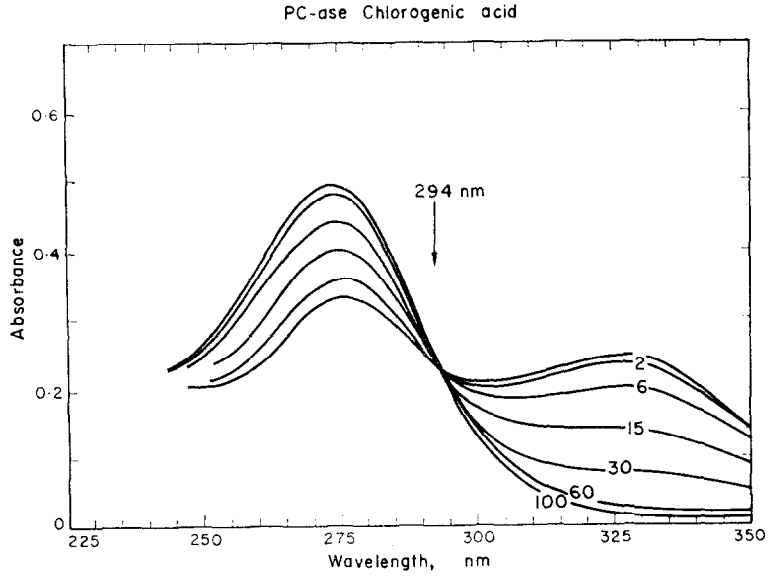


FIG. 2. SPECTRAL CHANGES DURING PCASE REACTION WITH CHLOROGENIC AND ENZYME, FINAL CONC. 0·58 mg/ml, ADDED TO BOTH CUVETTES.

Substrate, final concentration 3·33 × 10^{-5} M, added at zero time.

Reaction also occurred when Dopa and Dopamine were tested, but at an extremely slow rate so that the exceedingly high concentrations of enzyme required (near 1 mg/ml) approached the performance limits of the spectrophotometer, giving somewhat inexact values. The product from reaction with Dopa was characterized by an unstable absorption having ϵ greater than 3×10^3 (at about 255 nm). At alkaline pH there was a shift in peak position of about 10 nm to longer wavelength. With Dopamine as substrate there appeared to be a product with higher extinction than substrate but with a peak absorption close to that of substrate, so that during the reaction there was a rise in the peak height accompanied by a shift of maximum from 281 toward 275 nm. This result is difficult to interpret since the increase in absorption may have been due to the very slow darkening of Dopamine solutions which occurred at these high enzyme concentrations, possibly caused by non-specific protein catalyzed auto-oxidation or traces of catechol oxidase in the preparation.

Oxygen electrode studies. An oxygen electrode was used to further characterize the nature of the PCase reactions and confirm the stoichiometry during product formation from substrates. As standards of oxygen uptake, the reaction of PC with the enzyme under study and also the reaction of catechol with highly purified pyrocatechase were used. Table 1 indicates the stiochiometry of the oxidative reactions of the standard substrates and of CF.

TABLE 1. OXYGEN ELECTRODE READINGS DURING OXYGENASE REACTIONS

Enzyme	Pyrocatechase	Protocatechuate oxygenase	
Substrate	Catechol	Protocatechuate	Caffeate
		(scale deflection*/0·1 μmole substrate added)	
Date I		0·107 (0·0001 ml)†	0·128 (0·004 ml)
		0·118 (0·1 ml)	0·129 (0·004 ml)
		0·112 (0·1 ml)	0·116 (0·1 ml)
		0·110 (0·2 ml)	0·115 (0·2 ml)
		0·112 Av.	0·122 Av.
Date II	0·127 (0·04 ml)	0·131 (0·0002 ml)	
	0·145 (0·04 ml)	0·136 (0·0002 ml)	
	0·145 (0·04 ml)	0·135 (0·0002 ml)	
	0·139 Av.	0·133 Av.	

* Fraction of full scale deflection.
† Vol. of concentrated enzyme solution used (in parentheses).

The oxygen uptake during CF reaction with PCase was in agreement with 1 mole O_2/1 mole substrate, by comparison with the two known reactions tested. Reaction with CG, even at high concentrations of PCase, was too slow to give accurate stoichiometric data since both circuit drift and oxygen resorption into the reaction solution occur during prolonged periods. But here again, values approaching 1 mole O_2/1 mole substrate were obtained.

Reaction rates. The relative rates of reaction of the substrates PC, CF and CG with PCase were obtained from kinetic studies. The relative maximum rates (V_{max}) compared to that of PC were calculated as 0·013 and 0·00015 for CF and CG, respectively, with K_m values of 0·03 mM for PC (3), and 0·23 mM and 0·2 mM for CF and CG. The reactions with Dopa and Dopamine appeared even slower. It may be noted that neither ferulic acid nor isoferulic acid reacted with the enzyme at the highest concentration of enzyme used.

Metapyrocatechase

Reaction was observed with CF, but only when multimilligram quantities (near-substrate concentrations) of enzyme were used, thus introducing some instrumental limitations and inexactness of measurement in the presence of such high protein concentrations. The spectral changes during CF oxidation and the initial isosbestic points at about 247, 268 and 329 nm, which shift somewhat during the reaction, are seen in Fig. 3. The reaction

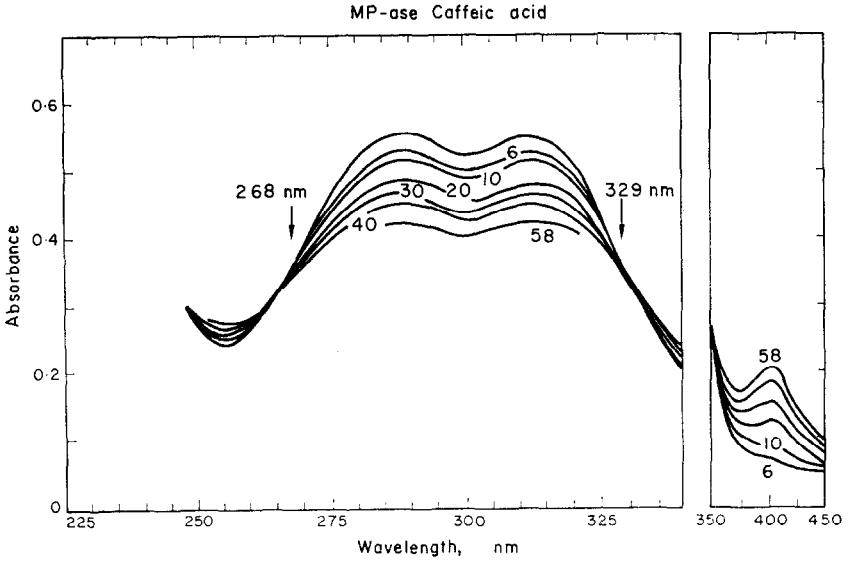


FIG. 3. SPECTRAL CHANGES DURING MPASE REACTION WITH CAFFEIC AND DIALYZED ENZYME, FINAL CONC. 1·0 mg/ml ADDED TO BOTH CUVETTES.

Substrate, final concentration $4{\cdot}0 \times 10^{-5}$ M, added at zero time.

product showed an absorption peak at 395 nm in 0·05 M potassium phosphate buffer at pH 7·5. Since the reaction did not go to completion and the product appeared somewhat unstable, the large ϵ value (*ca.* 2×10^4) could only be estimated relative to the decrease in substrate absorbance at 312 nm. The reaction solution in the cuvette was yellow in color. In pH 11 solution, there was essentially no shift of peak, but the absorptivity changed to a 40 per cent higher somewhat unstable value. Addition of acid (pH 1) entirely eliminated absorption peaks in the visible region and gave no high absorption peaks elsewhere.

Reaction with CG occurred but was barely measurable in several hours. Light absorption was diminished at the 325 nm peak, compared with only a slight decrease at the 277 nm peak. During reaction a new peak in the region of 405 nm appeared with ϵ probably $1{\cdot}5$–$2{\cdot}0 \times 10^4$ (estimated relative to the decrease in absorbance of CG at its 325 nm peak). Isosbestic points appeared at close to 275 nm (near the 277 nm substrate peak) and at 340 nm. A yellow coloration was discernible even from the small amount of reaction (less than 10 per cent of total reaction).

Dopamine was much more active than either of the above substrates in the MPase reaction. The spectral changes of Dopamine during reaction are shown in (Fig. 4). A primary product with λ_{max} at 384 nm appears to be rapidly converted to a secondary product having a symmetric peak at 418 nm with the particularly high $\epsilon > 3{\cdot}5 \times 10^4$. Whether the

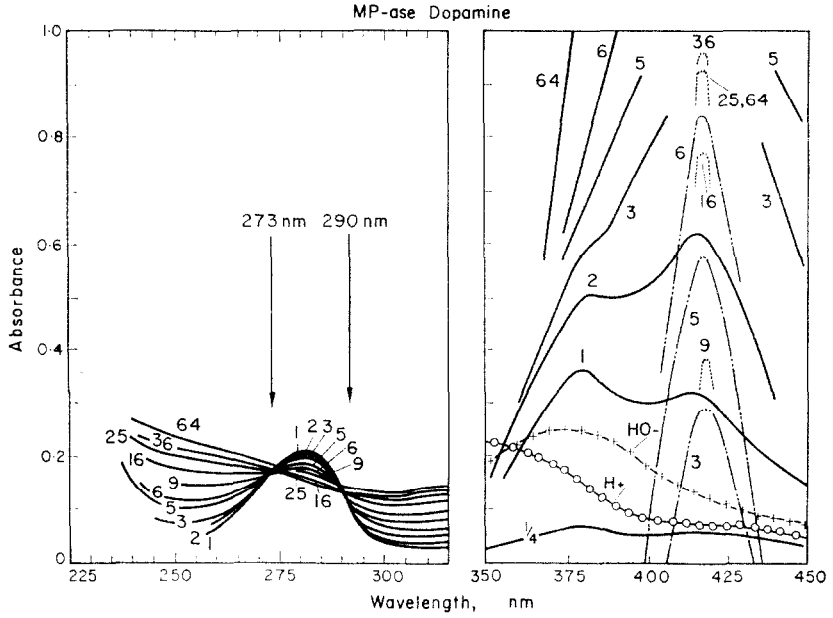


FIG. 4. SPECTRAL CHANGES DURING MPASE REACTION WITH DOPAMINE.

Dialyzed enzyme, final concentration 1·1 mg/ml, added to both cuvettes. Substrate, final concentration $8{\cdot}5 \times 10^{-5}$ M, added at zero time. Solid and broken lines, reaction mixture. (Broken lines, add 1·0 to absorbance value; dotted lines, add 2·0.) The effects of addition of NaOH (+, pH 11) or HCl (o, pH 1) to six-fold dilutions made after 4·25 hr of reaction are indicated.

secondary product arises by enzymatic or non-enzymatic conversion of the primary product is not known, but at a lower overall reaction rate brought about by decreasing the concentration of enzyme six-fold, the 384 nm peak hardly appeared; change of absorption from 384 to 418 nm was rapid even at the decreased enzyme level. The intensely yellow product was slightly unstable at room temperature (4 per cent loss/hr), equally so in the reaction mixture or in a six-fold dilution of it, so that there was a decrease of absorbance during the 1 hr of reaction under the conditions illustrated in the figure. Isosbestic points appeared at 273 and 290 nm during reaction with substrate. In pH 11 solution a shift occurred to a fairly stable peak at λ_{max} 374 nm having about 0·55 of the ϵ_{418} of the pH 7·5 product solution. This peak was, in turn, reversibly shifted by HCl addition (pH 1) to a stable peak at λ_{max} 354 nm with about 0·5 of the ϵ_{418} of the pH 7·5 solution.

No reaction with Dopa, ferulic acid, or isoferulic acid was observed in the experiments.

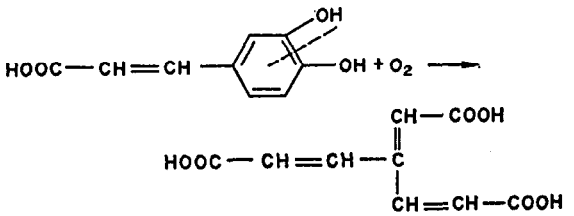
An insufficient amount of the enzyme was available for extensive rate studies with the slowly reacting substrates. Nozaki (unpublished) has determined the relative reaction rate of Dopamine at 4·5 mM as $1{\cdot}5 \times 10^{-4}$ that of catechol. A comparison of the enzyme reaction rate of CF with the rate of catechol (the maximal-rate substrate reported for the enzyme) was made at 0·06 mM, a rate-limiting concentration of both substrates under the measurement conditions. The rate against CF was found to be 2×10^{-6} the rate against catechol. Determination of K_m was not feasible because of the large amounts of enzyme needed, but a high affinity of CF for the enzyme is suggested by its marked competitive inhibition of the enzymatic oxidation of catechol, at three CF concentrations in the range

0·1–0·01 mM. The K_I value obtained (about 0·006 mM in reaction solutions containing 0·3 per cent acetone) was of the same order as the K_m of catechol in similar solution.

DISCUSSION

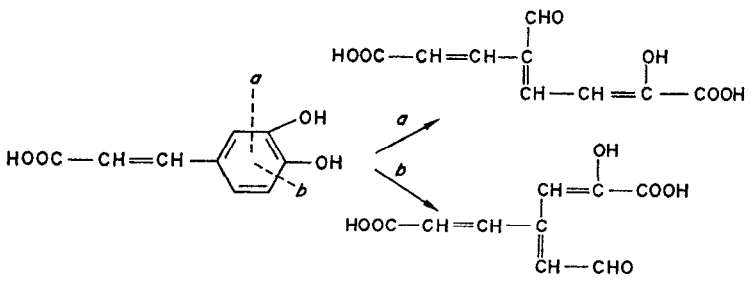
These findings establish the oxidative reactions of bacterial PCase and MPase with hydroxycinnamic acids and related compounds from higher plants. Recently a useful attribute of the substrate-cleaving reaction was demonstrated by adding PCase to apple juice to permanently prevent catechol oxidative darkening of its constituents.[5] Oxygenase enzymes such as described may also be present in higher plant tissues. However, it should be emphasized that the exceedingly low rates at which the bacterial enzymes reacted with the catechols are insignificant in themselves. Analogous cleavage enzymes, if they exist in higher plants, will most probably display quite different characteristics in reactions with substrates. The crystalline enzymes here studied, however, encompass relatively broad specificities with respect to the structure of the substrate side chain, even extending to quinic esters such as chlorogenic acid.

Intradiol cleavage of caffeic acid by PCase action would yield β-carboxyvinylmuconic acid (or, 4-carboxymethylene-2,5-heptadienoic acid):



With 3-caffeoylquinic acid as substrate in the reaction, the product would be the 3-quinic acid ester of the above muconic acid.

On the other hand, extradiol cleavage by MPase reacting with caffeic acid would be expected to yield one of two carboxyvinyl derivatives of α-hydroxymuconic semaldehyde. Cleavage would occur adjacent to the *o*-diol and at a position, viewed with respect to the side chain, either (a) proximal or (b) distal to the diol structure, depending on the specificity of the enzyme (product *a*, 5-formyl-2-hydroxy-2,4,6-octatrienedioic acid; product *b*, 4-(formylmethylene)-2-hydroxy-2,5-heptadienedioic acid):



[5] S. H. KELLY and B. J. FINKLE, *J. Sci. Food Agri.* **20**, 629 (1969).

Positive identification of the products of reaction with these oxygenases could not be made in the absence of authentic compounds, but the high purity of the well-characterized crystalline enzymes used and all additional data indicate oxidative cleavage in conformity with the known modes of reaction of the respective enzymes. The reactions with PCase yielded close to the stoichiometric uptake of 1 mole O_2/1 mole substrate, while the reactions of MPase gave a yellow product with a peak in the 400 nm region.[4] During reaction of MPase with Dopamine a rapid sequence of peaks occurred in the visible region, first at 384, then at 418 nm, of which the lower wavelength peak appeared much less prominently at a lower enzyme concentration. It seems possible that a comparable sequence of reaction products also occurred during the slow enzyme reactions of caffeic and chlorogenic acids, but that the initial product was so quickly transformed as not to appear in the spectra.

EXPERIMENTAL

Crystalline PCase (mol. wt. 700,000) was prepared from *Pseudomonas aeruginosa*[3] and stored in the refrigerator as a solution, 58 mg/ml, in 0·05 M tris-acetate buffer, pH 7·5. MPase (mol. wt. 140,000) prepared from *Pseudomonas arvilla* was stored as a crystalline suspension,[4] ~20 mg/ml, which was dissolved as needed in 0·05 M potassium phosphate buffer, pH 7·5, containing 10 per cent acetone as a stabilizer. When removal of acetone was required, the enzyme solution was dialyzed at 2° for several hours against acetone-free buffer. Highly purified pyrocatechase (E.C. 1.13.1.1) prepared by the method of Kojima *et al.*[6] was obtained through the kindness of Dr. T. Nakazawa.

Reactions were run at pH 7·5 in 0·05 M air-saturated solutions of tris-acetate buffer for PCase and pyrocatechase reactions, or potassium phosphate buffer for MPase reactions. Reaction spectra were recorded as difference spectra, with enzyme and buffer and sometimes additions of alkali or acid in both cuvettes. Protein content was determined spectrophotometrically.[3,4]

Synthetic caffeic acid[7] (mol. wt. 180) (obtained through the kindness of Dr. J. Corse) was recrystallized from water (dec 207–209°; $E^{312} = 1{\cdot}36 \times 10^4$) in 0·05 M potassium phosphate (or tris-acetate) buffer, pH 7·5. Stock solutions were made up in the minimum 0·05 M phosphate buffer to dissolve. Crystalline chlorogenic acid (mol. wt. 354) derived from coffee bean extracts[8] (dec 227–235°; $E^{325} = 8{\cdot}1 \times 10^3$ in the phosphate buffer), ferulic acid and isoferulic acid (also prepared by Dr. Corse) were stored in phosphate buffer solutions. Protocatechuic acid, catechol, Dopa (dec 275–277°; $E^{281} = 2{\cdot}5 \times 10^3$ in the phosphate buffer), and Dopamine-HCl (dec 245–247°; $E^{281} = 2{\cdot}5 \times 10^3$ in the phosphate buffer), obtained from commercial suppliers, were dissolved in water. These stock substrate solutions were generally stored frozen until just before use.

Spectra were determined using a Shimadzu Multipurpose Model MPS-50L or a Cary 15 spectrophotometer; the former instrument is capable of recording absorbances up to 3·0. A Hagihara rotating oxygen electrode,[9] connected to a Hitachi Model QPD-53 recorder and having a reaction chamber volume of 2·2 ml, was used for oxygen uptake studies.

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