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The Expression of Catechol Oxidase Activity during the Hydroxylation of *p*-Coumaric Acid by Spinach-Beet Phenolase

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1. The conditions under which oxygen consumption in excess of that required for the hydroxylation of *p*-coumaric acid to caffeic acid, catalysed by spinach-beet phenolase, can be suppressed, have been examined. 2. With dimethyltetrahydropteridine as electron donor, oxygen uptake was exactly equivalent to the caffeic acid produced, provided that *p*-coumaric acid was in excess, but with excess of reductant, oxygen uptake caused by the further oxidation of caffeic acid was also observed. 3. With equal concentrations of ascorbate and *p*-coumaric acid, equivalent oxygen uptake and caffeic acid production was found only in the first stages of the reaction, whereas with NADH substituted for ascorbate, oxygen uptake was in excess throughout. 4. When ascorbate was used, the period of the reaction over which this equivalence was found was decreased at high reaction rates and not observed at all with aged enzyme preparations; equivalence was restored by adding bovine serum albumin to these aged preparations. 5. Equivalence between oxygen consumption and caffeic acid production was observed with NADH, if small quantities of dimethyltetrahydropteridine were also added. 6. It is concluded that hydroxylation proceeds without the concomitant production of caffeic acid only if the enzyme is stabilized for hydroxylation by *p*-coumaric acid and the reductant, and is protected from attack by *o*-quinones.

The enzymic hydroxylation of *p*-coumaric acid to caffeic acid with a phenolase extracted from leaves of spinach beet (*Beta vulgaris* L.) is accompanied by further oxidation of the product by the catechol oxidase activity of the enzyme (Vaughan & Butt, 1969*b*). The hydroxylation reaction requires an electron donor such as ascorbate, which is also utilized in the second reaction in reducing the *o*-quinone that it produces. One molecule of oxygen is consumed in the production of each molecule of caffeic acid, and further oxygen is utilized in its oxidation to the *o*-quinone. The extent of this secondary catechol oxidase activity as the hydroxylation of *p*-coumaric acid proceeds can therefore be assessed by determining the oxygen uptake in excess of that required by hydroxylation, as measured by the amount of caffeic acid produced.

The effects of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (dimethyltetrahydropteridine), ascorbate and NADH as electron donors on the relationship between oxygen consumption and caffeic acid production have been investigated, leading to proposals on the action of these electron donors in the hydroxylation reaction. Conditions for hydroxylation to take place in living cells, in which caffeic acid or its derivatives, such as chlorogenic acid, accumulate without further oxidation are discussed.

Some of this work has been presented briefly by Vaughan & Butt (1969*a*).

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Materials and Methods

The reagents, analytical methods and definitions of enzyme units have been described by Vaughan & Butt (1969*b*). Except where indicated, the enzyme was prepared by the modified method of Vaughan & Butt (1970).

The hydroxylation reaction was followed by measuring the quantity of caffeic acid produced during aerobic incubation at 30°C. It was important to minimize the copper-catalysed autoxidation of ascorbate by the inclusion of EDTA (Butt & Hallaway, 1961) in the incubation mixture. The enzyme was incubated with *p*-coumaric acid (10μmol), EDTA (10μmol), $(NH_4)_2SO_4$ (1.5mmol), Na_2HPO_4 (100μmol)–citric acid (40μmol) buffer, pH5.3, and ascorbate, NADH or dimethyltetrahydropteridine in a total volume of 3.3ml in a shaking incubator under air at 30°C. The reaction was stopped by transferring 0.4ml portions to 0.5ml of 2% (w/v) trichloroacetic acid. The pH was carefully adjusted

to 3.2–3.4 with 3 M-NaOH and the caffeic acid content of the sample determined (Vaughan & Butt, 1969*b*).

Oxygen uptake during hydroxylation was determined with replicate flasks by the standard Warburg manometric technique.

Results

Dimethyltetrahydropteridine used as electron donor

When dimethyltetrahydropteridine was included in the incubation mixture at concentrations up to 1.0 mM, corresponding to 3 μmol in each flask, the amount of caffeic acid produced when the reductant was exhausted (when the flask contents became yellow) was almost exactly equivalent to the amount of electron donor supplied (Fig. 1). There was no indication that any dimethyltetrahydropteridine, unlike ascorbate and NADH (Vaughan & Butt, 1969*b*), had been consumed during catechol oxidase activity. The biphasic reaction course was a characteristic feature of hydroxylation with dimethyltetrahydropteridine and was again observed when oxygen uptake was measured during the hydroxylation of 10 μmol of *p*-coumaric acid with 2 μmol of reductant (Fig. 2). Essentially similar results were obtained with 3 μmol and 5 μmol of dimethyltetrahydropteridine in the reaction mixture. The amount of oxygen consumed was equal to the caffeic acid produced at all stages of the reaction. The addition of more reductant when the initial rapid phase had passed restored the rate of oxygen uptake, with an equivalent production of caffeic acid.

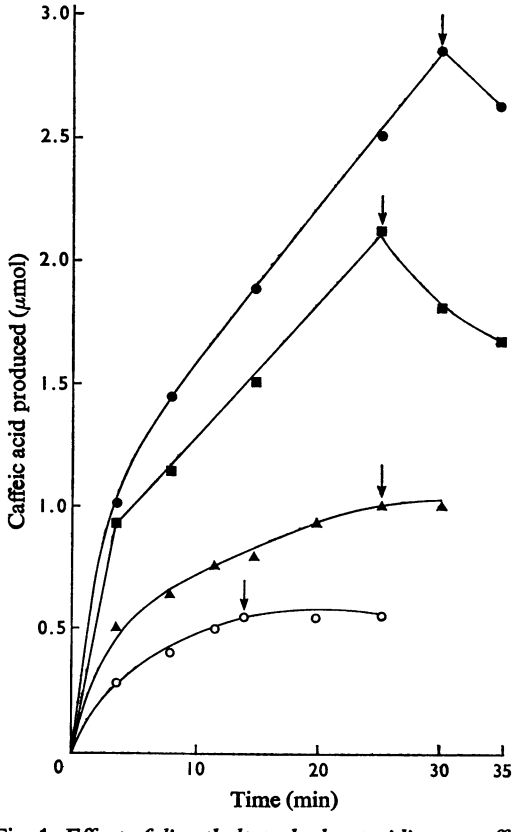

Fig. 1. *Effect of dimethyltetrahydropteridine on caffeic acid production*

Enzyme (93 m-units, purified to stage IV; Vaughan & Butt, 1969*b*) was incubated with *p*-coumaric acid (10 μmol) and 0.5 μmol (○), 1 μmol (▲), 2 μmol (■), or 3 μmol (●) of dimethyltetrahydropteridine 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. The incubation mixtures became yellow where indicated by the arrows. Caffeic acid was determined as described in the Materials and Methods section.

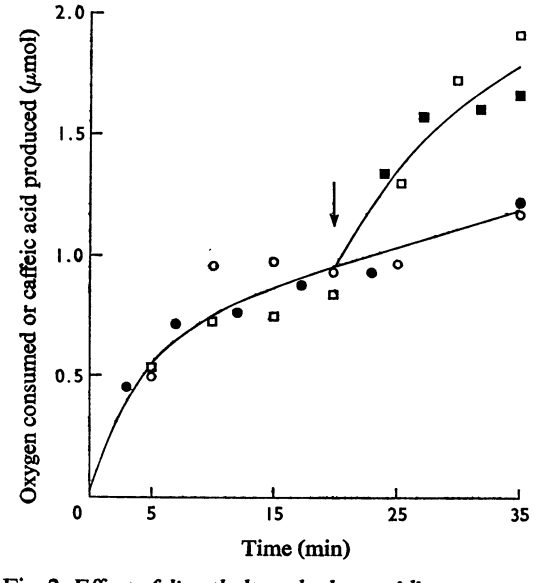

Fig. 2. *Effect of dimethyltetrahydropteridine on oxygen consumption and caffeic acid production*

Enzyme (40 m-units, stage VI; Vaughan & Butt, 1969*b*) was incubated with *p*-coumaric acid (10 μmol) and dimethyltetrahydropteridine (2 μmol) under the conditions described in Fig. 1, and caffeic acid production (●) and oxygen consumption (○) were measured in replicate flasks. A further 2 μmol of pteridine was added, where indicated by the arrow, to replicate flasks in which oxygen consumption (□) was measured, and caffeic acid (■) was determined after addition of further reductant.

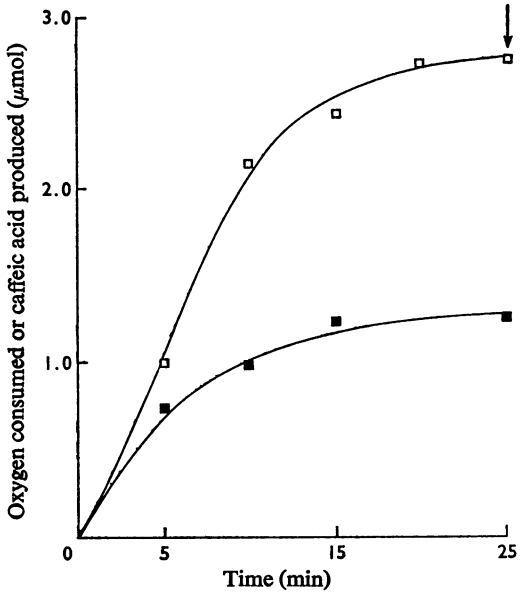

Fig. 3. *Effect of a low p-coumaric acid/dimethyltetrahydropteridine ratio on catechol oxidase activity*

Caffeic acid production (■) and oxygen consumption (□) were measured during incubation of 67m-units of enzyme with *p*-coumaric acid (2μmol) and dimethyltetrahydropteridine (5μmol) under the conditions described in Fig. 1. The incubation mixture became yellow where indicated by the arrow.

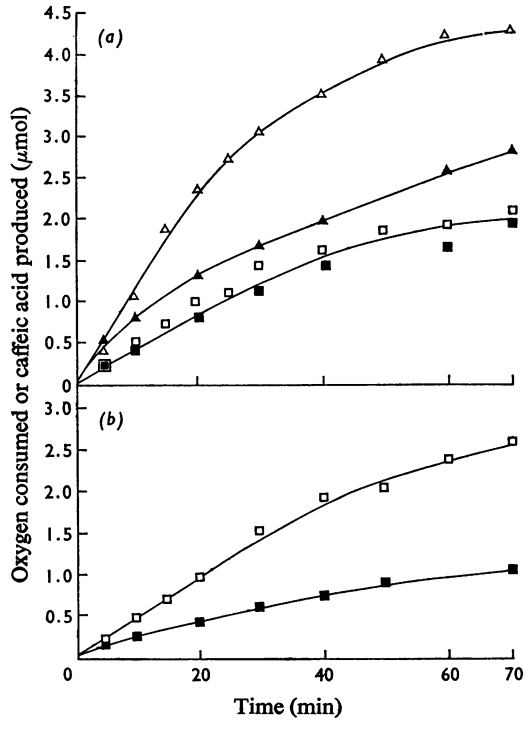

Fig. 4. *Effect of enzyme concentration and age of preparation on catechol oxidase activity during hydroxylation*

(*a*) Caffeic acid production (▲, ■) and oxygen consumption (△, □) when 112m-units (▲, △) and 54m-units (■, □) of freshly prepared enzyme (stage IV; Vaughan & Butt, 1970) were incubated with *p*-coumaric acid (10μmol), ascorbate (10μmol) and EDTA (10μmol) under the conditions described in Fig. 1. (*b*) Caffeic acid production (■) and oxygen consumption (□) during incubation with 54m-units of enzyme, after storage for 14 days at 0°C, under the conditions described for Fig. 4(*a*).

A marked divergence between oxygen consumption and caffeic acid production was observed when subsaturating amounts of *p*-coumaric acid were supplied. Thus, when 5μmol of dimethyltetrahydropteridine was used with only 2μmol of *p*-coumaric acid, the rate of oxygen uptake was in excess of caffeic acid production when not more than 10% of the substrate had reacted (Fig. 3). At the 'yellow point' when the reductant was exhausted (Vaughan & Butt, 1969*b*), 90% of the reductant could be accounted for by the combined hydroxylase and catechol oxidase reactions. The observations suggest that the catechol oxidase activity of the phenolase is suppressed during hydroxylation with dimethyltetrahydropteridine only when saturating concentrations of *p*-coumaric acid are supplied.

Ascorbate used as reductant

The equivalence between the rates of oxygen uptake and caffeic acid production was also dependent on the rate at which hydroxylation progressed, when ascorbate was used as reductant. With 10μmol of ascorbate and 10μmol of *p*-coumaric acid in the presence of 54m-units of enzyme (Vaughan & Butt, 1969*b*), oxygen consumption and caffeic acid production remained equivalent until 2.0μmol of caffeic acid was formed, at which stage the reaction was becoming slower (Fig. 4*a*). By contrast, with 112m-units of enzyme, the rate of caffeic acid production began to fall after no more than 0.5μmol of caffeic acid had been formed, even though oxygen consumption remained more or less constant until 3μmol of oxygen had been consumed.

After storage of the enzyme at 4°C, no equivalence between oxygen consumption and caffeic acid production was observed during the reaction. For

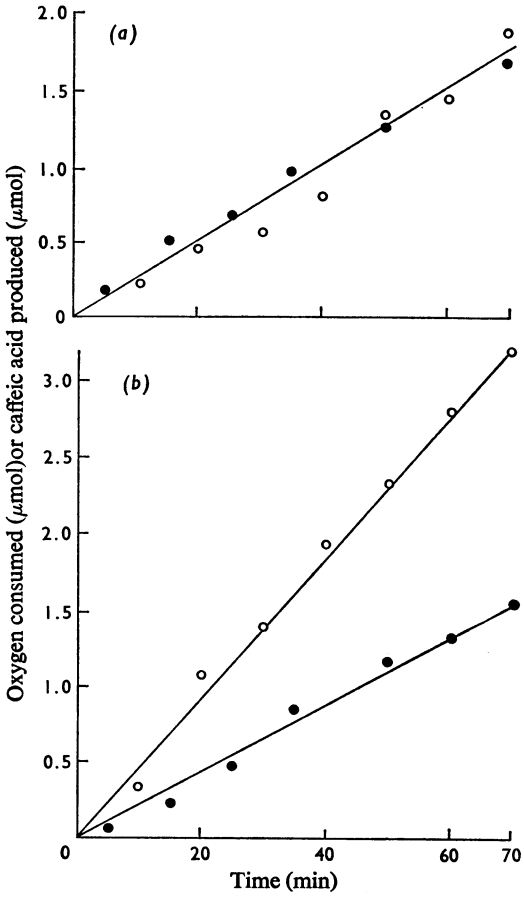

Fig. 5. *Effect of added caffeic acid on oxygen consumption and caffeic acid production during hydroxylation*

Enzyme (23m-units, stage VI; Vaughan & Butt, 1969*b*) was incubated with *p*-coumaric acid (10μmol) under the conditions described in Fig. 4(*a*) and caffeic acid production (●) and oxygen consumption (○) were determined. (*a*) 0, (*b*) 1.0μmol of caffeic acid was added immediately before *p*-coumaric acid.

example, with 54m-units of enzyme, which had been stored in Na_2HPO_4 (0.1M)–citric acid (0.05M) buffer, pH5.3, for 14 days, no change was found in the rate of oxygen uptake even though the rate of caffeic acid production fell by 50% (Fig. 4*b*). Similar declines in caffeic acid production without an equivalent fall in oxygen consumption after storage of the enzyme were observed at pH5.3 with 1.5 and 7μmol of *p*-coumaric acid, and at pH7.3, with 10μmol of *p*-coumaric acid. No evidence of bacterial contamination consequent on storage was found; with all

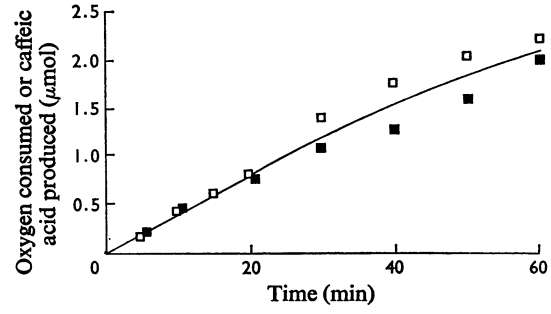

Fig. 6. *Effect of bovine serum albumin on oxygen consumption and caffeic acid production during hydroxylation*

An enzyme preparation (54m-units) that had been stored at 4°C in the refrigerator for 14 days was incubated with *p*-coumaric acid (10μmol) and bovine serum albumin (200μg) under the conditions described in Fig. 4(*a*), and caffeic acid production (●) and oxygen consumption (○) determined.

samples, oxygen uptake was observed only on the addition of substrate.

The maintenance of oxygen consumption at the same rate, even when the rate of caffeic acid production began to fall, suggests the possibility of competition between *p*-coumaric acid and caffeic acid for the enzyme, so that, as the latter was produced, catechol oxidase activity was favoured relative to hydroxylation activity. When 1μmol of caffeic acid was added before *p*-coumaric acid, the oxygen consumption during the subsequent 20min increased from 0.5 to 0.9μmol but with a barely significant fall in caffeic acid production, from 0.50 to 0.44μmol (Fig. 5). Similarly the addition of 0.1 and 0.5μmol of caffeic acid stimulated oxygen consumption in replicate flasks to 0.64 and 0.76μmol respectively during the subsequent 20min; caffeic acid production was unaffected. It therefore seems unlikely that the caffeic acid produced prevents its further formation by a simple competitive mechanism.

The evidence that the equivalence between caffeic acid production and oxygen consumption could be restored when 200μg of bovine serum albumin was added to 54m-units of an aged enzyme preparation (Fig. 6) suggests rather that inactivation of the hydroxylase activity is a consequence of some other reaction product from which the purified enzyme could be protected by the addition of foreign protein.

NADH used as reductant

At all stages of the reaction, oxygen consumption exceeded caffeic acid production, when either 5μmol

or 10 μmol of NADH was supplied as electron donor with 10 μmol of *p*-coumaric acid in the reaction mixture (Fig. 7), even though the amount of enzyme used was similar to that used in the experiments with ascorbate. When 200 μg of bovine serum albumin was included, hydroxylase activity was slightly stimulated, but no equivalence between oxygen consumption and caffeic acid production was observed.

If, however, even relatively small proportions of dimethyltetrahydropteridine were included with NADH, oxygen consumption and caffeic acid production were equivalent both in the presence of 200 μg of bovine serum albumin and in its absence (Fig. 8). Further, although more than 2 μmol of caffeic acid was produced when 1 μmol of pteridine was included with 5 μmol of NADH, hydroxylation ceased when much less caffeic acid had been produced than the total amount of reductant supplied.

Discussion

The relationship between the hydroxylase and catechol oxidase activities of phenolase can be understood from the mechanism for *ortho*-hydroxylation of monophenols originally proposed by Mason (1956). He suggested two stages: an initial activating reaction in which the cupric copper of the enzyme may be reduced to cuprous or the cuprous system changes its oxygenated state (Mason, 1957), followed by either hydroxylation of the monophenol or oxidation of the *o*-dihydric phenol; this latter oxidation represents the basic process for which catechol oxidase activity of the enzyme is essential. The second stage requires some critical special relationship between the copper atoms and the associated oxygen molecule for hydroxylation to take place, whereas the alternative catechol oxidase activity should be less dependent on the precise atomic configuration around the copper atoms. Irrespective of the mechanism, it is assumed that the role of the electron donor is limited to the reduction of the *o*-quinones produced at each stage.

The efficiency with which the *o*-quinones are reduced is likely to determine the effectiveness of the enzyme as a hydroxylase, since these may react directly with thiol and amino groups in protein

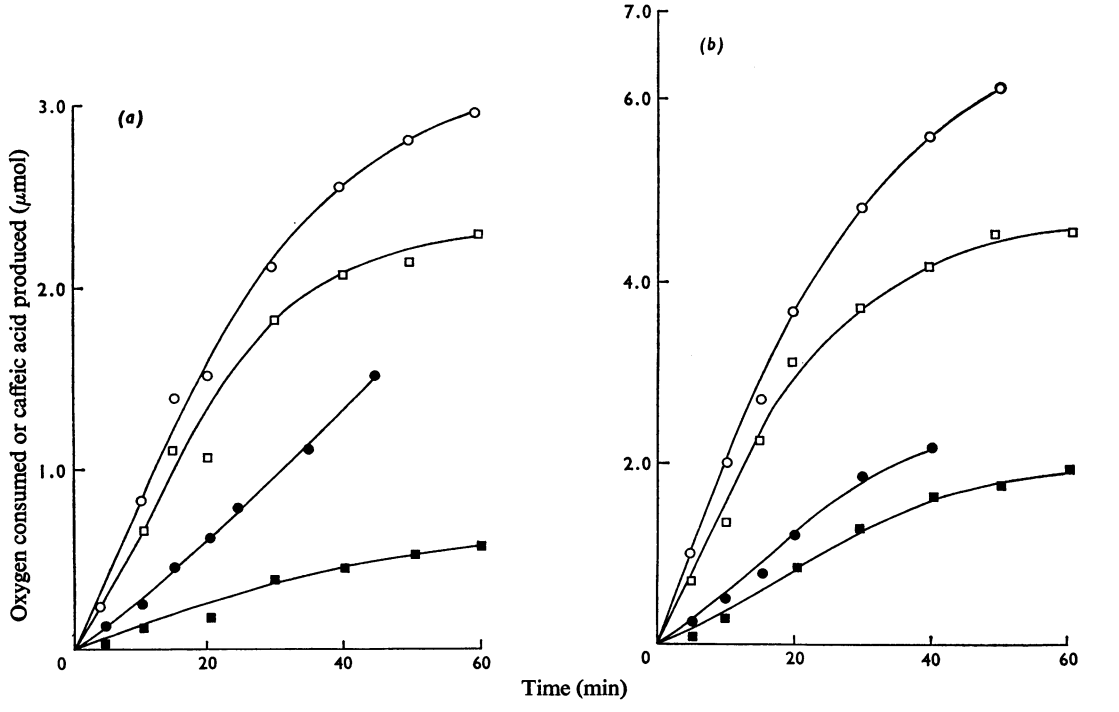

Fig. 7. *Effect of NADH on the relationship between caffeic acid production and oxygen consumption during hydroxylation*

(*a*) Enzyme (54 m-units, stage VI; Vaughan & Butt, 1969*b*) was incubated with *p*-coumaric acid (10 μmol) under the conditions described in Fig. 1 with the substitution of NADH (5 μmol) for dimethyltetrahydropteridine. (*b*) Enzyme (112 m-units) was used with NADH (10 μmol). Oxygen consumption (○, □) and caffeic acid production (●, ■) were measured in the absence (□, ■) and presence (○, ●) of bovine serum albumin (200 μg).

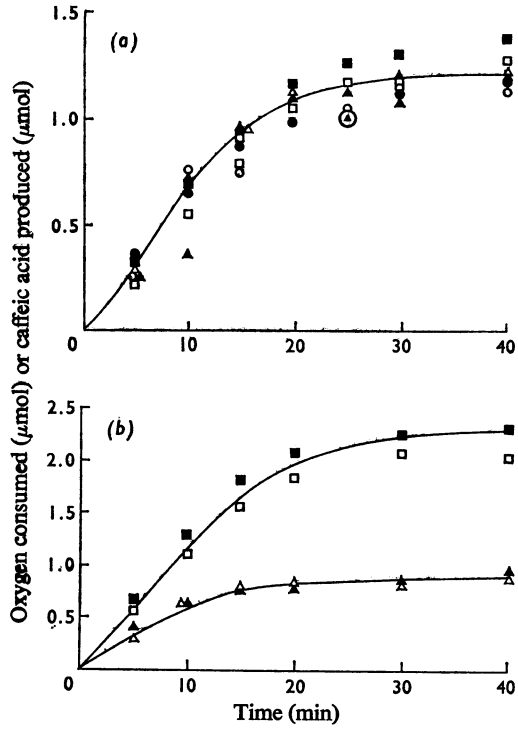

Fig. 8. *Effect of dimethyltetrahydropteridine on the relationship between caffeic acid production and oxygen consumption during hydroxylation, in the presence of NADH*

(*a*) Enzyme (34 m-units, stage VI; Vaughan & Butt, 1969*b*) was incubated with *p*-coumaric acid (10 μmol), dimethyltetrahydropteridine (3 μmol) and 0 (△, ▲), 3 (□, ■) and 10 μmol (○, ●) of NADH. The line is the best fit for the experimental points without NADH. (*b*) Bovine serum albumin (200 μg) was added to the incubation mixture described in (*a*), containing 1 μmol of dimethyltetrahydropteridine, without NADH (△, ▲) and with 5 μmol of NADH added (□, ■). Oxygen consumption (△, □) and caffeic acid production (▲, ■) were determined under the conditions described in Fig. 1 with the exceptions indicated.

(Pierpoint, 1969), and Wood & Ingraham (1965) have demonstrated the covalent labelling of protein by [1-^{14}C]phenol during its hydroxylation with mushroom phenolase, with ascorbate as reductant. The more exacting spatial requirement for hydroxylase activity renders it more sensitive to *o*-quinone attack than is catechol oxidase activity.

The increased catechol oxidase activity observed as hydroxylation progressed was not due to caffeic acid itself, since addition of the product did not inhibit hydroxylation, although competitive effects have been reported with tyrosinase from hamster melanoma (Pomerantz, 1964). It seems more likely that *o*-quinones, accumulating as the concentration of ascorbate or NADH fell, were responsible. The sensitivity of aged preparations may have been a consequence of the exposure of some groups to *o*-quinone attack, from which they could be protected by the provision of alternative groups in foreign protein. At faster reaction rates, achieved with higher enzyme concentrations, the *o*-quinone may have been less efficiently reduced and consequently have inactivated the hydroxylation reaction.

When dimethyltetrahydropteridine was used as reductant, the rate of hydroxylation fell as the reaction progressed, but with no corresponding increase in catechol oxidase activity. The reductant itself appeared to suppress the further oxidation of caffeic acid. The rate of hydroxylation decreased perhaps because the efficiency of *o*-quinone reduction had diminished especially as it was fully restored by the supply of further reductant. The rate of oxidation of catechol has been shown to be independent of the nature of the reductant (Vaughan & Butt, 1970) so that dimethyltetrahydropteridine here appears to exercise an additional function in hydroxylation, perhaps in maintaining the critical spatial relationships. This is effective only when *p*-coumaric acid is present in saturating concentrations.

It is difficult to find supporting evidence for these ideas among the results of earlier workers, most of whom determined hydroxylase (or cresolase) activity only by oxygen uptake in the presence of a monophenol, without measuring the amounts of diphenol produced. For example, both Kendal (1949) and Sato (1969) observed catechol oxidase activity from the earliest stages of the hydroxylation of tyrosine and *p*-coumaric acid respectively, with mushroom phenolase and ascorbate as reductant, but the effects due to the concentration or age of the enzyme preparation cannot be assessed from their data. Kendal (1949), however, reported that during the hydroxylation of several monophenols the production of diphenols was more sensitive to reaction inactivation than oxygen consumption.

The accumulation of caffeic acid or its derivatives, such as chlorogenic acid, is a common feature of many plant organs. The cells and tissues in which they are found are usually rich in phenolases, which convert them rapidly into brown polymeric products when the cells are damaged. This process is restrained in intact cells by the separate location of the phenols in vacuoles away from the cytoplasmic phenolase. Before their transport to the vacuole, however, the dihydric phenols must be maintained in the reduced state either by the continuous use of reductants or by the suppression of catechol oxidase activity. This latter appears to be feasible if relatively high propor-

tions of *p*-coumaric acid are present and tetrahydropteridines available as reductants. The observation that a high proportion of the label from [2-^{14}C]-cinnamic acid applied to leaf discs of spinach beet accumulated in *p*-coumaric acid and ferulic acid, but little in caffeic acid (Vaughan, 1968), and the demonstrations that the phenolase of sugar beet leaves (Mayer & Friend, 1960; Baldry *et al.*, 1970) and tetrahydropteridines in spinach leaves (Nugent *et al.*, 1966; Fujita & Myers, 1966) are both located in the chloroplasts, imply that these conditions may be fulfilled.

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