Biochem. J. (1970) **119**, 89–94
Printed in Great Britain

The Action of *o*-Dihydric Phenols in the Hydroxylation of *p*-Coumaric Acid by a Phenolase from Leaves of Spinach Beet (*Beta vulgaris* L.)

By P. F. T. VAUGHAN* and V. S. BUTT
Botany School, University of Oxford, South Parks Road, Oxford OX1 3RA, U.K.

(*Received* 30 *April* 1970)

1. Under defined conditions, the hydroxylation of *p*-coumaric acid catalysed by a phenolase from leaves of spinach beet (*Beta vulgaris* L.) was observed to develop its maximum rate only after a lag period. 2. By decreasing the reaction rate with lower enzyme concentrations or by increasing it with higher concentrations of reductants, the length of the lag period was inversely related to the maximum rate subsequently developed. 3. Low concentrations of caffeic acid or other *o*-dihydric phenols abolished this lag period. With caffeic acid, the rate of hydroxylation was independent of the reductant employed. 4. Hydroxylation was inhibited by diethyldithiocarbamate, but with low inhibitor concentrations hydroxylation recovered after a lag period. This lag could again be abolished by the addition of high concentrations of caffeic acid or other *o*-dihydric phenols. 5. Catechol oxidase activity showed no lag period, and did not recover from diethyldithiocarbamate inhibition. 6. The purified enzyme contained 0.17–0.33% copper; preparations with the highest specific activity were found to have the highest copper content. 7. The results are interpreted to suggest that the oxidation of *o*-dihydric phenols converts the enzymic copper into a species catalytically active in hydroxylation. This may represent the primary function for the catechol oxidase activity of the phenolase complex. The electron donors are concerned mainly, but not entirely, in the reduction of *o*-quinones produced in this reaction.

The enzymic hydroxylation of *p*-coumaric acid to caffeic acid with a phenolase extracted from leaves of spinach beet (*Beta vulgaris* L.) requires an electron donor, such as ascorbate, NADH or dimethyltetrahydropteridine (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine; Vaughan & Butt, 1969). Mushroom and other phenolase preparations can catalyse the hydroxylation of monophenols (Nelson & Dawson, 1944), tyrosine (Krueger, 1950) and *p*-coumaric acid (Sato, 1969) without any added reductant, but the reaction course then shows an induction period which can be eliminated by the addition of ascorbate or caffeic acid. The spinach-beet enzyme was found to remain inactive when caffeic acid was added without any electron donor, but a lag period was observed when NADH or dimethyltetrahydropteridine was used as reductant.

The conditions under which this lag period can be observed with the spinach-beet enzyme were investigated, and its elimination by caffeic acid or other *o*-dihydric phenols was demonstrated. The reversal of diethyldithiocarbamate inhibition by *o*-dihydric phenols was examined, leading to suggestions on the relation of catechol oxidase activity to the function of enzymic copper in the mechanism of hydroxylation.

Part of this work has already been presented briefly by Vaughan & Butt (1968).

* Present address: Department of Biochemistry, University of Glasgow, Glasgow W.2, U.K.

MATERIALS AND METHODS

The reagents, most of the analytical methods and definition of enzyme units have been described by Vaughan & Butt (1969). The protein content of fractions obtained during enzyme purification was measured spectrophotometrically at 260 and 280 nm (Warburg & Christian, 1941), and that of the purest fractions eluted from CM-cellulose columns by the more sensitive method of Bailey (1962).

Determination of copper. Samples (10 ml) of enzyme that had been eluted from CM-cellulose columns and then dialysed for 5 h against 1 mM-Na_2HPO_4–0.5 mM-citric acid buffer, pH 5.3, were heated with 0.3 ml of conc. H_2SO_4, 0.3 ml of conc. HNO_3 and 0.3 ml of 60% (w/v) $HClO_4$ until fuming had ceased. A further 1.0 ml of HNO_3 and 0.1 ml of $HClO_4$ were then added and the mixture was heated to complete the digestion. The residue was transferred to stoppered graduated tubes. The digestion flasks were washed successively with 1.0 ml

and 0.5 ml of deionized water and 1.0 ml of aq. NH_3 (sp.gr. 0.88), and the washings were added to the graduated tubes.

The copper content of these samples was determined by a modification of the method of Stark & Dawson (1958). The sample in each tube was mixed with 1.0 ml of aq. NH_3 (sp.gr. 0.88) and 0.5 ml of freshly prepared 40% (v/v) acetaldehyde. The bluish-purple colour that developed after 30 min was measured in a Beckman DB spectrophotometer at 540 nm.

To obtain accurate determinations it was necessary to accompany the measurement of each batch of digestions with samples containing 0, 0.5 and 1.0 μg of copper, which gave a linear relationship. All determinations of copper content were made within this range.

Preparation of the enzyme. Most of the experiments were carried out with the enzyme prepared as described by Vaughan & Butt (1969), and purified as an eluate from DEAE-cellulose columns (stage IV). Further purification was necessary to obtain a solution with sufficiently high protein concentration to allow determination of the copper content. This was achieved by modifying the earlier procedure so that the solution after dialysis (stage III) was passed through a CM-cellulose column (2.2 cm × 33 cm) treated as described by Vaughan & Butt (1969). After the extinction of the effluent at 280 nm had fallen to a very low value the column was eluted with a linear increase in the NaCl concentration up to 0.75 M.

RESULTS

Action of o-dihydric phenols on progress of hydroxylation. The lag period before the hydroxylation of *p*-coumaric acid developed its maximum rate was demonstrated most clearly when smaller quantities of the enzyme were used, with dimethyltetrahydropteridine as electron donor (Fig. 1). The lag periods were about 30s, 2min and 5min with 93, 37 and 19m-units of enzyme respectively; the corresponding maximum rates of caffeic acid production were 0.18, 0.07 and 0.04 μmol/min. This inverse relationship between the length of the lag period and the rate of caffeic acid production was further demonstrated by the severe shortening of the lag period when saturating concentrations of dimethyltetrahydropteridine (1 mM) or NADH (6.7 mM) were added.

When 1 nmol of caffeic acid was added with 1 μmol of dimethyltetrahydropteridine in the reaction mixture before the addition of *p*-coumaric acid, the lag period was shorter. It was further decreased with 0.01 μmol of caffeic acid and was not detectable when 0.1 μmol of caffeic acid was added (Fig. 2). The maximum rate achieved by the reaction was in each case increased.

These effects were not specific for caffeic acid. The lag period was also abolished when 0.1 μmol of protocatechuic acid, catechol, 4-methylcatechol or 3,4-dihydroxy-L-phenylalanine were substituted for caffeic acid (Fig. 3). The maximum rates of hydroxylation achieved in the presence of each diphenol were different, but were sustained only with protocatechuic acid. Nevertheless, the fastest initial rate was observed with 0.1 μmol of caffeic acid.

By using sufficiently small quantities of the

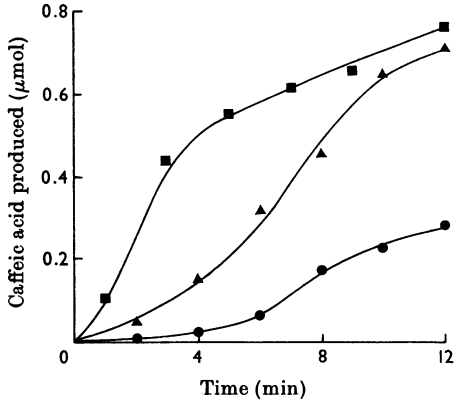

Fig. 1. Effect of enzyme concentration on caffeic acid production. Enzyme (purified to Stage IV; Vaughan & Butt, 1969) [■, 93 m-units; ▲, 37 m-units; ●, 19 m-units] was incubated with *p*-coumaric acid (10 μmol) and dimethyltetrahydropteridine (1 μmol) in 0.1 M-Na_2HPO_4–0.05 M-citric acid buffer, pH 5.3, containing $(NH_4)_2SO_4$ (1.5 mmol) at 30°C in air. Total volume was 3.3 ml.

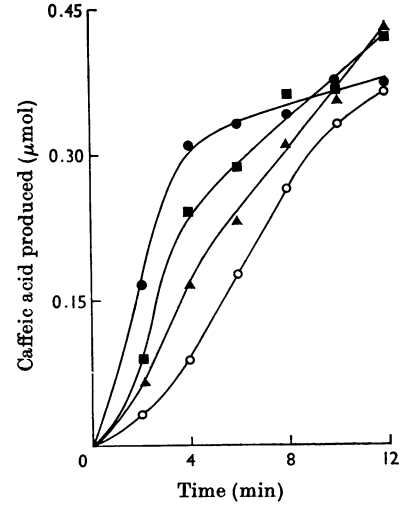

Fig. 2. Effect of caffeic acid on the progress of hydroxylation. Enzyme (37 m-units) was incubated with 0 (○), 0.001 (▲), 0.01 (■) or 0.1 (●) μmol of caffeic acid added immediately before *p*-coumaric acid (10 μmol) under the reaction conditions described for Fig. 1.

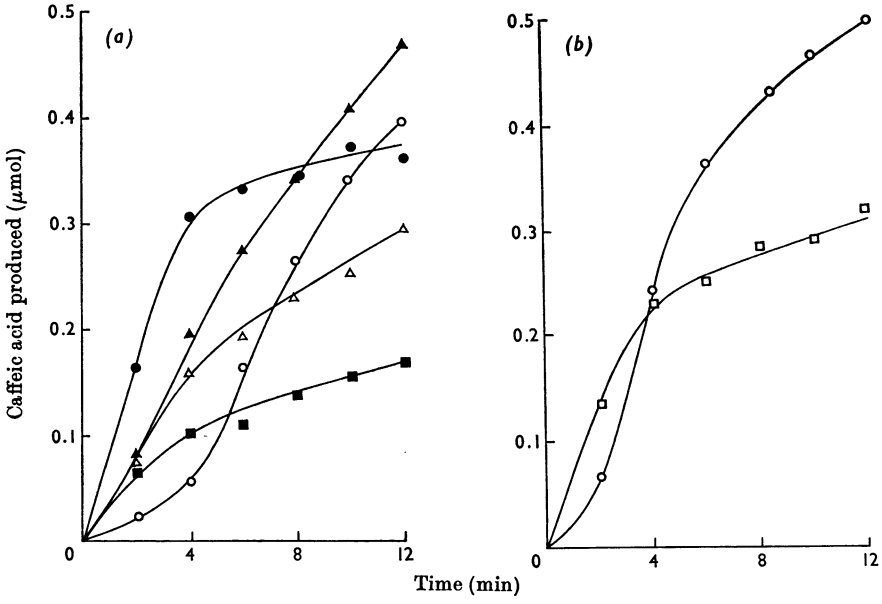

Fig. 3. Effect of *o*-dihydric phenols on the progress of hydroxylation. (*a*) Enzyme (37 m-units) was incubated without addition (○), or with 0.1 μmol of caffeic acid (●), 0.1 μmol of protocatechuic acid (▲), 0.1 μmol of catechol (■) or 0.1 μmol of 4-methylcatechol (△), added immediately before *p*-coumaric acid (10 μmol). (*b*) Enzyme (42 m-units) was incubated without addition (○) or with 0.1 μmol of 3,4-dihydroxy-L-phenylalanine (□) added immediately before *p*-coumaric acid (10 μmol). The other conditions in (*a*) and (*b*) were as described for Fig. 1.

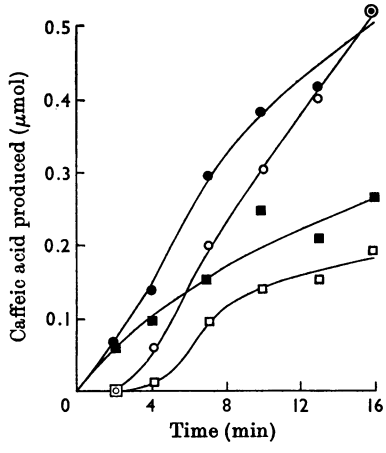

Fig. 4. Effect of caffeic acid on progress of hydroxylation, with ascorbate or NADH as electron donors. Enzyme (33 m-units) was incubated with 10 μmol of ascorbate (○), 5 μmol of NADH (□), 10 μmol of ascorbate and 0.1 μmol of caffeic acid (●) or 5 μmol of NADH and 0.1 μmol of caffeic acid (■), each added before *p*-coumaric acid (10 μmol). Other conditions were as described for Fig. 1.

enzyme in the reaction mixture, initial lag periods could also be observed with 10 μmol of ascorbate or 5 μmol of NADH as electron donors; these lags could be eliminated by the inclusion of 0.1 μmol of caffeic acid (Fig. 4). The maximum rates of hydroxylation were the same with each reductant when caffeic acid had been added beforehand, although they were quite different in its absence.

Reversal of diethyldithiocarbamate inhibition by o-dihydric phenols. The hydroxylase activity of the phenolase was completely inhibited by 1 mM-diethyldithiocarbamate. When lower concentrations of the inhibitor were used, the reaction was halted at first, but then increased to a steady rate. This effect was observed over a range of concentrations of diethyldithiocarbamate from 0.01 to 0.05 mM with 5 μmol of NADH and 1 μmol of dimethyltetrahydropteridine together as reductant (Fig. 5), and from 0.005 to 0.01 mM-diethyldithiocarbamate when 10 μmol of ascorbate was used. As the inhibitor concentration was increased, the period before the inhibition was reversed became longer. When the enzyme was incubated with 0.02 mM-diethyldithiocarbamate and reductant for periods of up to 30 min before the addition of substrate, the subsequent course of the reaction was not affected; the inhibitor was therefore stable under these conditions (Hallaway, 1959).

The reaction course suggested some autocatalytic effect of a product of the reaction in destroying the effect of the inhibitor. This was confirmed by the

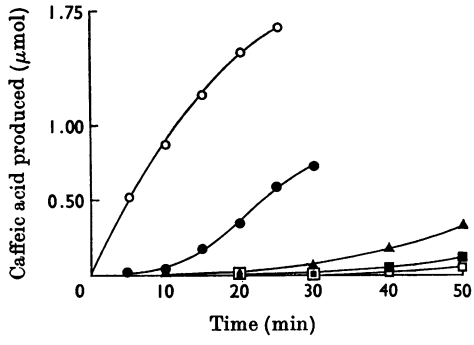

Fig. 5. Effect of diethyldithiocarbamate on the progress of hydroxylation. Enzyme (33 m-units) was incubated with dimethyltetrahydropteridine (1 μmol), NADH (5 μmol), and *p*-coumaric acid (10 μmol), in 0.1 M-Na_2HPO_4–0.05 M-citric acid buffer, pH 5.3, containing $(NH_4)_2SO_4$ (1.5 mmol) at 30°C in air, in the presence of 10 nM- (●), 20 nM- (▲), 30 nM- (■) or 50 nm- (□) diethyldithiocarbamate, or without inhibitor (○). Total volume was 3.3 ml.

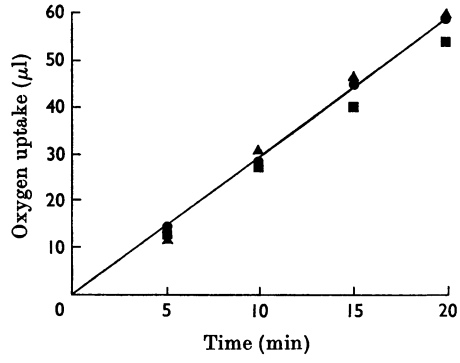

Fig. 7. Effect of different reductants on catechol oxidase activity. Ascorbate (5 μmol) (●), NADH (5 μmol) (■) or dimethyltetrahydropteridine (5 μmol) (▲) was incubated with the enzyme (85 m-units), catechol (0.75 μmol) and $(NH_4)_2SO_4$ (1.5 mmol) in 0.1 M-Na_2HPO_4–0.05 M-citric acid buffer, pH 5.3, at 30°C in air. Total volume was 3.0 ml. The line is the best fit for the experimental points for ascorbate.

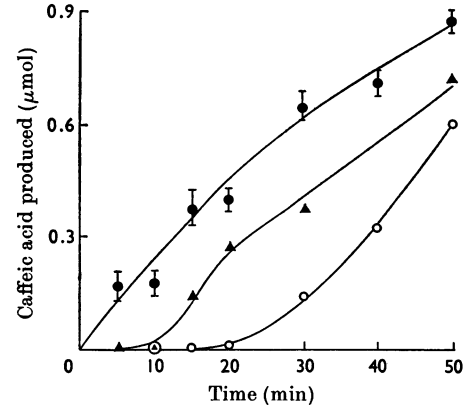

Fig. 6. Effect of caffeic acid on the progress of hydroxylation in the presence of diethyldithiocarbamate. Enzyme (33 m-units) was incubated with 20 nM-diethyldithiocarbamate under the conditions described for Fig. 5. 1.0 μmol of caffeic acid (▲), 3.0 μmol of caffeic acid (●) or no caffeic acid (○) was added immediately before *p*-coumaric acid (10 μmol). The bars indicate the S.E.M. of five determinations.

decrease in the lag period when 1 μmol of caffeic acid was added to the reaction mixture before *p*-coumaric acid, and its abolition by 3 μmol of caffeic acid (Fig. 6). The delay in the reaction was also completely eliminated by 3 μmol of protocatechuic acid or 3 μmol of catechol.

However, whereas the delay was abolished by *o*-dihydric phenols, appreciable inhibition of hydroxylation persisted. The reaction rate was decreased by 75%, 93% and 83% by 3 μmol of caffeic acid, protocatechuic acid and catechol respectively. This was shown to be due largely to the high concentrations of diphenol necessary to overcome the diethyldithiocarbamate inhibition. In the absence of any inhibitor and with 5 μmol of NADH and 1 μmol of dimethyltetrahydropteridine as reductant, 5 μmol of catechol decreased the rate of hydroxylation by 62%, whereas with 10 μmol of ascorbate as reductant, 2.5 and 5 μmol of protocatechuic acid inhibited the reaction by 31% and 61% respectively.

Reaction course of catechol oxidase activity. In contrast with the hydroxylation reaction, the direct oxidation of caffeic acid catalysed by the phenolase in the presence of reductants showed no lag period. The reaction course was linear with time with caffeic acid and with catechol, and the rate was independent of the reductant employed (Fig. 7). Severe inhibition was observed with 10 mM- and 5 mM-diethyldithiocarbamate, with 10 μmol of ascorbate as reductant, but no recovery was found over 60 min (Fig. 8).

Copper content of the purified enzyme. The modified extraction procedure for the enzyme gave a recovery of 20% of the activity of the crude homogenate with a 1000-fold increase in specific activity (Table 1). The protein concentration was invariably higher than that obtained with the original method. The relative hydroxylase and catechol oxidase activities did not vary significantly, although erratic variations, such as those

shown in Table 1, were evident during the purification of some extracts.

The determination of the copper content of the purest preparations ranged from 0.17% to 0.33% (w/w) of the protein content (Table 2). Duplicate measurements with the same sample gave identical values; preparations with the highest specific activity invariably had the highest copper contents.

Spectrophotometric examination of enzyme preparations revealed no absorption peak in the 610nm region such as is found with some copper proteins, even when the purest and most concentrated samples, containing about 0.2mg of protein/ml, were used.

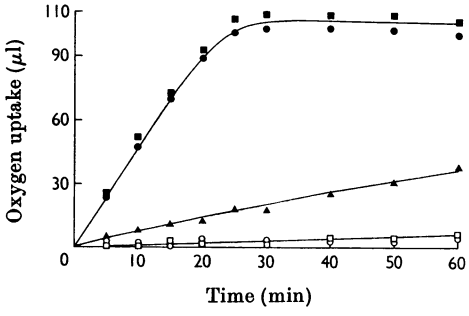

Fig. 8. Effect of diethyldithiocarbamate on catechol oxidase activity. Enzyme (35 m-units) was incubated with caffeic acid (0.9 μmol), ascorbate (10 μmol), 0.1 M-Na_2HPO_4–0.05 M-citric acid buffer, pH 5.3, and $(NH_4)_2SO_4$ in the presence of 1 μM- (■), 5 μM- (▲), 10 μM- (○) or 100 μM- (□) diethyldithiocarbamate, without inhibitor (●). Other conditions were as described for Fig. 7.

DISCUSSION

The copper content of enzyme preparations with the highest specific activity was similar to those reported for other phenolases. These range from 0.15% for phenolase from broad bean leaves (Robb, Mapson & Swain, 1965) to 0.32% for tea phenolase (Gregory & Bendall, 1966), with intermediate values for phenolases from potato (Kubowitz, 1937), mushroom (Kertesz & Zito, 1965) and the mealworm, *Tenebrio molitor* (Heyneman, 1965). In the absence of other criteria of purity, these values suggest that there is little contaminant copper or protein in these preparations.

Table 1. *Purification of phenolase from leaves of spinach beet by using the modified procedure*

Details of the modified purification procedure are described in the Materials and Methods section. The units of activity are those given by Vaughan & Butt (1969).

	Total volume (ml)	Activity (m-units/ml)		Protein content (mg/ml)	Sp. activity (m-units/mg of protein)		Hydroxylase/catechol oxidase activity ratio	Recovery (% of crude macerate)
		Hydroxylase	Catechol oxidase		Hydroxylase	Catechol oxidase		
I Crude macerate	1086	71	400	50	1.4	8	0.18	100
II Precipitate from 0.35–0.7-satd. $(NH_4)_2SO_4$	46	1070	4800	140	7.6	33	0.22	63
III Heat treatment and dialysis	74	650	1400	10	65	140	0.46	62
IV Eluate from CM-cellulose column	58	300	580	0.19	1580	3000	0.52	22

Table 2. *Copper content of purified enzyme*

Enzyme samples (10 ml) were prepared by dialysis of the eluate from CM-cellulose columns, and were assayed for protein (Bailey, 1962), copper (Stark & Dawson, 1958) and hydroxylase activity (Vaughan & Butt, 1969).

Sample	Protein content (μg/ml)	Hydroxylase activity (m-units/ml)	Sp. activity (m-units/mg of protein)	Copper content (μg/ml)	Copper content (% of protein)
1	37	133	3600	0.06	0.17
2	25	97	3900	0.04	0.18
3	50	200	4000	0.10	0.20
4	26	137	5300	0.08	0.31
5	15	74	5000	0.05	0.33

The elimination of the lag period, observed when low enzyme concentrations were used in the reaction, by the addition of catalytic quantities of *o*-dihydric phenol suggests that these phenols probably act as the immediate electron donors in the hydroxylation of *p*-coumaric acid. Since neither the added electron donors nor caffeic acid are alone effective, it is suggested that the electron donors act by reducing the *o*-quinone as it is produced, and which otherwise inhibits the hydroxylation (cf. Bordner & Nelson, 1939).

NADH, dimethyltetrahydropteridine and ascorbate all sustain oxygen consumption above that required for the oxidation of the catalytic traces of *o*-dihydric phenol added. In its role as electron donor, therefore, the *o*-dihydric phenol must undergo cyclic oxidation and reduction. The catechol oxidase activity is thus obligatorily linked with the hydroxylation process, although the former studied in isolation may appear to have different characteristics. For instance, Pomerantz & Warner (1967) demonstrated a cofactor function for 3,4-dihydroxy-L-phenylalanine in tyrosine hydroxylation catalysed by hamster melanoma tyrosinase, but higher concentrations were necessary for its substrate function in the absence of tyrosine.

The rates of catechol oxidation were surprisingly similar with different electron donors, since these same donors give different rates of *p*-coumaric acid hydroxylation. This may be due to the different conditions of catechol oxidation or to a differential effectiveness in the reduction of *o*-quinones during hydroxylation, or the electron donors might exert a second function during hydroxylation in addition to the reduction of *o*-quinones.

The progressive reversal of diethyldithiocarbamate inhibition during hydroxylation indicated some competition between a product of the reaction, presumably caffeic acid, and diethyldithiocarbamate for the enzymic copper. This was further substantiated by the decreased inhibition by this reagent when *o*-dihydric phenols were added before the substrate. Mayer & Friend (1960) reported a similar observation when some reversal of the inhibition of *p*-cresol oxidation by 1mM-diethyldithiocarbamate occurred with sugar beet chloroplasts, although these authors did not examine the possible instability of the inhibitor under their conditions. No reversal of diethyldithiocarbamate inhibition was observed during catechol oxidation, but in the presence of an excess of ascorbate or other reductant the catechol concentration did not change appreciably during the reaction.

Pierpoint (1966) suggested that the *o*-quinone produced by the oxidation of chlorogenic acid with tobacco phenolase reacts with diethyldithiocarbamate. The presence of excess of electron donor, however, makes it unlikely that this mechanism would explain the reversal of inhibition observed during the hydroxylation of *p*-coumaric acid but not during catechol oxidation.

The requirement for trace quantities of *o*-dihydric phenol to initiate the hydroxylation of *p*-coumaric acid is similar to that for the hydroxylation of other monophenols catalysed by the phenolases from mushroom, potato and broad bean. It is suggested that *o*-dihydric phenols interact with enzymic copper to produce a reactive species that effects the hydroxylation of *p*-coumaric acid. Any accumulation of *o*-quinone inhibits this first stage. The catechol oxidase activity of the enzyme is therefore an integral part of the hydroxylation mechanism (see Mason, 1957) in producing this active species, but in the absence of a monophenol or as the concentration of *o*-dihydric phenol increases during hydroxylation, this active species can also oxidize *o*-dihydric phenols so that the catechol oxidase activity appears distinct from hydroxylation.

The authors thank Mr D. A. Baker for his skilled technical assistance, and the Science Research Council for the award of a Studentship to P. F. T. V.

REFERENCES

Bailey, J. L. (1962). *Techniques in Protein Chemistry*, p. 293. Amsterdam: Elsevier Publishing Co.

Bordner, C. A. & Nelson, J. M. (1939). *J. Am. chem. Soc.* **61**, 1507.

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

Hallaway, M. (1959). *Biochim. biophys. Acta*, **36**, 538.

Heyneman, R. A. (1965). *Biochem. biophys. Res. Commun.* **21**, 162.

Kertesz, D. & Zito, R. (1965). *Biochim. biophys. Acta*, **96**, 447.

Krueger, R. C. (1950). *J. Am. chem. Soc.* **72**, 5582.

Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.

Mason, H. S. (1957). *Adv. Enzymol.* **19**, 79.

Mayer, A. M. & Friend, J. (1960). *J. exp. Bot.* **11**, 141.

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

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

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

Robb, D. A., Mapson, L. W. & Swain, T. (1965). *Phytochemistry*, **4**, 731.

Sato, M. (1969). *Phytochemistry*, **8**, 353.

Stark, G. R. & Dawson, C. R. (1958). *Analyt. Chem.* **30**, 191.

Vaughan, P. F. T. & Butt, V. S. (1968). *Biochem. J.* **107**, 7P.

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

Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.