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# Kinetic Studies on the Hydroxylation of *p*-Coumaric Acid to Caffeic Acid by Spinach-Beet Phenolase

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1. A spectrophotometric assay is described that enables the hydroxylation of *p*-coumaric acid to caffeic acid, catalysed by spinach-beet phenolase, to be followed continuously. 2. Initial-velocity and inhibitor studies indicate that the order of substrate addition is oxygen, *p*-coumaric acid and electron donor, with an irreversible step separating the binding of each substrate. 3. Caffeic acid is most likely to act as electron donor at the active site; other electron donors, such as ascorbic acid, NADH and dimethyltetrahydropteridine, function mainly to recycle cofactor amounts of caffeic acid. 4. A reaction scheme, consistent with these data, is proposed.

The hydroxylation of *p*-coumaric acid to caffeic acid is an important step in the biosynthesis of lignins, flavonoids and coumarins in higher plants, and can be catalysed by a phenolase preparation (EC 1.14.18.1) purified from spinach-beet (*Beta vulgaris* L.) leaves. Previous studies (Vaughan & Butt, 1969) showed that the reaction catalysed:

$$p\text{-Coumaric acid} + O_2 + AH_2 \rightarrow \text{caffeic acid} + H_2O + A$$

has the stoicheiometry of a mixed-function oxidase (Mason, 1955) in which $AH_2$ represents electron donors such as NADH, ascorbic acid or dimethyltetrahydropteridine (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine). In the presence of these electron donors a short lag period was observed before hydroxylation reached its maximum rate, which could be eliminated by caffeic acid or other *o*-dihydric phenol (Vaughan & Butt, 1970). This implied that *o*-dihydric phenol acted as the electron donor at the catalytic site of phenolase to generate an enzyme species effective in hydroxylation. It was suggested that the main function of the other electron donors was the chemical reduction of *o*-quinone resulting from the catechol oxidase activity of phenolase (Nelson & Dawson, 1944). Further studies showed that ascorbic acid, NADH and dimethyltetrahydropteridine affected the extent to which catechol oxidase activity accompanied hydroxylation, and it was suggested that these electron donors alter the ratio of catechol oxidase to hydroxylase activities of phenolase as well as recycling *o*-dihydric phenols (Vaughan & Butt, 1972).

Although steady-state kinetic analyses have been carried out on the catechol oxidase activity of mushroom (Duckworth & Coleman, 1970), prune (Ingraham, 1957) and tea (Gregory & Bendall, 1966) phenolases no similar study has been carried out on the hydroxylase activity of phenolases.

The present paper presents a steady-state kinetic analysis of the hydroxylation of *p*-coumaric acid which aims to establish the order of substrate addition to the enzyme and to determine the effect of the nature of the electron donor on the mechanism of hydroxylation. It also describes a continuous spectrophotometric assay for the measurement of initial rates of caffeic acid production, in the presence of these electron donors.

## Materials and Methods

### *Reagents*

Benzoic acid, *p*-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. All other fine chemicals and definition of enzyme units have been described (Vaughan & Butt, 1969). *p*-Coumaric acid, caffeic acid, benzoic acid, *p*-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid were recrystallized from aq. ethanol (40%, v/v) in the presence of charcoal.

### *Enzyme purification*

Phenolase was prepared by the modified method of Vaughan & Butt (1970) and stored as an $(NH_4)_2SO_4$ precipitate at pH 7.3 and 3°C. The enzyme retained full activity under these conditions for at least 2 years. Before use the enzyme was diluted tenfold with bovine serum albumin (2mg/ml), a precaution necessary to stabilize the purified enzyme at this dilution.

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*Enzyme assays*

*Hydroxylation assay.* Hydroxylation was followed by measuring the quantity of caffeic acid produced during incubation of enzyme at 30°C in the presence of $O_2$ (0.056–1.12mM), *p*-coumaric acid (0.166–3.33mM) and ascorbic acid (0.055–3.33mM), dimethyltetrahydropteridine (0.13–1.0mM) or NADH (0.01–0.05mM) in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, and 0.5M-$(NH_4)_2SO_4$, in a total volume of 3ml. EDTA (3.33mM) was included in all assays with ascorbic acid to minimize copper-catalysed autoxidation (Butt & Hallaway, 1961). Catalase (200 units/ml) was included in all reaction mixtures containing dimethyltetrahydropteridine to remove $H_2O_2$ produced by autoxidation of the reduced pteridine (Nielsen, 1969). The reaction was started by the addition of enzyme and the cell contents were mixed by agitation with air or $O_2+N_2$ mixtures for 5s. Assays were carried out in a Beckman DB spectrophotometer, and initial rates were determined by measuring the change in $E_{340}$ during the linear part of the reaction (2–5min, depending on substrate concentration used). Changes in extinction were recorded as digital output by means of an Optilab Multilog and Printer Driver linked to an Addo-X Printer (Techmation Limited, 58 Edgware Way, Edgware, Middx. HA8 8JP, U.K.). To correct for absorbance due to dimethyltetrahydropteridine and NADH at 340nm, duplicate cuvettes were prepared and the reaction was followed at 340 and 370nm with dimethyltetrahydropteridine and at 240 and 370nm for NADH. Changes in extinction at these wavelengths were related to caffeic acid concentration by using the expressions derived in the Appendix.

*Determination of molar extinction coefficients.* The molar extinction coefficients of *p*-coumaric acid and caffeic acid at 240, 340 and 370nm were measured on standard solutions made up in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, containing 0.5M-$(NH_4)_2SO_4$. Dimethyldihydropteridine was formed as suggested by Nielsen (1969), by the addition of 0.3M-$H_2O_2$ and catalase (600units) to dimethyltetrahydropteridine in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, and 0.5M-$(NH_4)_2SO_4$ in air at 30°C. When $O_2$ evolution ceased the extinction was measured at 340 and 370nm.

The proportion of $NAD^+$ and NADH in a known weight of commercial sample [Sigma (London) Chemical Co., London S.W.6, U.K.] was calculated from the absorbance of solutions at pH7.5 by using literature values for the extinction coefficients of NADH at 340nm and $NAD^+$ at 259nm (Morris & Redfearn, 1969). Standard solutions could now be used to calculate the molar extinction coefficients for NADH at 240 and 370nm and $NAD^+$ at 240nm in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, and 0.5M-$(NH_4)_2SO_4$. The molar extinction coefficients obtained by these procedures are given in Table 1.

*Catechol oxidase assay.* Catechol oxidase activity was determined by measuring $O_2$ uptake with a Rank oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) coupled to a variable-input Servoscribe chart recorder (Smiths Industries, Wembley, Middx., U.K.). A relationship between $O_2$ content of the assay medium and full-scale chart deflexion was obtained by the method of Robinson & Cooper (1970). The assay mixture containing caffeic acid (0.033–1.33mM) was preincubated for 15min at 30°C before starting the reaction by the addition of enzyme.

*Variation of oxygen concentration in assay mixture.* The ratio of $O_2$-free $N_2$/air or pure $O_2$ in a gas mixture was varied by using gas-flow meters (G. A. P. Meters Ltd., Basingstoke, Hants., U.K.). The gases were passed into a mixing vessel and then through a closed flask containing all reaction components except enzyme and electron donor at 30°C. The percentage of $O_2$ in this flask was measured with a Beckman oxygen electrode (Beckman Instruments Ltd., Glenrothes, Fife, U.K.). The gas mixture was bubbled through the assay cuvette (covered with Parafilm), at 30°C for 5min, before the addition of enzyme. This time was judged sufficient to equilibrate the assay mixture, as no reaction was observed when $O_2$-free $N_2$ (British Oxygen Company, Polmadie Estate, Glasgow, U.K.) was passed through the reaction mixture for this period.

The amount of dissolved $O_2$ was converted into molar concentrations by assuming that the $O_2$ content of $O_2$-saturated water was 0.12mM at 30°C (*Handbook of Chemistry and Physics*, 33rd edn., 1951–

Table 1. *Molar extinction coefficients for p-coumaric acid, caffeic acid, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, NADH and NAD⁺ at wavelengths used in the spectrophotometric assay*

Molar extinction coefficients were determined by procedures described in the Materials and Methods section.

| Component | $\lambda$ (nm) | $10^{-3}\times\varepsilon_\lambda$ (litre·mol$^{-1}$·cm$^{-1}$) |
|---|---|---|
| *p*-Coumaric acid | 240 | 2.37 |
| | 340 | 0.38 |
| | 370 | 0.0076 |
| Caffeic acid | 240 | 10.9 |
| | 340 | 2.99 |
| | 370 | 0.066 |
| 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine | 340 | 0.37 |
| | 370 | 0.18 |
| NADH | 240 | 6.92 |
| | 370 | 2.4 |
| $NAD^+$ | 240 | 8.67 |

Table 2. *Comparison between spectrophotometric and colorimetric methods for caffeic acid determination*

*p*-Coumaric acid (10$\mu$mol) was incubated with ascorbate (20$\mu$mol), NADH (20$\mu$mol) or dimethyltetrahydropteridine (6$\mu$mol) and enzyme in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, and 0.5M-$(NH_4)_2SO_4$ in a total volume of 7.0ml under the conditions of Vaughan & Butt (1969). Caffeic acid content of 0.5ml portions at the times stated was estimated by either the colorimetric method (a) (Vaughan & Butt, 1969) or the spectrophotometric method (b). Results are expressed as $\mu$mol/3.5ml and are corrected for zero blank.

| Incubation time (min) | Ascorbate | | NADH | | Dimethyltetrahydropteridine | |
|---|---|---|---|---|---|---|
| | (a) | (b) | (a) | (b) | (a) | (b) |
| 2 | 0.10 | 0.11 | 0.07 | 0.06 | 0.15 | 0.15 |
| 4 | 0.18 | 0.19 | 0.14 | 0.13 | 0.19 | 0.17 |
| 6 | 0.26 | 0.25 | 0.23 | 0.21 | 0.22 | 0.24 |
| 8 | 0.33 | 0.33 | 0.33 | 0.32 | 0.29 | 0.28 |
| 10 | 0.36 | 0.36 | 0.40 | 0.40 | 0.35 | 0.35 |

52, p. 1481; Chemical Rubber Publishing Co., Cleveland, Ohio) and that there is a linear relationship between $O_2$ concentration and percentage of $O_2$ in the gas phase over the concentration range studied.

*Inhibitor studies.* Enzyme was incubated with 1.67mM-*p*-coumaric acid, 1.67mM-ascorbic acid and *p*-hydroxybenzoic acid (0.66–2.6mM), benzoic acid (0.33–3.33mM), 3,4-dihydroxybenzoic acid (0.084–1.67mM), CO (0.017–0.067mM), bathocuproinesulphonate (0.084–0.33mM) or diethyldithiocarbamate (0.5–1.5$\mu$M) under the standard conditions of the hydroxylation assay. CO concentrations were obtained by the addition of the relevant amount of a saturated aqueous solution of the gas at 20°C, assumed to be 1mM (*Handbook of Chemistry and Physics*, 1951–52). The concentrations of ascorbic acid and *p*-coumaric acid used in these studies were at least twelve and six times greater than their respective Michaelis constants.

*Data processing.* To check for general agreement between initial-velocity data and the respective rate equations appropriate double-reciprocal plots were made (Rudolph *et al.*, 1968). Data were then fitted to the rate equation by the least-squares method (Wilkinson, 1961) by using FORTRAN programs kindly supplied by Professor W. W. Cleland.

## Results

### *Colorimetric and spectrophotometric determination of caffeic acid*

The difference between the colorimetric (Vaughan & Butt, 1969) and spectrophotometric determinations of caffeic acid is less than 10% with ascorbic acid, dimethyltetrahydropteridine or NADH as electron donor (Table 2). The spectrophotometric method was therefore used as a routine to determine initial rates of caffeic acid production. With ascorbic acid and dimethyltetrahydropteridine as electron donors, the method is satisfactory for *p*-coumaric acid concentrations up to 3.33mM. The high total absorbance of NADH and *p*-coumaric acid at 240nm, however, limits the method to relatively low amounts of those substrates (0.33mM-NADH and 0.92mM-*p*-coumaric acid). Since these concentrations are not appreciably greater than their respective Michaelis constants, no detailed initial-velocity studies were carried out with NADH as electron donor.

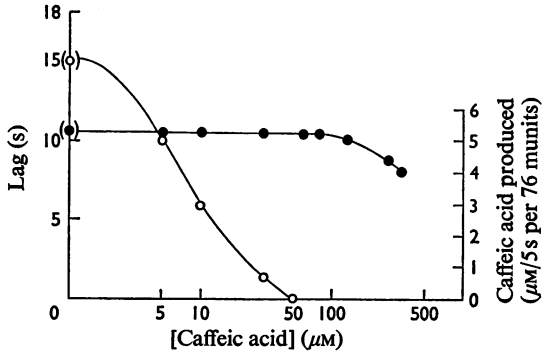



Fig. 1. *Effect of caffeic acid on hydroxylation*

Caffeic acid production (●) and lag-time (○) when 76m-units ($\mu$mol/min) of enzyme (stage IV; Vaughan & Butt, 1970) were incubated with *p*-coumaric acid (5$\mu$mol), ascorbic acid (10$\mu$mol), EDTA (10$\mu$mol) and the amounts of caffeic acid indicated, in 0.1M-$Na_2HPO_4$–0.05M-citric acid buffer, pH6.5, containing $(NH_4)_2SO_4$ (1.5mmol) at 30°C in air are shown. The points in parentheses on the vertical axis represents the hydroxylation rate and lag-time in the absence of added caffeic acid. The initial rates are expressed as $\mu$M (i.e. $\mu$mol of caffeic acid produced/litre)/5s per 76munits. Values are given as $\mu$M rather than nmol/ml or nmol/assay by substitution of the change in extinction, at the wavelength of assay, into the relevant expressions derived in the Appendix. This is done in one step by using a conversion factor on the multilog and so results are expressed directly as $\mu$M at 5s intervals with 76munits of enzyme per assay.

Table 3. *$K_m$ and $V_{max.}$ values for all substrates in the presence of different electron donors*

For details see the text. For definition of units of $V_{max.}$ values see the legend to Fig. 1. Values are means ± S.E.M.

| Electron donor | Variable substrate | $K_m$ (mM) | $V_{max.}$ ($\mu$M-caffeic acid/5s per 76munits) |
|---|---|---|---|
| Ascorbic acid | Oxygen | 0.067±0.004 | 6.13±0.08 |
| | *p*-Coumaric acid | 0.268±0.027 | 5.25±0.15 |
| | Ascorbic acid | 0.133±0.014 | 5.70±0.08 |
| Dimethyltetrahydropteridine | Oxygen | 0.051±0.007 | 10.80±0.25 |
| | *p*-Coumaric acid | 0.192±0.018 | 10.19±0.21 |
| | Dimethyltetra-hydropteridine | 0.131±0.014 | 11.08±0.34 |
| NADH | Oxygen | 0.041±0.006 | 5.42±0.17 |
| | *p*-Coumaric acid | 0.366±0.041 | 5.84±0.23 |
| | NADH | 0.208±0.035 | 4.17±0.28 |

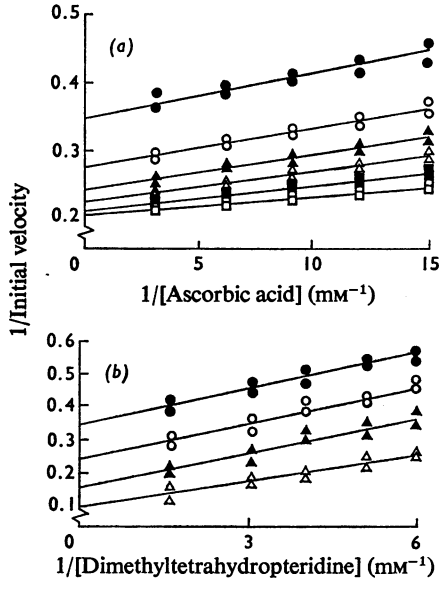



Fig. 2. *Initial-velocity pattern with electron donors as variable substrates*

(*a*) Reciprocal plots of initial velocity against ascorbic acid concentrations at a series of fixed $O_2$ and *p*-coumaric acid concentrations held in a constant ratio. The respective $O_2$ and *p*-coumaric acid concentrations were 0.056mM, 0.21mM (●); 0.085mM, 0.31mM (○); 0.112mM, 0.42mM (▲); 0.168mM, 0.63mM (△); 0.224mM, 0.83mM (■); and 0.448mM, 1.67mM(□). (*b*) Reciprocal plots of initial velocities against dimethyltetrahydropteridine concentrations at a series of fixed *p*-coumaric acid and $O_2$ concentrations held in a constant ratio. The respective *p*-coumaric acid and $O_2$ concentrations were 0.42mM, 0.056mM (●); 0.61mM, 0.084mM (○); 0.83mM, 0.112mM (▲); and 1.67mM, 0.224mM (△). Throughout and in subsequent Figures initial velocities are expressed as caffeic acid produced ($\mu$M/5s per 76munits of enzyme; see legend to Fig. 1).

*Action of caffeic acid on hydroxylation*

Caffeic acid (50$\mu$M) was sufficient to eliminate the slight lag observed during hydroxylation in the presence of ascorbic acid and dimethyltetrahydropteridine. No stimulation of hydroxylation was observed even when the concentration of caffeic acid, added initially, was raised to 80$\mu$M; above this concentration slight inhibition occurred (Fig. 1).

The Michaelis constant for caffeic acid, acting as a cofactor in the elimination of the lag period, was estimated to be $1.6\times10^{-6}$M by the method of Pomerantz & Warner (1967). This compares with a Michaelis constant of $3.3\times10^{-4}$M for caffeic acid as a substrate for the catechol oxidase activity of phenolase.

*Evaluation of kinetic constants*

Kinetic constants for each substrate in the presence of saturating concentrations of the respective other two substrates were evaluated by direct fit of initial-velocity data to the equation for a rectangular hyperbola (Table 3).

*Initial-velocity studies*

Initial-velocity patterns were obtained by the method of Rudolph *et al.* (1968), in which each substrate, in turn, is varied against different amounts of the other two substrates, held in the ratio of their Michaelis constants. Parallel initial-velocity patterns were observed with $O_2$ or *p*-coumaric acid as the variable substrate and either ascorbic acid or dimethyltetrahydropteridine as reducing agents. In the presence of either ascorbic acid (Fig. 2*a*) or dimethyltetrahydropteridine (Fig. 2*b*) the parallel patterns observed at low concentrations of $O_2$ and *p*-coumaric acid appear to converge at the highest concentrations of substrates used. The assignment of

Table 4. *Computer analysis of initial-velocity data*

Initial-velocity data were fitted to the rate equation for Ping Pong mechanism (model a) or sequential mechanism (model b) as given in the text. General assay conditions were as described in the Materials and Methods section and the substrate concentrations (mM) used in each experiment were as follows. Expt. 1: *p*-coumaric acid (0.166–1.33) against fixed $O_2$ and ascorbate concentrations in the range (0.056, 0.25–0.224, 1.0) respectively. Expt. 2: as in Fig. 2(*a*). Expt. 3: $O_2$ (0.056–0.4) against fixed *p*-coumaric acid and ascorbate concentrations in the range (0.21, 0.125–1.67, 1.0) respectively. Expt. 4: *p*-coumaric acid (0.166–1.33) against fixed $O_2$ and dimethyltetrahydropteridine concentration in the range (0.056, 0.21–0.224, 0.83) respectively. Expt. 5: as in Fig. 2(*b*). Expt. 6: $O_2$ (0.056–0.4) against fixed *p*-coumaric acid and dimethyltetrahydropteridine concentrations in the range (0.41, 0.21–1.67, 0.83) respectively. Values are means ± S.E.M.

| Expt. no. | Model | $K_A$ (mM) | $K_B$ (mM) | $V$ (μM/5s per 76munits) | $K_{iA}$ (mM) | Variance |
|---|---|---|---|---|---|---|
| 1 | (a) | 0.168±0.008 | 0.0617±0.003 | 5.78±0.121 | | 0.007 |
| | (b) | 0.17±0.016 | 0.0624±0.005 | 5.8±0.174 | −0.0039±0.019 | 0.0078 |
| 2 | (a) | 0.0259±0.0022 | 0.065±0.0028 | 6.19±0.116 | | 0.017 |
| | (b) | 0.0167±0.0031 | 0.0502±0.0046 | 5.82±0.14 | 0.0276±0.0098 | 0.0144 |
| 3 | (a) | 0.0157±0.0014 | 0.279±0.0094 | 5.93±0.087 | | 0.0121 |
| | (b) | 0.0134±0.0022 | 0.261±0.0156 | 5.83±0.112 | 0.0056±0.0045 | 0.012 |
| 4 | (a) | 0.656±0.104 | 0.239±0.043 | 18.84±2.33 | | 0.132 |
| | (b) | 0.502±0.147 | 0.194±0.049 | 16.67±2.54 | 0.0559±0.0561 | 0.131 |
| 5 | (a) | 2.29±1.13 | 1.605±0.784 | 78.85±36.6 | | 0.0436 |
| | (b) | 0.422±0.141 | 0.35±0.096 | 23.12±4.51 | 0.118±0.036 | 0.0291 |
| 6 | (a) | 0.293±0.027 | 0.519±0.06 | 16.2±1.13 | | 0.0436 |
| | (b) | 0.244±0.040 | 0.431±0.078 | 14.74±1.37 | 0.024±0.02 | 0.043 |

Table 5. *Computer analysis of initial-velocity data in the presence of* 0.067 *mM-caffeic acid*

Experimental conditions as in Expts. 1, 2 and 3 respectively in Table 4, except for the inclusion of 0.067 mM-caffeic acid in each case. Values are means ± S.E.M.

| Expt. no. | Model | $K_A$ (mM) | $K_B$ (mM) | $V$ (μM/5s per 76munits) | $K_{iA}$ (mM) | Variance |
|---|---|---|---|---|---|---|
| 1 | (a) | 0.174±0.013 | 0.036±0.0042 | 6.26±0.217 | | 0.0333 |
| | (b) | 0.0685±0.0098 | 0.014±0.0024 | 5.33±0.114 | 0.629±0.149 | 0.0088 |
| 2 | (a) | 0.031±0.0062 | 0.204±0.022 | 10.4±0.905 | | 0.0232 |
| | (b) | 0.0023±0.0045 | 0.082±0.018 | 6.84±0.58 | 0.0338±0.0119 | 0.0145 |
| 3 | (a) | 0.0197±0.0013 | 0.209±0.0073 | 5.64±0.074 | | 0.0104 |
| | (b) | 0.0197±0.0022 | 0.209±0.0126 | 5.64±0.100 | 0.00007±0.0047 | 0.0106 |

a parallel initial-velocity pattern with $O_2$ and *p*-coumaric acid was substantiated by direct fit to the data to the rate equations for Ping Pong (model a) and sequential mechanisms (model b) (Cleland, 1963).

$$v = \frac{V_1 AB}{K_b A + K_a B + AB} \quad (a)$$

and

$$v = \frac{V_1 AB}{K_{ia} K_b + K_b A + K_a B + AB} \quad (b)$$

(in which $K_{ia}$ is the apparent dissociation constant of the EA complex and $K_a$ and $K_b$ are Michaelis constants). The negative value for $K_{ia}$ (Table 4; Expt. 1) and the close agreement between the $K_{ia}$ values and their standard errors (Table 4; Expts. 3, 4 and 6), support the assignment of initial-velocity data for $O_2$ and *p*-coumaric acid to Ping Pong (model a) rate equations rather than sequential (model b). In contrast, with ascorbic acid or dimethyltetrahydropteridine as variable substrates, the significant $K_{ia}$ values and lower variance (Table 4; Expts. 2 and 5) suggest that the data fit the rate equation for a sequential addition of substrates better than the Ping Pong rate equation.

In the presence of non-inhibitory concentrations of caffeic acid (0.067 mM), a parallel initial-velocity pattern was observed with $O_2$ as the variable substrate, whereas the initial-velocity patterns with ascorbic

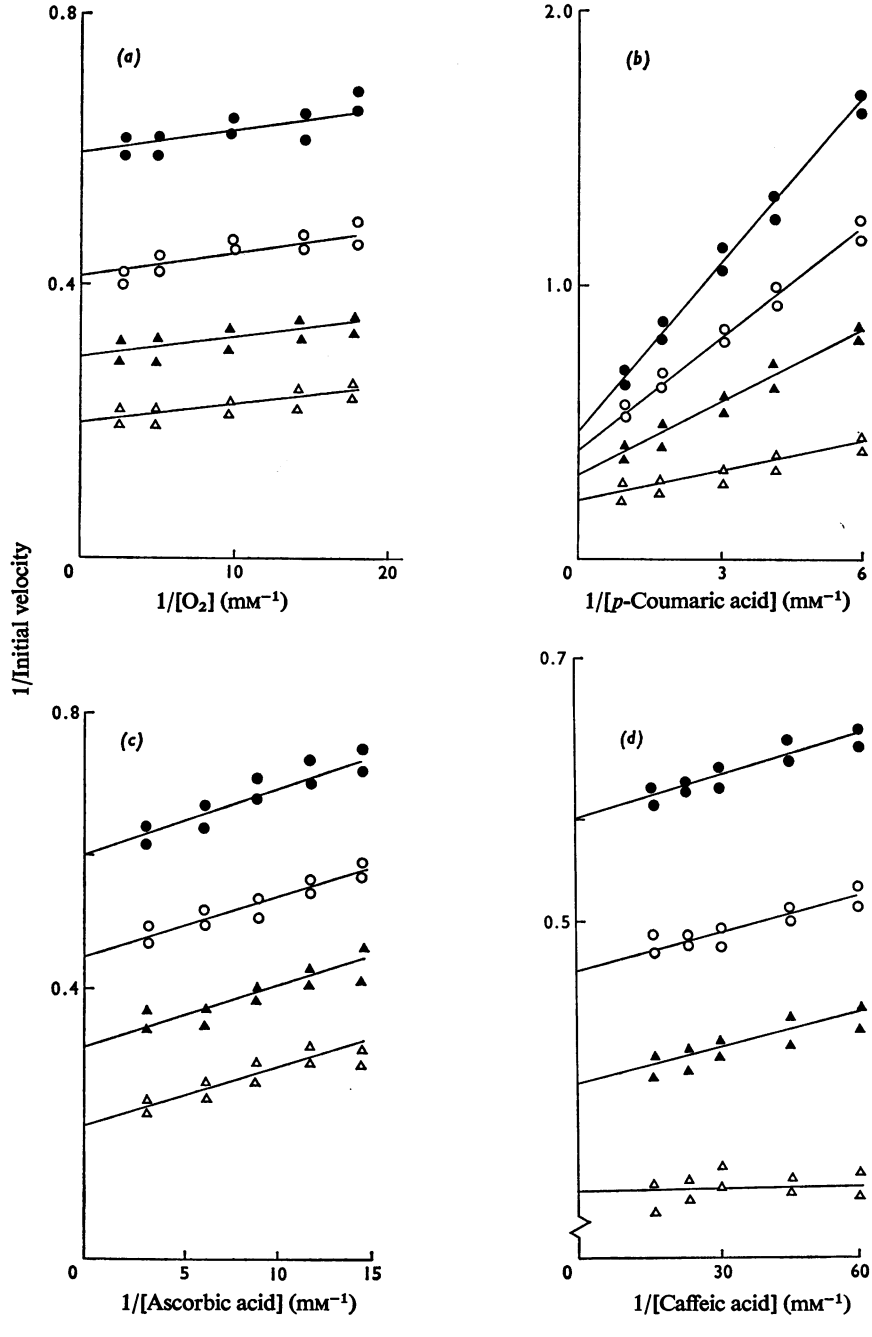



Fig. 3. *Inhibition patterns with p-hydroxybenzoic acid*

Reciprocal plots of initial velocity against (*a*) $O_2$ concentrations in the presence of 1.67mM-*p*-coumaric acid and 1.67mM-ascorbic acid; (*b*) *p*-coumaric acid concentations in the presence of 1.67mM-ascorbic acid and air; (*c*) ascorbic acid concentration in the presence of 1.67mM-*p*-coumaric acid and air; (*d*) caffeic acid concentrations in the presence of 1.67mM-*p*-coumaric acid, 1.67mM-ascorbic acid and air. In each case either no (△), 0.84mM-(▲), 1.67mM-(○) or 2.5mM-(●) *p*-hydroxybenzoic acid was also included in the assay medium.

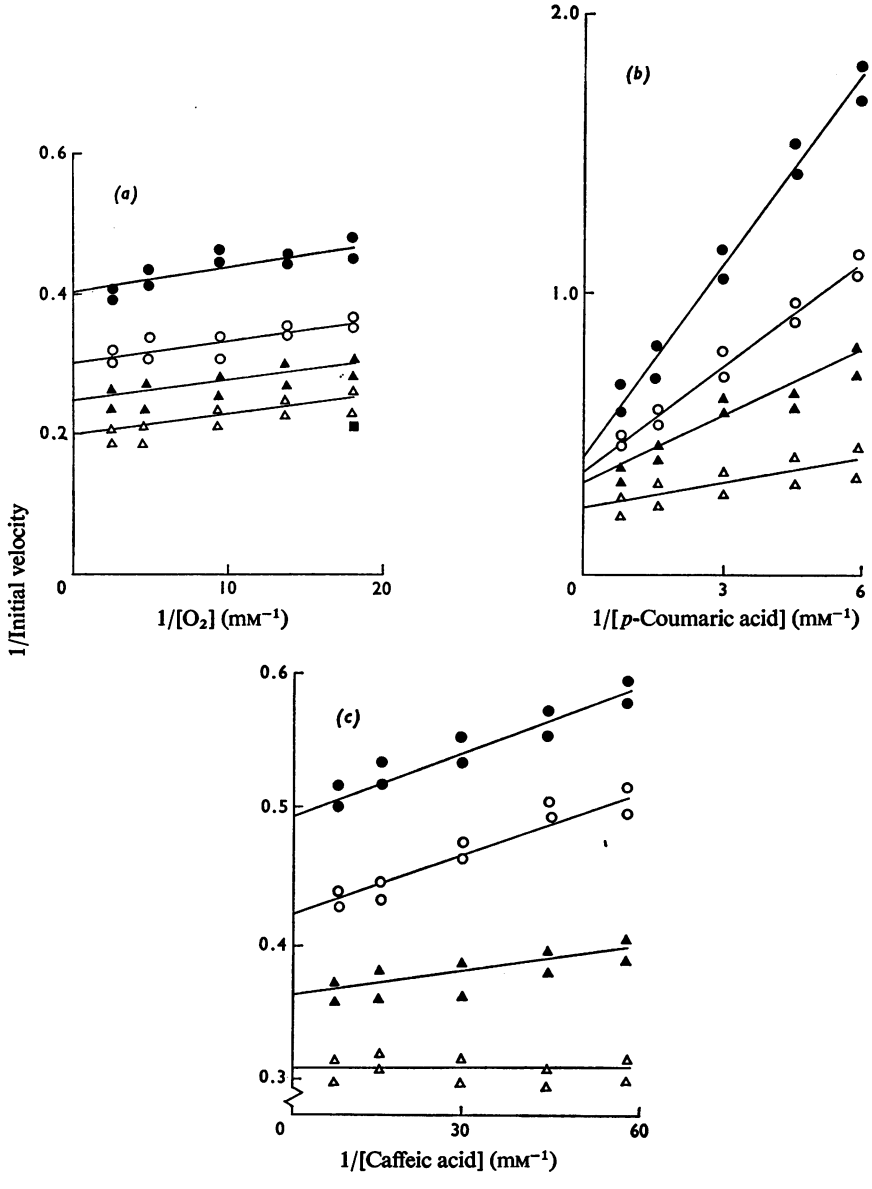



Fig. 4. *Inhibition patterns with benzoic acid*

Reciprocal plots of initial-velocity data against (*a*) $O_2$, (*b*) *p*-coumaric acid and (*c*) caffeic acid in the presence of either no (△), 0.5mM- (▲), 1.0mM- (○) or 1.67mM- (●) benzoic acid. Other conditions were as in Figs. 3(*a*), 3(*b*) and 3(*d*) respectively.

acid or *p*-coumaric acid were convergent. With $K_{ia}$ and variance as criteria, inclusion of caffeic acid does not affect the closeness of fit of initial-velocity data to the Ping Pong mechanism, with $O_2$ as variable substrate (Table 5; Expt. 3). In contrast, the lower variance and significant $K_{ia}$ value observed with *p*-coumaric acid (Table 5; Expt. 1) or ascorbic acid (Table 5; Expt. 2) as variable substrates supports the inclusion of a $K_{ia}$ term in the rate equations for these substrates in the presence of caffeic acid.

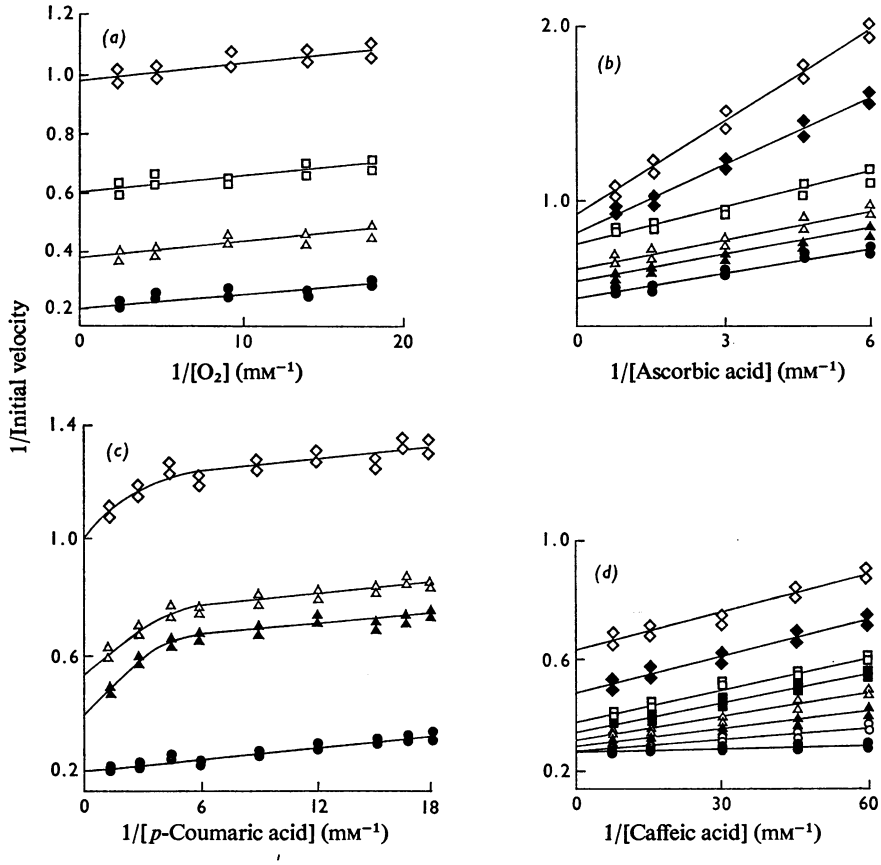



Fig. 5. *Inhibition patterns with* 3,4-*dihydroxybenzoic acid*

Reciprocal plots of initial-velocity data against (*a*) $O_2$, (*b*) *p*-coumaric acid, (*c*) ascorbic acid and (*d*) caffeic acid in the presence of either no (●), 0.084mM-(○), 0.167mM-(▲), 0.33mM-(△), 0.5mM-(■), 0.667mM-(□), 1.167mM-(◆) or 1.67mM-(◇) 3,4-dihydroxybenzoic acid. Other conditions were as in Figs. 3(*a*), 3(*b*), 3(*c*) and 3(*d*) respectively.

*Inhibitor studies*

All the results are recorded as double-reciprocal plots (Figs 3–8). $K_i$ intercept and $K_i$ slope values, where applicable, are shown in Table 6.

## Discussion

*Initial-velocity studies*

The observation that preincubation with low concentrations of caffeic acid does not alter the subsequent rate of caffeic acid production shows that the slope of the linear portion of the time-course, determined in the absence of added caffeic acid, is a reliable measure of initial rate of hydroxylation under the condition of assay. The parallel initial-velocity patterns observed when either $O_2$ or *p*-coumaric acid were the variable substrates, together with the better fit of initial-velocity data to the rate equation with no $K_{ia}$ term, is consistent with a double displacement, or Ping Pong mechanism (Cleland, 1963). This mechanism is characterized by release of product before the addition of the second or third substrate, and involves the formation of a stable modified form of the enzyme, without the occurrence of any kinetically significant amounts of a ternary complex. The occurrence of parallel plots, however, is not conclusive evidence for a Ping Pong mechanism, since any irreversible step between binding of two substrates would result in a parallel initial-velocity pattern. One example of this is seen for the oxidation of 3-hydroxyanthranilate (Ogasawara *et al.*, 1966), where the large free-energy change associated with the reduction of $O_2$ has been suggested to account for

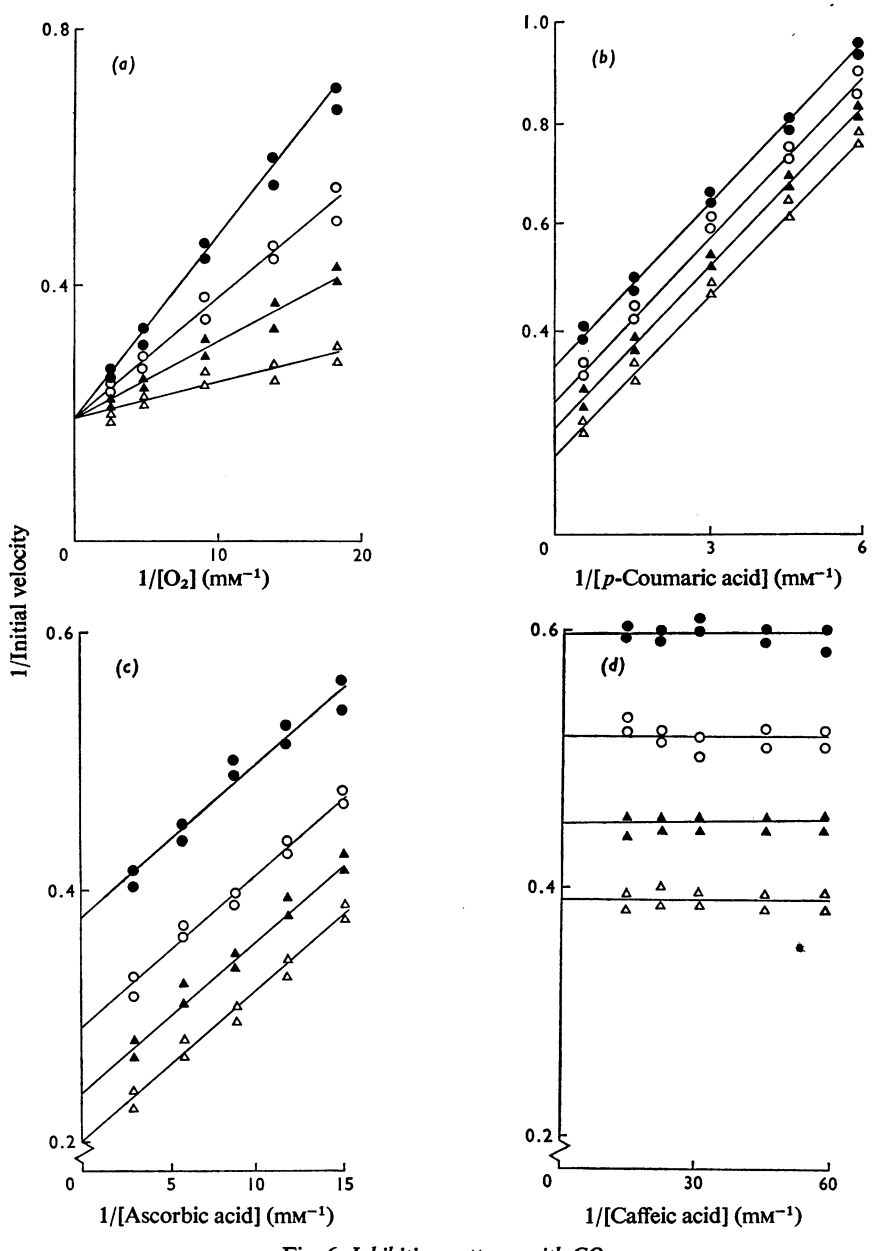



Fig. 6. *Inhibition patterns with CO*

Reciprocal plots of initial velocity against (*a*) $O_2$, (*b*) *p*-coumaric acid, (*c*) ascorbic acid and (*d*) caffeic acid in the presence of no (△), 0.017mM- (▲), 0.034mM- (○) or 0.067mM- (●) CO. Other conditions were as in Figs. 3(*a*), 3(*b*), 3(*c*) and 3(*d*) respectively.

the parallel pattern observed for a mechanism thought to be sequential on other grounds.

The occurrence of convergent initial-velocity patterns when non-inhibitory amounts of caffeic acid were included with *p*-coumaric acid or ascorbic acid as variable substrates suggested that caffeic acid establishes a reversible connexion between these two substrates. This is the predicted behaviour for a

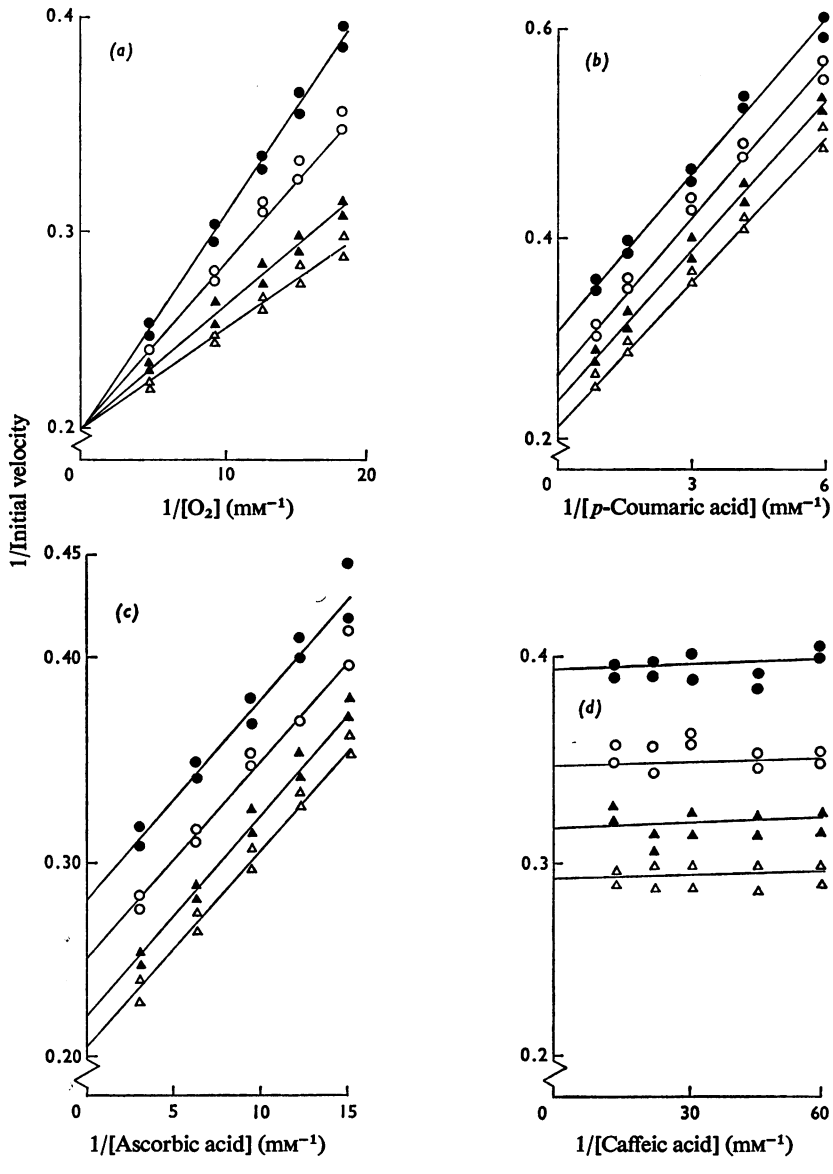



Fig. 7. *Inhibition pattern with bathocuproinesulphonate*

Reciprocal plots of initial velocity against (*a*) $O_2$, (*b*) *p*-coumaric acid, (*c*) ascorbic acid and (*d*) caffeic acid in the presence of no (△), 0.084mM- (▲), 0.167mM- (○) or 0.33mM- (●) bathocuproinesulphonate. Other conditions were as in Figs. 3(*a*), 3(*b*), 3(*c*) and 3(*d*) respectively.

classic Ping Pong step (Cleland, 1970) and suggests that caffeic acid is released between the binding of *p*-coumaric acid and electron donor.

These initial-velocity studies are consistent with a mechanism in which *p*-coumaric acid reacts with an oxygenated enzyme species to liberate caffeic acid as product, before caffeic acid, acting as an electron donor, adds to the enzyme. It is reasonable to assume that $O_2$ binds to the enzyme before *p*-coumaric acid and that the irreversible step detected between the binding of $O_2$ and the other substrates is associated with the free energy involved in the reduction of molecular $O_2$ to a species active in hydroxylation.

The kinetic mechanism for *p*-coumaric acid

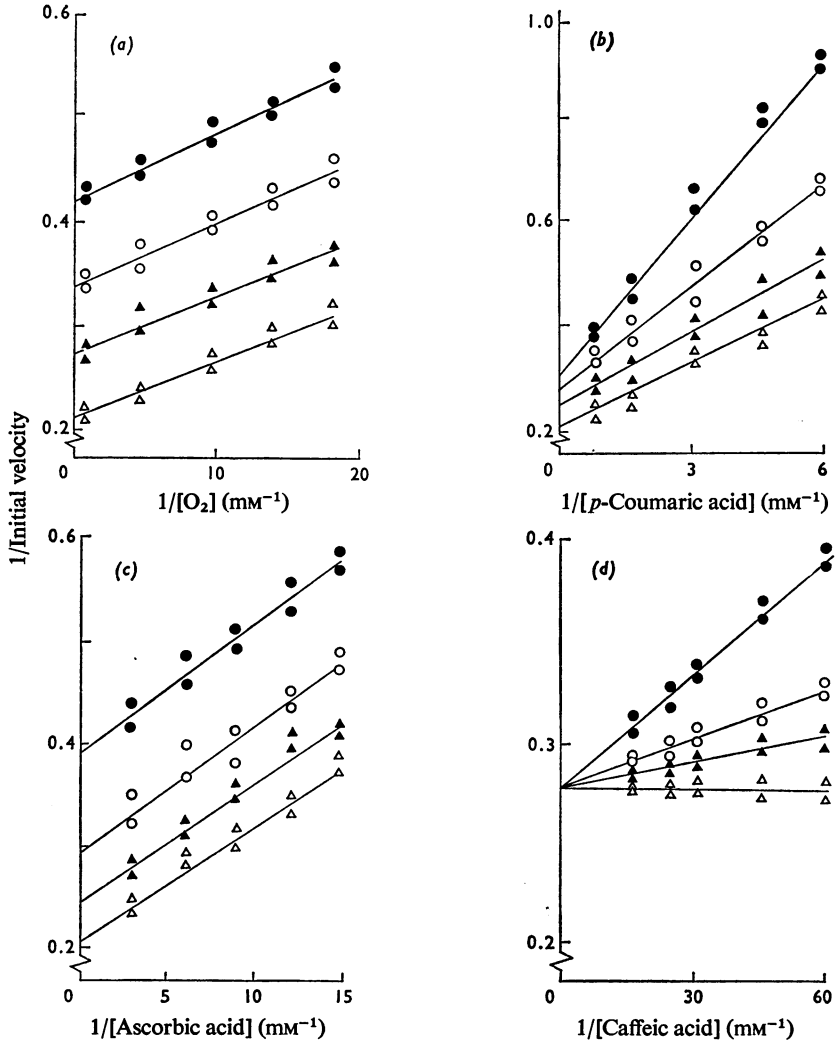



Fig. 8. *Inhibition pattern with diethyldithiocarbamate*

Reciprocal plots of initial velocity against (*a*) $O_2$, (*b*) *p*-coumaric acid, (*c*) ascorbic acid and (*d*) caffeic acid in the presence of no (△), 0.5 $\mu$M- (▲), 1.0 $\mu$M- (○) and 1.5 $\mu$M- (●) diethyldithiocarbamate. Other conditions were as in Figs. 3(*a*), 3(*b*), 3(*c*) and 3(*d*) respectively.

hydroxylation would thus be (using the notation of Cleland, 1963) either Hexa Uni Ping Pong or Bi Uni Uni Uni Ping Pong, with an irreversible step separating the addition of the first two substrates.

*Inhibitor studies*

It is not possible to carry out product-inhibition studies on this reaction since water is present in an excess, caffeic acid is also a substrate for the catechol oxidase activity of the enzyme and *o*-quinone is reduced to *o*-dihydric phenol by the ascorbic acid or dimethyltetrahydropteridine present in the assay medium. Therefore competitive inhibitors or weak alternative substrates were used to obtain information on reaction mechanism (Fromm, 1967). The following assumptions were made when applying this approach to the hydroxylation of *p*-coumaric acid. (i) The order of substrate addition is oxygen, *p*-coumaric acid and electron donor. (ii) Caffeic acid acts as an electron donor at the active site, the main role of ascorbic acid being the chemical reduction

Table 6. *Inhibition constants*

For details see the text. N.L., Non-linear; N.A., slope or intercept not altered by inhibitor.

| Inhibitor | Variable substrate | $K_i$ (intercept) ($\mu$M) | $K_i$ (slope) ($\mu$M) |
|---|---|---|---|
| CO | Oxygen | N.A. | 16.2 |
| | *p*-Coumaric acid | 62.0 | N.A. |
| | Ascorbic acid | 150.0 | N.A. |
| | Caffeic acid | 338.0 | N.A. |
| Bathocuproinesulphonate | Oxygen | N.A. | 284.0 |
| | *p*-Coumaric acid | 561.0 | N.A. |
| | Ascorbic acid | 825.0 | N.A. |
| | Caffeic acid | 277.0 | N.A. |
| Diethyldithiocarbamate | Oxygen | 1.13 | N.A. |
| | *p*-Coumaric acid | 11.9 | N.L. |
| | Ascorbic acid | N.L. | N.A. |
| | Caffeic acid | N.A. | N.L. |
| Benzoic acid | Oxygen | 450.0 | N.A. |
| | *p*-Coumaric acid | 700.0 | 50.0 |
| | Caffeic acid | 750.0 | N.L. |
| *p*-Hydroxybenzoic acid | Oxygen | 350.0 | N.A. |
| | *p*-Coumaric acid | 500.0 | 150 |
| | Ascorbic acid | 375.0 | N.A. |
| | Caffeic acid | 750.0 | N.L. |
| 3,4-Dihydroxybenzoic acid | Oxygen | 520.0 | N.A. |
| | *p*-Coumaric acid | N.L. | N.L. |
| | Ascorbic acid | N.L. | N.L. |
| | Caffeic acid | N.L. | N.L. |

of the *o*-quinone of caffeic acid to caffeic acid (Vaughan & Butt, 1970). (iii) Oxygen binds to copper.

The inhibition pattern observed with CO and bathocuproinesulphonate agrees with that predicted for a Hexa Uni Ping Pong mechanism (Table 7), and thus provides further support for the presence of an irreversible step between the binding of $O_2$ and the other two substrates.

The observation that diethyldithiocarbamate acts as a competitive inhibitor to caffeic acid and not $O_2$, although it confirms previous results (Vaughan & Butt, 1970), is unexpected on the basis of the results with other copper inhibitors. Brady *et al.* (1972) have interpreted the different effects of bathocuproinesulphonate and diethyldithiocarbamate as inhibitors of tryptophan 2,3-dioxygenase on the basis of their behaviour as Cu(I)- (Diehl & Smith, 1958) and Cu(II)- (McFarlane, 1932) specific complexing agents respectively. This would imply that $O_2$ binds to Cu(I) whereas caffeic acid binds to the enzyme in the Cu(II) form. However, the classification of valency-specific chelators is based on studies with metal ions in solution and it is questionable whether this concept is valid when applied to copper atoms bound to proteins. A more likely explanation, in this case, is that competitive inhibition is the result of reaction between the *o*-quinone of caffeic acid (generated from the caffeic acid added to the reaction mixture) and diethyldithiocarbamate, as reported by Pierpoint (1966) from studies on the effect of diethyldithiocarbamate on oxidation of chlorogenic acid with tobacco phenolase.

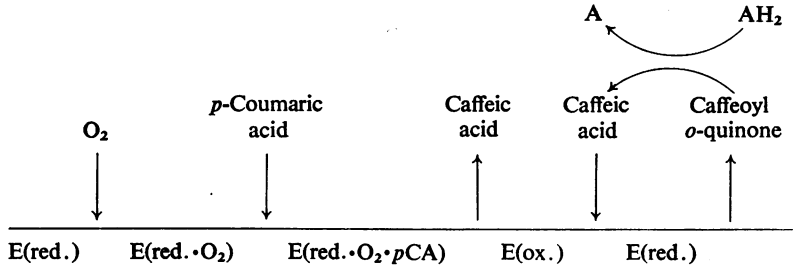
The non-competitive inhibition patterns observed for *p*-hydroxybenzoic acid and benzoic acid with *p*-coumaric acid as the variable substrate (Figs. 3 and 4) are also unexpected since, on structural grounds, *p*-hydroxybenzoic acid would be expected to be a competitive inhibitor, and benzoic acid was found to be a competitive inhibitor to phenolic substrates with mushroom phenolase (Duckworth & Coleman, 1970).

These kinetic studies suggest that spinach-beet phenolase contains at least three phenol-binding sites, one for *o*-dihydric phenols acting as cofactors in hydroxylation, and one each for monohydric and *o*-dihydric phenols acting as a substrate for the hydroxylase or catechol oxidase reactions respectively. The anomalies in the inhibition patterns observed with both monohydric and *o*-dihydric phenol alternative substrates could then be accounted for on the basis that the substrate sites do not have complete specificity, so that some binding of monophenol to the *o*-dihydric phenol site occurs and vice versa. The

Table 7. *Predicted and observed alternate substrate and competitive-inhibition patterns*

For details see the text. C, Competitive; NC, non-competitive; UC, uncompetitive inhibition.

| Inhibitor | Variable substrate | Predicted Model (a) | Predicted Model (b) | Observed |
|---|---|---|---|---|
| CO | Oxygen | C | C | C |
| | *p*-Coumaric acid | UC | NC | UC |
| | Caffeic acid | UC | UC | UC |
| | Ascorbic acid | UC | UC | UC |
| Bathocuproinesulphonate | Oxygen | C | C | C |
| | *p*-Coumaric acid | UC | NC | UC |
| | Caffeic acid | UC | UC | UC |
| | Ascorbic acid | UC | UC | UC |
| Diethyldithiocarbamate | Oxygen | C | C | UC |
| | *p*-Coumaric acid | UC | NC | NC |
| | Caffeic acid | UC | UC | C |
| | Ascorbic acid | UC | UC | UC |
| Benzoic acid | Oxygen | UC | UC | UC |
| | *p*-Coumaric acid | C | C | NC |
| | Caffeic acid | UC | UC | NC → UC |
| *p*-Hydroxybenzoic acid | Oxygen | UC | UC | UC |
| | *p*-Coumaric acid | C | C | NC |
| | Caffeic acid | UC | UC | NC → UC |
| | Ascorbic acid | UC | UC | UC |
| 3,4-Dihydroxybenzoic acid | Oxygen | UC | UC | UC |
| | *p*-Coumaric acid | UC | UC | UC → NC |
| | Caffeic acid | C | C | C → NC |
| | Ascorbic acid | C | C | Curved UC |




Scheme 1. *Graphical representation of reaction sequence for p-coumaric acid hydroxylation*

$AH_2$, Electron donors such as ascorbic acid, NADH or dimethyltetrahydropteridine.

proposal that spinach-beet phenolase contains more than one phenol-binding site agrees with similar suggestions for tyrosinases from mouse melanoma (Pomerantz & Warner, 1967) and mushroom (Kendal, 1949; Duckworth & Coleman, 1970).

A kinetic mechanism based on these results is summarized in Scheme 1. It is proposed that $O_2$ binds to a reduced form of the enzyme E(red.) to form an oxygenated species, similar to oxytyrosinase reported to be a catalytic intermediate of mushroom tyrosinase (Jolley *et al.*, 1974). A further postulate is that combination of $O_2$ with the enzyme leads to a free-energy decrease, sufficiently large to account for the irreversibility of the step between $O_2$ and *p*-coumaric acid. Caffeic acid is liberated to form an oxidized stable enzyme form, E(ox.), which is subsequently reduced, probably by caffeic acid bound to the cofactor site. In this Scheme reductants such as ascorbic acid act mainly to recycle cofactor amounts of caffeic acid. The similar $K_m$ values for ascorbic acid and dimethyl-

tetrahydropteridine, together with their similar $V_{max.}$ values (Table 3), provide additional support for this view.

The postulate that $O_2$ binds to a reduced form of the enzyme agrees with the report that up to 15% of resting mushroom tyrosinase exists as a stable reversibly oxygenated form (Jolley *et al.*, 1974). However, the complex inhibition patterns suggest that the mechanism may be more complex than that proposed here. In particular the proposal for three phenol-binding sites, together with the effect of monophenol on the binding of *o*-dihydric phenol, and vice versa, requires more direct study than the kinetic experiments described in this paper.

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## References

Brady, F. O., Monaco, M. E., Forman, H. J., Schutz, G. & Fiegelson, P. (1972) *J. Biol. Chem.* **247**, 7915–7922

Butt, V. S. & Hallaway, M. (1961) *Arch. Biochem. Biophys.* **92**, 24–32

Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 104–137

Cleland, W. W. (1970) *Enzymes*, 3rd edn., **2**, 1–65

Diehl, H. & Smith, G. F. (1958) *The Copper Reagents, Cuproine, Neocuproine and Bathocuproine*, pp. 39–44, G. F. Smith Chemical Co., Columbus, Ohio

Duckworth, H. W. & Coleman, J. E. (1970) *J. Biol. Chem.* **245**, 1613–1625

Fromm, H. J. (1967) *Biochim. Biophys. Acta* **139**, 221–230

Gregory, R. P. F. & Bendall, D. S. (1966) *Biochem. J.* **101**, 569–581

Ingraham, L. L. (1957) *J. Am. Chem. Soc.* **79**, 666–669

Jolley, R. L., Evans, L. H., Makino, N. & Mason, H. S. (1974) *J. Biol. Chem.* **249**, 335–345

Kendal, L. P. (1949) *Biochem. J.* **44**, 442–454

Mason, H. S. (1955) *Adv. Enzymol.* **16**, 105–184

McFarlane, W. O. (1932) *Biochem. J.* **26**, 1022–1033

Morris, J. G. & Redfearn, E. R. (1969) in *Data for Biochemical Research*, 2nd edn. (Dawson, R. M. C., Elliott, D., Elliott, W. H. & Jones, K. M., eds.), pp. 191–215, Oxford University Press, London

Nelson, J. M. & Dawson, C. R. (1944) *Adv. Enzymol.* **4**, 99–152

Nielsen, K. H. (1969) *Eur. J. Biochem.* **7**, 360–369

Ogasawara, N., Gander, J. E. & Henderson, L. M. (1966) *J. Biol. Chem.* **241**, 613–619

Pierpoint, W. S. (1966) *Biochem. J.* **98**, 567–580

Pomerantz, S. H. & Warner, M. C. (1967) *J. Biol. Chem.* **242**, 5308–5314

Robinson, J. & Cooper, J. M. (1970) *Anal. Biochem.* **33**, 390–399

Rudolph, F. B., Purich, D. I. & Fromm, H. J. (1968) *J. Biol. Chem.* **243**, 5539–5545

Vaughan, P. F. T. & Butt, V. S. (1969) *Biochem. J.* **113**, 109–115

Vaughan, P. F. T. & Butt, V. S. (1970) *Biochem. J.* **119**, 89–94

Vaughan, P. F. T. & Butt, V. S. (1972) *Biochem. J.* **127**, 641–647

Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324–332

## APPENDIX

# Relationship between Caffeic Acid Concentration and Change in Absorbance at Wavelengths of Assay

The relationship between caffeic acid concentration and change in absorbance is derived from eqn. (i):

$$\Delta E\lambda_{nm}=\Sigma\varepsilon_i^{\lambda}\cdot C_i^t-\Sigma\varepsilon_i^{\lambda}\cdot C_i^0 \qquad (i)$$

where $\lambda_{nm}$ is wavelength (nm) at which absorbance change is measured, $\varepsilon_i^{\lambda}$ is molar extinction coefficient for each species absorbing at $\lambda_{nm}$, and $C_i^0$ and $C^t$ are the concentrations (mol/litre) of species at time=0 and $t$s respectively.

### Ascorbic Acid

$\lambda$=340nm. *p*-Coumaric acid (pCA) and caffeic acid (caff) are the only components in the assay absorbing at 340nm.

From eqn. (i):

$$E_{340}=\varepsilon_{pCA}^{340}\cdot C_{pCA}^t+\varepsilon_{caff}^{340}\cdot C_{caff}^t-\varepsilon_{pCA}^{340}\cdot C_{pCA}^0-\varepsilon_{caff}^{340}\cdot C_{caff}^0$$

From the stoicheiometry of the hydroxylation reaction (Vaughan & Butt, 1969):

$$C_{pCA}^t=C_{pCA}^0-C_{caff}^t \qquad (ii)$$

Since $C_{caff}^0=0$:

$$\Delta E_{340}=\varepsilon_{caff}^{340}\cdot C_{caff}^t-\varepsilon_{pCA}^{340}\cdot C_{caff}^t$$

and

$$C_{caff}^t=\Delta E_{340}/(\varepsilon_{caff}-\varepsilon_{pCA})$$

By substitution of molar extinction coefficients from Table 2, $\therefore C_{caff}^t=\Delta E_{340}/2.61\times10^3$M.

### Dimethyltetrahydropteridine

In addition to caffeic acid and *p*-coumaric acid dimethyldihydropteridine absorbs at 340nm. Absorbance changes at 370nm due to dimethyldihydropteridine are therefore determined and used to correct the 340nm absorbance change for dimethyldihydropteridine (DMDP).

From eqns. (i) and (ii):

$$\Delta E_{340}=\varepsilon_{caff}^{340}\cdot C_{caff}^t-\varepsilon_{pCA}^{340}\cdot C_{caff}^t+\varepsilon_{DMDP}^{340}(C_{DMDP}^t-C_{DMDP}^0) \qquad (iii)$$

$$\Delta E_{370}=\varepsilon_{caff}^{370}\cdot C_{caff}^t-\varepsilon_{pCA}^{370}\cdot C_{caff}^t+\varepsilon_{DMDP}^{370}(C_{DMDP}^t-C_{DMDP}^0) \qquad (iv)$$

Since $C_{DMDP}^0=0$, from eqn. (iii):

$$C_{DMDP}^t=\frac{\Delta E_{340}-C_{caff}^t(\varepsilon_{caff}^{340}-\varepsilon_{pCA}^{340})}{E_{DMDP}^{340}} \qquad (v)$$

Substitution of eqn. (v) into eqn. (iv) and rearrangement gives:

$$C_{caff}^t=\frac{\varepsilon_{DMDP}^{340}\cdot\Delta E_{370}-\varepsilon_{DMDP}^{370}\cdot\Delta E_{340}}{\varepsilon_{DMDP}^{340}(\varepsilon_{caff}^{370}-\varepsilon_{pCA}^{370})-\varepsilon_{DMDP}^{370}(\varepsilon_{340}^{caff}-\varepsilon_{pCA}^{340})}$$

Substitution of values for molar extinction coefficients from Table 2 gives $C_{caff}^t=(0.40\Delta E_{340}-0.85\Delta E_{370})\times10^{-3}$M

### NADH

Convenient wavelengths for following hydroxylation in the presence of NADH were 240nm and 370nm.

From eqns. (i) and (ii), and as $C_{NAD}^t=C_{NADH}^0-C_{NADH}^t$, then:

$$\Delta E_{370}=C_{caff}^t(\varepsilon_{caff}^{370}-\varepsilon_{pCA}^{370})-\varepsilon_{NADH}^{370}\cdot C_{NAD}^t \qquad (vi)$$

and

$$\Delta E_{240}=C_{caff}^t(\varepsilon_{caff}^{240}-\varepsilon_{pCA}^{240})-C_{NAD}^t(\varepsilon_{NADH}^{240}-\varepsilon_{NAD}^{240}) \qquad (vii)$$

From eqn. (vi):

$$C_{NAD}^t=\frac{C_{caff}^t(\varepsilon_{caff}^{370}-\varepsilon_{pCA}^{370})-\Delta E_{370}}{E_{NADH}^{370}}$$

Substitution in eqn. (vii) and rearrangement gives:

$$C_{caff}^t=\frac{\varepsilon_{NADH}^{370}\cdot\Delta E_{240}+(\varepsilon_{NAD}^{240}-\varepsilon_{NADH}^{240})\Delta E_{370}}{\varepsilon_{NADH}^{370}(\varepsilon_{caff}^{240}-\varepsilon_{pCA}^{240})+(\varepsilon_{NAD}^{240}-\varepsilon_{NADH}^{240})(\varepsilon_{caff}^{370}-\varepsilon_{pCA}^{370})}$$

Substitution of values for molar extinction coefficients from Table 2 gives $\therefore C_{caff}^t=(1.16\Delta E_{240}+0.85\Delta E_{370})\times10^{-4}$M.

### References

Vaughan, P. F. T. & Butt, V. S. (1969) *Biochem. J.* **113**, 109–115