Biochem. J. (1969) **113**, 109
Printed in Great Britain

The Hydroxylation of *p*-Coumaric Acid by an Enzyme from Leaves of Spinach Beet (*Beta vulgaris* L.)

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(*Received* 22 *January* 1969)

1. An enzyme from the leaves of spinach beet (*Beta vulgaris* L.) that catalyses the hydroxylation of *p*-coumaric acid to caffeic acid in the presence of ascorbate has been purified about 1000-fold on a protein basis. 2. It is activated by high concentrations of ammonium sulphate and sodium chloride. 3. The preparation shows both hydroxylase and catechol oxidase activities, in a constant ratio throughout the purification procedure; they are similarly activated by salts. 4. Ascorbate acts as a reductant in quantities equivalent to the caffeic acid produced by hydroxylation. 5. Ascorbate can be replaced by tetrahydrofolic acid, NADH, NADPH or 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, but not by caffeic acid. Among these, the pteridine is the most effective, but the reaction is not inhibited by aminopterin. In experiments with saturating concentrations of NADH and the pteridine, these reductants compete in the reaction and are equivalent on a molar basis. 6. No cofactor has been separated from the enzyme by prolonged dialysis. 7. The relation of the enzyme to other hydroxylases and phenolases is discussed.

The hydroxylation of *p*-coumaric acid (4-hydroxycinnamic acid) to caffeic acid (3,4-dihydroxycinnamic acid) is a central reaction in the metabolism of phenolic substances in plants. This has been established by the use of ^{14}C-labelled intermediates in the synthesis of lignin (Brown, 1966), coumarins and flavonoids (Neish, 1964), and chlorogenic acid (Steck, 1968).

These observations have so far stimulated no more than limited studies with isolated enzymes. Patil & Zucker (1965) demonstrated that potato phenolase (EC 1.10.3.1) catalysed the hydroxylation, and Sato (1966) reported the reaction with chloroplast preparations.

In the work reported here, a phenolase from leaves of spinach beet (*Beta vulgaris* L.) was purified and some properties of the enzymic hydroxylation were studied. The hydroxylation of monohydric phenols to *o*-dihydric phenols has been reported with phenolase preparations from many sources (Mason, 1957), but much of the work has been difficult to interpret because of the dual function of these enzymes, which catalyse both hydroxylation and the further oxidation of the *o*-dihydric phenols to their *o*-quinones (catechol oxidase activity). Both reactions consume molecular oxygen. Ascorbate is normally used as a reductant in the hydroxylation, but also reduces the *o*-quinone subsequently produced. Unless the contribution due to catechol oxidase activity can be determined, estimates of hydroxylation activity based on the utilization of either molecular oxygen or ascorbate can be no more than semiquantitative. The accumulation of caffeic acid, when excess of reductant and molecular oxygen are available, is used in this paper to give precise measurements of the hydroxylation activity of the enzyme.

Part of this work was presented briefly by Vaughan & Butt (1967).

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METHODS AND MATERIALS

Reagents

p-Coumaric acid and caffeic acid (Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were recrystallized from aq. ethanol. Catechol (British Drug Houses Ltd., Poole, Dorset) was crystallized at least twice from toluene. Ascorbic acid (Biochemical grade; British Drug Houses Ltd.) was used without further purification.

2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (dimethyltetrahydropteridine; California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) was stored at $-10°$ under an atmosphere of N_2. Aminopterin (4-aminopteroylglutamic acid), NADH, NADPH and tetrahydrofolic acid were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6.

The purest reagents available and deionized water were used in all solutions to decrease the concentration of contaminant copper to the minimum.

Analytical methods

Determination of caffeic acid. Samples (2·0 ml.), containing up to 1 μmole of caffeic acid in aqueous solution at pH 3·2–3·4 (adjusted with NaOH or HCl as necessary), were treated with 2 ml. of 5% (v/v) acetic acid and 2 ml. of 0·5% (w/v) $NaNO_2$. After the solution had stood at room temperature for 5 min., the red colour developed was measured in a Beckman DB spectrophotometer at 525 nm. by a modification of the method of Hoepfner (1932). A linear relationship between extinction and caffeic acid concentration was observed over this range, and each batch of measurements was accompanied by a standard sample of 0·8 μmole of caffeic acid.

Determination of p-*coumaric acid.* Standard samples (0·3 ml.), containing up to 0·5 μmole of *p*-coumaric acid, were added to 3·0 ml. of water and 1·0 ml. of $HgSO_4$ reagent (prepared by filtering a solution of 30 g. of $HgSO_4$ in 200 ml. of 2·5 M-H_2SO_4) and heated on a boiling-water bath for 10 min. After 1 hr. at room temperature, 0·8 ml. of 0·5% (w/v) $NaNO_2$ was added and, after 20 min., the deep-pink colour was measured at 490 nm. Over this range a linear relationship between extinction and *p*-coumaric acid content was observed when the method of Lugg (1937), modified by Krueger (1950) for tyrosine, was used.

When *p*-coumaric acid was determined after a period of incubation with the enzyme, heating led to the formation of a precipitate, which was removed by centrifugation. The precipitate was heated further with 1·0 ml. of water and 1·0 ml. of $HgSO_4$ reagent on a boiling-water bath for 10 min., and again centrifuged. The supernatants from each centrifugation were combined before the addition of $NaNO_2$ in the determination procedure.

Determination of protein. During purification of the enzyme, protein was estimated spectrophotometrically at 260 and 280 nm. by using the data of Warburg & Christian (1941).

Determination of oxygen uptake. Standard Warburg manometric technique was employed to measure O_2 uptake. Flasks contained 3 ml. of liquid under air at 30°.

Assays of enzyme activity. (*a*) Hydroxylation activity was assayed by measuring the quantity of either caffeic acid produced or *p*-coumaric acid consumed during aerobic incubation at 30°.

The enzyme was incubated with *p*-coumaric acid (10 μmoles), ascorbate (10 μmoles), $(NH_4)_2SO_4$ (1·5 m-moles) and Na_2HPO_4 (100 μmoles)–citric acid (40 μmoles) buffer, pH 5·3, in a total volume of 3 ml. in a shaking incubator under air at 30°. The reaction was stopped at 0, 10 or 20 min. after the addition of *p*-coumaric acid by transferring the contents of each flask to 0·5 ml. of 10% (w/v) trichloroacetic acid in a centrifuge tube. The pH of each tube was carefully adjusted to 3·2–3·4 with 3 M-NaOH, and any precipitate was removed by centrifugation for 10 min. The caffeic acid content of the supernatant fluid was determined.

In assays of the fractions from DEAE- and CM-cellulose columns, a final volume of 3·3 ml. of incubation mixture was used, from which 0·4 ml. portions were each transferred to 0·5 ml. of 2% (w/v) trichloroacetic acid. The caffeic acid content was determined by using 1 ml. of the sample and 2 ml. of acetic acid–$NaNO_2$ mixture.

One unit of hydroxylation activity is defined as the quantity that catalyses the production of 1 μmole of caffeic acid or the utilization of 1 μmole of *p*-coumaric acid/min. under these conditions.

(*b*) Catechol oxidase activity was measured by the O_2 uptake of a 3 ml. incubation mixture containing the enzyme with catechol (0·75 μmole), ascorbate (10 μmoles), $(NH_4)_2SO_4$ (1·5 m-moles) and Na_2HPO_4 (100 μmoles)–citric acid (40 μmoles) buffer, pH 5·3, at 30° under air. The O_2 uptake was linear with time.

One unit of catechol oxidase is defined as the quantity that catalyses the uptake of 1 μmole of O_2/min. under these conditions.

Preparation of the enzyme

The enzyme was prepared from freshly gathered leaves of spinach beet (*Beta vulgaris* L.). About 180 g. of leaf material was obtained after 150 leaves had been washed thoroughly with distilled water and the petiole and main veins had been removed. All subsequent operations were carried out at temperatures below 5°.

Stage I: extraction of crude enzyme. The leaves were macerated in three portions, each with 300 ml. of 10 mM-Na_2HPO_4–5 mM-citric acid buffer, pH 5·3, in a Waring Blendor for about 2 min. The macerated mixtures were combined and squeezed through cheesecloth. The liquid was centrifuged at 2000 *g* in an MSE Mistral 4L refrigerated centrifuge for 90 sec. The supernatant was diluted, when necessary, to give a protein concentration below 60 mg./ml., and solid ascorbic acid was added to a final concentration of 10 mM.

Stage II: fractionation with ammonium sulphate. Solid $(NH_4)_2SO_4$ (analytical grade; 210 g./l.) was added in small amounts to the stirred solution to give a 35% saturated solution; drops of 3 M-NaOH were added as necessary to maintain pH 5·3. The solution was equilibrated for 30 min. with occasional stirring and then centrifuged at 10000 *g* for 15 min. in a Servall Superspeed refrigerated centrifuge. Further solid $(NH_4)_2SO_4$ was added carefully to the supernatant (230 g./l.) to obtain 70% saturation, the pH being checked and adjusted as above, and the solution was finally left for 30 min. before centrifugation at 10000 *g* for 15 min. The precipitate was suspended in 30 ml. of Na_2HPO_4–NaH_2PO_4 buffer (10 mM with respect to phosphate), pH 7·3.

Stage III: heat treatment and dialysis. The resuspended precipitate from $(NH_4)_2SO_4$ fractionation was heated at 60° for 10 min. It was then centrifuged at 10000 *g* for 10 min., and the supernatant was dialysed successively against deionized water (2 l.) for 2 hr., and Na_2HPO_4–NaH_2PO_4 buffer (1 mM with respect to phosphate), pH 7·3 (2 l.), for 3 hr.

Stage IV: ion-exchange chromatography on DEAE-cellulose. The dark-brown solution was passed through a column (2 cm. × 25 cm.) of DEAE-cellulose (Whatman DE-22), which had been pretreated in accordance with the manufacturer's instructions and equilibrated with Na_2HPO_4–NaH_2PO_4 buffer (1 mM with respect to phosphate), pH 7·3. The column was washed with 1·5 vol. of this buffer, and the proteins were then eluted with a linear gradient of 50 mM-Na_2HPO_4–25 mM-citric acid and 0·15 M-Na_2HPO_4–75 mM-citric acid, pH 5·3. Fractions (5 ml.) were collected automatically. Some of the brown colour was eluted in the first inactive fractions, but the active fractions were pale yellow.

Stage V: precipitation with ammonium sulphate. The active fractions from the DEAE-cellulose columns were combined and solid $(NH_4)_2SO_4$ was added (650 g./l.) to 90% saturation. After the precipitate had equilibrated for 30 min., it was centrifuged at 10000 g for 15 min., dissolved in 10 ml. of 1 mM-Na_2HPO_4–5 mM-citric acid buffer, pH 5·3 (10 ml.), and finally dialysed against this buffer for 5 hr.

Stage VI: ion-exchange chromatography on CM-cellulose. CM-cellulose (Whatman CM-22) was pretreated with alkali and acid in accordance with the manufacturer's instructions, equilibrated with 1 mM-Na_2HPO_4–5 mM-citric acid buffer, pH 5·3, and packed in a column (1·0 cm. × 20 cm.). The enzyme solution was passed through the column, followed by 2 vol. of 0·1 M-Na_2HPO_4–50 mM-citric acid buffer, pH 5·3, with a linear increase in NaCl concentration to 1·5 M. The yellow colour was eluted before the application of NaCl, and the active extract, collected in fractions (3·0 ml.), was colourless.

RESULTS

Table 1 summarizes the stages in the purification of the enzyme. The final purification achieved was greater than 1000-fold, although the recovery was less than 10% of the total activity of the original homogenate. This increase in specific activity was achieved with an equivalent increase in the specific activity of catechol oxidase. The ratio of hydroxylase to catechol oxidase activities remained virtually unchanged at each stage, so that both activities appear to be components of the same protein.

Unless attention was paid to the operational details of stage II, results were variable. If the enzyme was to be precipitated by ammonium sulphate concentrations above 35% saturation, an equilibration period with 35% saturated ammonium sulphate was essential to release the enzyme from some bound form that was otherwise sedimented. The precipitate from lower concentrations of ammonium sulphate contains the crude enzyme, heavily contaminated with particles. The equilibrium procedure released the enzyme, but only rarely was more than 50% of the homogenate activity recovered.

Considerable activity was lost after dialysis at stage III, unless salts were added to the assay system. Fig. 1 shows the effect of adding ammonium sulphate or sodium chloride after dialysis. Maximum activity was observed with a 0·5 M concentration of either salt; 0·5 M-ammonium sulphate was therefore included in the assay mixture. Hydroxylase and catechol oxidase activities showed similar proportionate changes over the range of concentrations examined. The addition of ammonium sulphate to a boiled enzyme preparation did not induce any hydroxylation.

Requirement for ascorbate in hydroxylation. With ascorbate in the reaction mixture, the hydroxylation of *p*-coumaric acid proceeded with an equivalent production of caffeic acid (Fig. 2) until the reaction mixture suddenly developed a yellow colour. Caffeic acid then began to disappear, despite some further conversion of *p*-coumaric acid. The addition of a further 10 μmoles of ascorbate as soon as the mixture became yellow initiated the resumption of hydroxylation at the original rate, suggesting that the yellowing was due to reactions following the exhaustion of the ascorbate supply. Hydroxylation was found not to proceed in the absence of ascorbate, for which small quantities of caffeic acid could not substitute (cf. Bordner & Nelson, 1939; Gregg & Nelson, 1940).

If it is assumed that the ascorbate has been exhausted at the yellowing point by the combined hydroxylase and catechol oxidase activities of the

Table 1. *Purification of phenolase from the leaves of spinach beet*

The units of activity and details of the purification procedure are given in the Methods section.

Stage	Total volume (ml.)	Activity (m-units/ml.)		Protein content (mg./ml.)	Specific activity (m-units/mg. of protein)		Ratio of activities (hydroxylase/catechol oxidase)	Recovery (% of activity in crude homogenate)
		Hydroxylase	Catechol oxidase		Hydroxylase	Catechol oxidase		
I. Crude homogenate	1200	28	90	41	0·68	2·2	0·31	[100]
II. Precipitate from 35–70%-satd. $(NH_4)_2SO_4$	40	367	1170	58	6·4	20	0·31	44
III. Heat treatment and dialysis	61	200	1270	8	25	158	0·16	36
IV. Eluate from DEAE-cellulose	140	87	310	1·2	73	258	0·28	36
V. $(NH_4)_2SO_4$ precipitate after dialysis	22	400	1400	3·6	110	390	0·29	26
VI. Eluate from CM-cellulose	14·8	160	430	0·22	730	1950	0·37	7

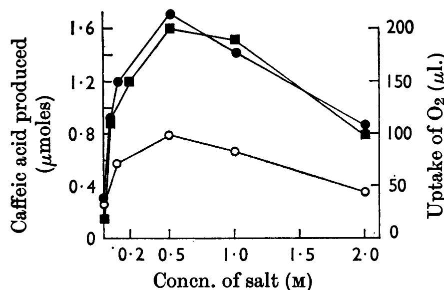


Fig. 1. Effect of $(NH_4)_2SO_4$ and NaCl on the hydroxylation and catechol oxidase activity of the purified enzyme after stage III. Hydroxylation activity was measured by incubating the enzyme (567m-units) with 10μmoles of p-coumaric acid and 10μmoles of ascorbate in 10mM-Na_2HPO_4–5mM-citric acid buffer, pH 5·3, for 30min. at 30° in air, with various concentrations of $(NH_4)_2SO_4$ (●) or NaCl (○). The total volume was 3·0ml. Catechol oxidase activity was measured by incubating the enzyme with 0·75μmoles of catechol and 10μmoles of ascorbate in the buffer for 30min. with various concentrations of $(NH_4)_2SO_4$ (■).

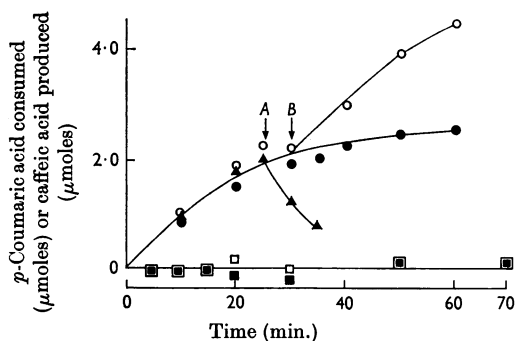


Fig. 2. Equivalence between the utilization of p-coumaric acid and caffeic acid production, and the requirement for ascorbate in the reaction. p-Coumaric acid (5μmoles) was incubated with the enzyme (90m-units), $(NH_4)_2SO_4$ (1·5m-moles), ascorbate and buffer as in Fig. 1. ●, p-Coumaric acid consumption; ▲, caffeic acid production. The incubation mixture became yellow at the arrow (*A*), and in a replicate vessel, in which only the consumption of p-coumaric acid was measured (○), a further 10μmoles of ascorbate was added at the arrow (*B*). p-Coumaric acid consumption in the absence of ascorbate (□) and when ascorbate was replaced by 1μmole of caffeic acid (■) was determined in replicate vessels.

enzyme, the oxygen consumed at that stage of the reaction can also be accounted for by the two reactions. If only one molecule of oxygen is used in the hydroxylation of each molecule of p-coumaric acid (Mason, 1957), the oxygen consumed in hydroxylation is given by the caffeic acid formed. Any excess is due to catechol oxidase activity and accounted for by an equivalent utilization of ascorbate. With these assumptions, Table 2 apportions the ascorbate used at the yellowing point between the hydroxylation and catechol oxidase reactions. With a range of ascorbate concentrations, the ascorbate used in hydroxylation was always equivalent on a molar basis to the caffeic acid formed.

Effects of various reductants. A number of other reductants were also found effective in the hydroxylation reaction as substitutes for ascorbate (Table 3). Among these, dimethyltetrahydropteridine was found to be more effective than ascorbate at all concentrations studied, and only NADPH was generally less active.

The effect of a range of concentrations of dimethyltetrahydropteridine and NADH was examined (Fig. 3). The maximum rate was much higher with dimethyltetrahydropteridine than with NADH, and was achieved at a lower concentration of reductant.

The difference between these two reductants was less marked when the time-course of hydroxylation was followed (Fig. 4). The 5min. values used in Fig. 3 include lag periods, which were much greater with NADH than with dimethyltetrahydropteridine. Nevertheless, the greater rate was observed with dimethyltetrahydropteridine; this was not further increased when NADH was included in the reaction mixture, but in this case it persisted even after dimethyltetrahydropteridine, when supplied alone, had been exhausted. The effectivenesses of NADH and dimethyltetrahydropteridine when supplied together were clearly not additive.

The coupled effects of these two reagents were examined with 1μmole of dimethyltetrahydropteridine and a fivefold range of NADH concentration to determine how far effects other than their reducing action might be involved. By using measurements and assumptions identical with those for Table 2, it was clear that the total reductant necessary to account for the oxygen consumption of the combined hydroxylase and catechol oxidase activities when the mixture became yellow was almost equal to that supplied, especially at the lower concentrations of NADH. A serious discrepancy appeared only when NADH was considerably in excess (Table 4).

Although these results imply that the reductants act on some common site on the enzyme, prolonged dialysis failed to remove from the enzyme any cofactor with which they might react. After dialysis for 15hr. at 4° against 40mM-Na_2HPO_4–20mM-citric acid buffer, pH 5·3, the enzyme produced 1·74μmoles and 1·54μmoles of caffeic acid from 10μmoles of p-coumaric acid in 20min. under

Table 2. *Caffeic acid production and ascorbate utilization in the hydroxylation of* p-*coumaric acid*

p-Coumaric acid (10μmoles) was incubated with various concentrations of ascorbate in 0·5M-$(NH_4)_2SO_4$–0·1M-Na_2HPO_4–50mM-citric acid buffer, pH 5·3, in a total volume of 3·0ml., with the enzyme in air at 30°, until the reaction mixture became yellow.

A Ascorbate supplied (μmoles)	*B* O_2 consumed at yellowing point (μmoles)	*C* Caffeic acid produced (μmoles)	*D* O_2 uptake not accounted for by *C* (*B*−*C*) (μmoles)	*E* Ascorbate equivalent to *D* (μmoles)	*F* Ascorbate taking part in hydroxylation (*A*−*E*) (μmoles)
5·0	3·2	1·8	1·4	2·8	2·2
2·5	1·8	1·2	0·6	1·2	1·3
1·5	1·1	0·7	0·4	0·8	0·7
1·0	0·8	0·5	0·3	0·6	0·4

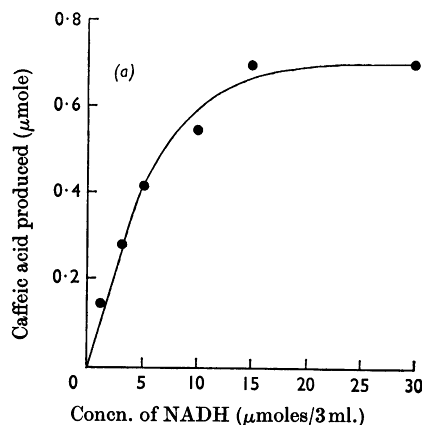
Table 3. *Effectiveness of reductants in the hydroxylation of* p-*coumaric acid*

Incubation conditions were as described in Table 2, with the substitution of various quantities of other reductants for ascorbate in a total volume of 3ml. The rates of reaction were determined as μmoles of caffeic acid produced/enzyme unit in 30min., and are expressed relative to the rate obtained with 10μmoles of ascorbate.

Reductant	Relative rate of reaction — Concn. of reductant (μmoles/3ml.) 20	10	5	1
Ascorbate	—	1·0	1·0	0·7
Dimethyltetrahydropteridine	—	2·1	2·4	1·5
Tetrahydrofolic acid	—	—	—	0·7
NADH	1·6	1·1	0·9	0·3
NADPH	—	—	0·6	0·3

the standard conditions with 3μmoles of dimethyltetrahydropteridine and 20μmoles of NADH respectively; after the enzyme had been stored at 4° for 15hr. without dialysis, 1·88μmoles and 1·49μmoles of caffeic acid were produced.

Unlike the hydroxylases for phenylalanine (Nair & Vining, 1965*b*) and cinnamic acid (Nair & Vining, 1965*a*), the hydroxylation of *p*-coumaric acid was not inhibited by aminopterin. With either 10μmoles of ascorbate or 10μmoles of ascorbate with 1μmole of dimethyltetrahydropteridine as reductant, 1mM-aminopterin stimulated the production of caffeic acid by nearly 50%, but when 1μmole of dimethyltetrahydropteridine alone was used aminopterin had no effect and no inhibition was observed at any stage of the reaction.



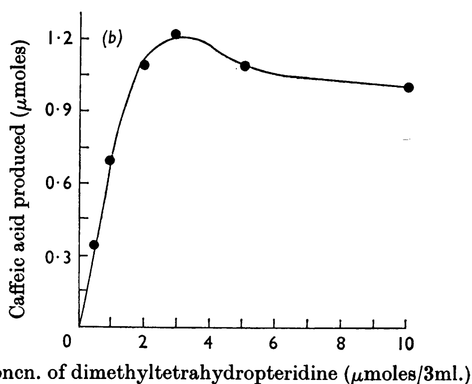


Fig. 3. Effect of the concentration of reductants on the rate of caffeic acid production. The enzyme (93m-units) was incubated for 5min. with *p*-coumaric acid (10μmoles) and various concentrations of (*a*) NADH or (*b*) dimethyltetrahydropteridine, instead of ascorbate, under the conditions given in Fig. 2.

DISCUSSION

Most plant and mammalian enzymes used in the study of the *ortho*-hydroxylation of monohydric phenols also show catechol oxidase activity. The two activities appear to be functions of the same enzyme, although certain *o*-diphenol oxidases, such as those from tea (Gregory & Bendall, 1966), sweet potato (Eiger & Dawson, 1949) and tobacco (Clayton, 1959), show no capacity to catalyse the hydroxylation. However, preparations capable of

catalysing the hydroxylation only, such as those from brain and adrenal medulla of a number of mammals (Nagatsu, Levitt & Udenfriend, 1964) and from *Pseudomonas putida* (Hosokawa & Stanier, 1966), have been reported. The enzyme reported here appears to fall in the first-mentioned class for, throughout the purification procedure, the ratio of hydroxylase activity to catechol oxidase activity remained virtually constant. They were similarly affected by the range of ammonium sulphate concentrations used, and both required ascorbate. No significant separation or differential inactivation of the two reactions was achieved.

Ascorbate has been most frequently used as the electron donor in the study of this class of hydroxylases. The equivalence between ascorbate consumption and caffeic acid production suggests that the enzyme acts as a mixed-function oxidase, in which molecular oxygen is used equally in the hydroxylation reaction and the oxidation of the reductants (Mason, Fowlks & Peterson, 1955). Certain phenolase preparations have been reported to require no accessory electron donor; the hydroxylation commenced after a lag period, which was shortened by ascorbate or substituted catechols. In the hydroxylation of *p*-coumaric acid, no reaction was observed without ascorbate over a prolonged period even when caffeic acid was added, but the ascorbate requirement could be satisfied by a number of reductants.

These electron donors could be effective in the reduction of an ion or cofactor bound to the enzyme. This appears likely, since saturating concentrations of dimethyltetrahydropteridine and NADH show no additive effect, and the reaction proceeds at the rate of the more effective reductant. The differences in the activities of the different reductants are insufficient to suggest that any particular one is more likely to be the natural reductant, but they have sufficiently different structures to suggest that their action is probably non-enzymic. Nevertheless, no diffusible cofactor could be removed from the purified enzyme by dialysis.

Although dimethyltetrahydropteridine is the most effective of the reductants, it is unlikely to act in the same way as in phenylalanine hydroxylase (Kaufman, 1959), for which a catalytic amount of the oxidized reductant was reduced by NADPH through the action of an associated enzyme. This

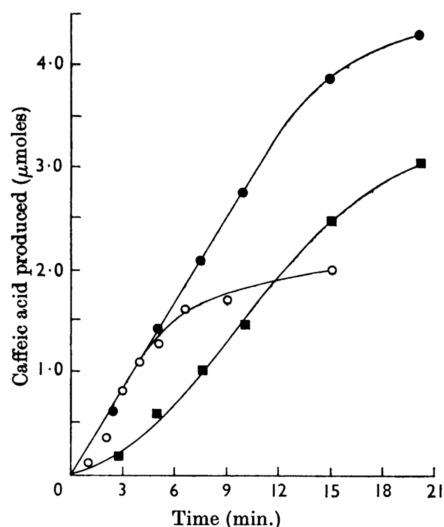


Fig. 4. Effect of saturating concentrations of NADH and dimethyltetrahydropteridine, separately and together, on the rate of caffeic acid production. The enzyme (93 m-units) was incubated with *p*-coumaric acid (10 μmoles) and either 20 μmoles of NADH (■) or 3 μmoles of dimethyltetrahydropteridine (○), or 20 μmoles of NADH and 3 μmoles of dimethyltetrahydropteridine together (●), instead of ascorbate, under the conditions given in Fig. 2.

Table 4. *Relationship between caffeic acid produced and supply of reductant in the hydroxylation of* p-*coumaric acid*

Incubation conditions were as in Table 2, with 1 μmole of dimethyltetrahydropteridine and various quantities of NADH as reductant in a total volume of 3 ml. until the reaction mixture became yellow. In column *C*, 1 μmole of O_2, and in column *E*, 1 μmole of reductant is assumed to be consumed for each μmole of caffeic acid formed.

Pteridine supplied (μmole)	NADH supplied (μmoles)	*A* O_2 consumed at yellowing point (μmoles)	*B* Caffeic acid produced (μmoles)	*C* O_2 uptake not accounted for by *B* (*B*−*A*) (μmoles)	*D* Reductant equivalent to *C* (μmoles)	*E* Total reductant consumed (*B*+*D*) (μmoles)
1·0	2·0	2·5	1·7	0·8	1·6	3·3
1·0	3·0	3·2	2·2	1·0	2·0	4·2
1·0	4·0	3·4	2·2	1·2	2·4	4·6
1·0	5·0	3·8	2·5	1·3	2·6	5·1
1·0	10·0	6·5	3·5	3·0	6·0	9·5

hydroxylase and the tyrosine hydroxylase from brain and adrenal medulla (Nagatsu *et al.* 1964) and plant preparations catalysing the hydroxylation of phenylalanine and cinnamic acid (Nair & Vining, 1965*a*,*b*) were severely inhibited by aminopterin, which was ineffective on the *p*-coumaric acid hydroxylase described here. Pomerantz (1966) also found this pteridine to be more effective than ascorbate in the hydroxylation of tyrosine with mammalian tyrosinase.

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

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