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CINNAMIC ACID HYDROXYLASE IN SPINACH*

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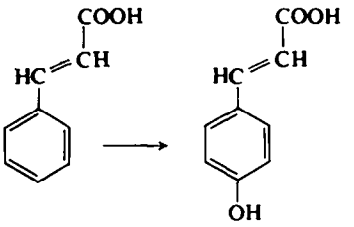
Abstract—An acetone precipitate from an extract of spinach leaves catalysed the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid. The enzyme was unstable and could not be purified. Crude preparations had a pH optimum of 4·6 and showed an absolute requirement for an external electron donor. Tetrahydrofolic acid and a reduced pyridine nucleotide coenzyme were necessary for maximum activity. Aminopterin was a potent inhibitor of the hydroxylating system. No requirement for metal ions could be demonstrated but inhibition by *p*-chloromercuribenzoate suggests that a sulfhydryl group participates in the reaction. These properties, and the course of the reaction when cofactors were added separately, indicate a similarity between this enzyme and the phenylalanine hydroxylase of mammalian liver. The crude spinach preparation also catalysed the conversion of L-phenylalanine to tyrosine, and addition of L-phenylalanine inhibited cinnamic acid hydroxylase activity.

INTRODUCTION

THE higher plants form a variety of hydroxylated cinnamic acid derivatives, and biosynthetically related compounds such as flavonoids and lignin are widely distributed in the plant kingdom. Where the manner of their formation has been studied it has been established that L-phenylalanine is an important intermediate.[1,2] This amino acid is converted to *trans*-cinnamic acid, probably by the action of the enzyme phenylalanine ammonia lyase,[3] then hydroxylated and, where necessary, substituted in an appropriate manner. A less prevalent route involves the enzyme tyrosine ammonia lyase which converts L-tyrosine directly to *trans-p*-coumaric acid.[4]

Although the hydroxylation of cinnamic acid, either as the free acid or in a bound form, is an important step in the biosynthetic pathway to phenolic compounds derived from phenylalanine little is yet known about the enzymes involved. This paper reports the presence, in an acetone precipitate of an extract from spinach leaves, of an enzyme which will catalyse the conversion of *trans*-cinnamic acid to *p*-coumaric acid (Fig. 1) when supplemented with an external electron donor.



Cinnamic acid *p*-Coumaric acid

FIG. 1. REACTION CATALYSED BY CINNAMIC ACID HYDROXYLASE.

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[1] A. C. NEISH, *Ann. Rev. Plant Physiol.* **11**, 55 (1960).
[2] M. N. ZENK, *Z. Naturforsch* **196**, 83 (1964).
[3] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[4] A. C. NEISH, *Phytochem.* **1**, 1 (1961).

RESULTS AND DISCUSSION

The ability of spinach leaf extracts to catalyse ring hydroxylation of *trans*-cinnamic acid was assessed by testing the reaction mixture for the presence of phenols using a modification of the colorimetric method of Booth and Boyland.[5] The mixture was incubated aerobically at 30°. A reduced pyridine nucleotide coenzyme was included because of the known requirement of mixed function oxidases for an external electron donor.[6] An acetone precipitable fraction from an aqueous extract of spinach leaves was found to be active, and retained its activity for at least two months on storage at −20°. When the powder was suspended in

TABLE 1. EFFECT OF COFACTORS ON THE HYDROXYLATION OF CINNAMIC ACID

Supplement*	*p*-Coumaric acid formed (μmoles)
None	0
NADH	0·300
NADPH	0·300
THFA	0·210
Ascorbic acid	0
FAD	0
NADH + THFA	0·540
NADH + ascorbic acid	0·390
NADH + FAD	0·327
THFA + ascorbic acid	0·225
NADH + THFA + ascorbic acid	0·570

* The reaction mixture contained citrate-phosphate buffer pH 4·6 (1·9 ml); cinnamic acid (1·0 μmole); enzyme extract (1·0 ml); cofactor(s); redistilled water to 3·0 ml. Cofactors were added in the following amounts: NADH, NADPH, FAD, ascorbic acid, 0·5 μmoles; THFA, 0·2 μmoles. The mixture was incubated at 30° for 20 min.

water or buffers, however, enzyme activity was rapidly lost. Addition of glutathione, ethylenediamine tetraacetic acid, or ascorbic acid failed to stabilize it. Since attempts to purify the preparation were unsuccessful and little activity remained when the aqueous suspension was kept for 1 hr at 0–5°, or even after centrifugation in the cold to remove cell debris, experiments were carried out with aqueous suspension of the acetone powder.

By paper chromatography of the reaction mixture when cinnamic acid-α-^{14}C was used as substrate it was ascertained that a single product was formed. This was identified as *p*-coumaric acid.

The requirement of the system for an external electron donor was absolute (Table 1). Reduced nicotinamide adenine dinucleotide (NADH) or its 2′-phosphate (NADPH) were equally effective, tetrahydrofolic acid (THFA) somewhat less so. Ascorbic acid was ineffective, but a combination of NADH with ascorbic acid resulted in an increased rate of reaction. This latter effect might result from the increased availability of NADH which would occur if

[5] J. BOOTH and E. BOYLAND, *Biochem. J.* **66**, 73 (1957).
[6] H. S. MASON, *Adv. Enzymol.* **19**, 79 (1957).

ascorbic acid were inhibiting other NADH requiring oxidases.[7] Addition of ascorbic acid with THFA had little effect. FAD, which has been reported to serve with NADH as a cofactor in the hydroxylation and decarboxylation of salicylic acid,[8] was without action.

Increasing the concentration of NADH in the reaction mixture beyond 0·17 mM did not increase the reaction rate, but THFA at 0·068 mM combined with 0·17 mM NADH caused a marked stimulation. This cofactor supplement was adopted in the standard assay procedure. A similar result was obtained by Kaufman[9] with the phenylalanine hydroxylase system of

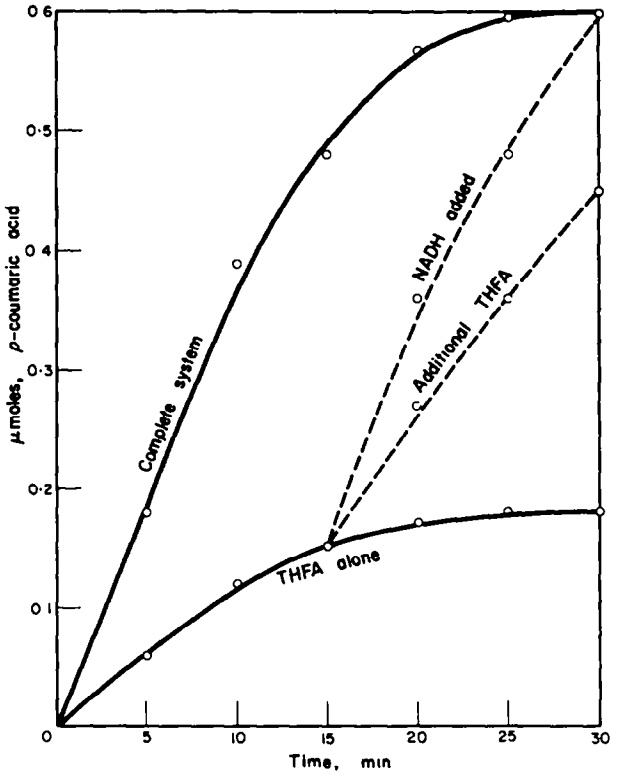

FIG. 2. KINETIC STUDY OF THE FORMATION OF *p*-COUMARIC ACID.

Standard assay conditions were used, except for variations in the addition of cofactors. All additions of NADH and THFA were of 0·5 and 0·2 μmoles respectively. The second addition was made after 15 min. Each experimental point represents a separate reaction.

animal liver. Kaufman and coworkers have subsequently shown[10] that the immediate electron donor in this reaction is a reduced pteridine which acts catalytically only when continuously reduced by an auxiliary electron donor and a second enzyme. In the phenylalanine hydroxylase system NADPH was more active in this role than NADH. A kinetic examination of the hydroxylation of cinnamic acid showed that in the presence of added THFA alone the reaction rate was initially slower than when both NADH and THFA were present, and after ten minutes declined rapidly (Fig. 2). A second addition of NADH or

[7] F. BERNHEIM, K. M. WILBUR and C. B. KENASTON, *Arch. Biochem. Biophys.* **38**, 177 (1952).
[8] M. KATAGIRI, S. YAMAMOTO and O. HAYAISHI, *J. Biol. Chem.* **237**, PC 2413 (1962).
[9] S. KAUFMAN, *Biochim. Biophys. Acta* **27**, 428 (1958).
[10] S. KAUFMAN, in *Oxygenases* (Edited by O. HAYAISHI), p. 129, Academic Press, New York and London (1962).

THFA after 15 minutes resulted in resumption of activity at a rate faster than the initial value. With NADH the amount of phenol formed at 30 min was as high as when the cofactors were added together at the beginning of the experiment. This latter result contrasts with the observation by Kaufman and coworkers that the oxidized pteridine cofactor in the phenylalanine hydroxylating system is unstable in the presence of phosphate and, in the absence of a reduced pyridine nucleotide, rearranges to the 7,8-dihydropteridine which is inactive. Several possible explanations for this difference might be offered but it seems unwise to speculate on results obtained with a crude enzyme system.

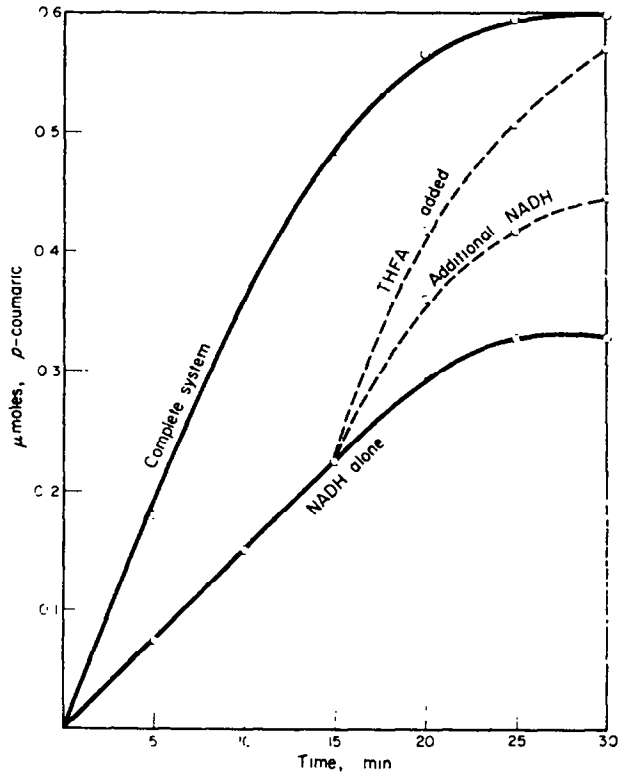

FIG. 3. KINETIC STUDY OF THE FORMATION OF *p*-COUMARIC ACID.

Experimental details were similar to those described for Fig. 2.

When the reaction mixture contained only NADH the rate of hydroxylation was slower than with the complete system and ceased after approximately 25 min. A second supplement of either NADH or THFA after 15 min caused an appreciable rate increase and a higher final conversion of cinnamate; THFA caused the greater degree of stimulation. The results, although not unequivocal, may be considered to support the need for a reduced pteridine cofactor as the initial electron acceptor in the reaction. Further support is provided by the powerful inhibitory action of aminopterin (Table 2). Kaufman has shown a similar effect of antifolic acid compounds on phenylalanine hydroxylase.[11]

Addition of metal salts to the reaction mixture caused no increase in activity; the enzyme

[11] S. KAUFMAN and B. LEVENBERG, *J. Biol. Chem.* **234**, 2683 (1959).

was also insensitive to metal chelating agents. In this respect it differed from the aryl hydroxylase described by Mitoma and coworkers.[12] Hg^2, Cu^2, and Co^2 ions inhibited the reaction (Table 2), as did *p*-chloromercuribenzoate (PCMB). Since the latter effect was reversed by glutathione (GSH) a sulfhydryl group may participate in the hydroxylating system.

The optimum pH for the reaction was found to be 4·6 (Fig. 4). With the assay method used the rate at which *p*-coumaric acid was formed showed no increase for substrate concentrations above 0·33 mM, but no evidence was obtained to indicate whether the reaction was inhibited by the substrate or its product, or limited by a cofactor deficiency.

TABLE 2. EFFECT OF METAL SALTS AND OTHER SUPPLEMENTS ON CINNAMIC ACID HYDROXYLASE ACTIVITY

Supplement*	*p*-Coumaric acid formed (μmoles)	Inhibition (%)
None	0·570	—
$HgCl_2$	0·090	84·2
$MnSO_4.H_2O$	0·570	—
$MgSO_4.7H_2O$	0·570	—
$ZnSO_4.7H_2O$	0·360	36·9
$Co(NO_3)_2$	0·090	84·2
$Fe_2(SO_4)_3$	0·390	31·6
$FeSO_4.7H_2O$	0·570	—
o-Phenanthroline	0·570	—
α-α′-Dipyridyl	0·570	—
Diethyldithiocarbamate	0·480	15·8
Azide	0·540	5·3
p-Chloromercuribenzoate (PCMB)	0·060	89·5
PCMB and reduced glutathione (GSH)	0·450	21·1
Aminopterin	0·000	100·0
L-Phenylalanine	0·060	90·0

* Standard assay conditions were used. Supplements were added to give a concentration of 5×10^{-4} M, except GSH which was used at 10^{-3} M.

The cinnamic acid hydroxylating system of spinach bears a notable resemblance to the phenylalanine hydroxylase present in mammalian liver. Aryl hydroxylases are recognized to have low substrate specificity[11,13] and the possibility that phenylalanine is the principal substrate of this enzyme *in vivo* must be considered. Bloch[14] has pointed out that the biosynthesis of tyrosine via phenylalanine does not seem to represent an important metabolic route in micro-organisms and higher plants. No report could be found in the literature concerning the ability of spinach leaves to convert phenylalanine to tyrosine. However, it has been shown[15,16] that in *Salvia splendens*, *Triticum vulgare* and *Fagopyrum tartaricum* there is a small, but appreciable conversion which is probably due to ring hydroxylation. When the acetone powder from spinach was incubated with phenylalanine-*U*-^{14}C a small amount of radioactive product with the properties of tyrosine was detected. The inhibition of cinnamic

[12] C. MITOMA, H. S. POSNER, H. C. REITZ and S. UDENFRIEND, *Arch. Biochem. Biophys.* **61**, 431 (1956).
[13] J. RENSON, H. WEISSBACH and S. UDENFRIEND, *J. Biol. Chem.* **237**, 2261 (1962).
[14] K. BLOCH, *Federation Proc.* **21**, 1058 (1962).
[15] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 531 (1959).
[16] O. L. GAMBORG and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 1277 (1959).

acid hydroxylation by L-phenylalanine also suggests a competition between substrates for the same active centre.

Levy and Zucker[17] have proposed that the aromatic hydroxylation reactions which must occur during biosynthesis of chlorogenic acid from precursors such as phenylalanine and cinnamic acid take place after the esterification of cinnamic acid with quinic acid. According to this scheme cinnamylquinic acid, rather than cinnamic acid, would be the true substrate for a hydroxylase. Further studies on the substrate specificities of purified enzyme preparations will be required to settle this point. However, since the enzyme in spinach does catalyse hydroxylation of cinnamic acid itself, a pathway in which the esterification of phenylpropanoid

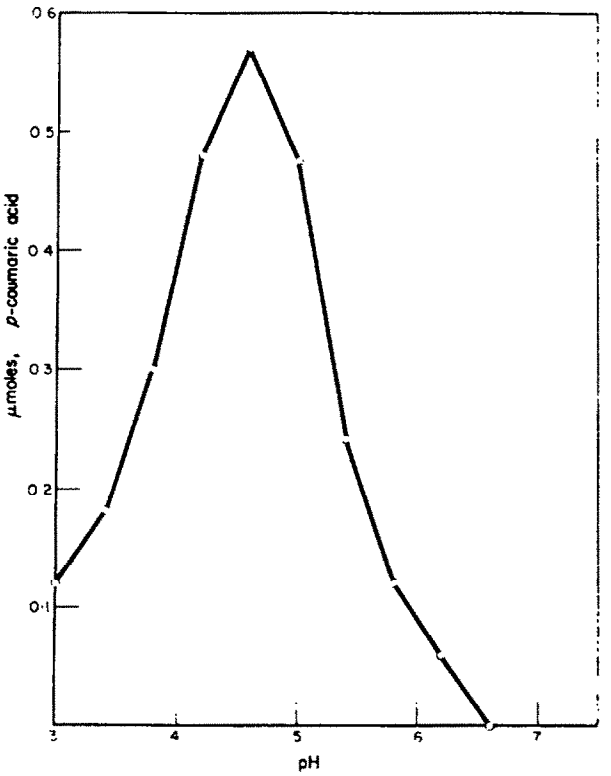

FIG. 4. EFFECT OF pH ON HYDROXYLATION OF CINNAMIC ACID.

The standard assay conditions were modified by using 0·1 M citric acid–0·2 M sodium phosphate buffers of pH values varying between 3·0 and 7·4.

acids represents the final step in the biosynthetic sequence, as first proposed in a general way by McCalla and Neish,[18] should not be excluded.

EXPERIMENTAL

Assay

The reaction mixture consisted of: 0·1 M citric acid–0·2 M sodium phosphate buffer pH 4·6 (1·0 ml); *trans*-cinnamic acid (1·0 μmole), NADH (0·5 μmole); THFA (0·2 μmole);

[17] C. C. LEVY and M. ZUCKER, *J. Biol. Chem.* **235**, 2418 (1960).
[18] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. Physiol.* **37**, 573 (1959).

enzyme extract (1·0 ml); redistilled water to a total volume of 3·0 ml. The mixture was incubated at 30° for 20 min. Trichloroacetic acid (0·5 ml of 20% w/v aqueous solution) was added and the insoluble material separated by centrifugation. An aliquot (1 ml) of the supernatant solution was removed for estimation of phenols as follows:

To the test solution was added 1 ml of a freshly prepared solution of sulphanilic acid diazotate (20 mg of sulphanilic acid in 10 ml of 0·1 N HCl treated at 0° with 8 mg of $NaNO_2$ in 1·6 ml of water. The mixture was made alkaline with Na_2CO_3 (1 ml of a 20% w/v aqueous solution) and the absorbance at 520 mμ measured after 15 min. Suitable controls and a reagent blank were included. The response to increasing concentration of *p*-coumaric acid in the test solution was linear.

Preparation of the Enzyme Extract

Spinach leaves (250 g fresh weight) were frozen at −20° and crushed with a pestle and mortar. The powder was stirred into water (250 ml) at 0–5° and the suspension filtered through cheese cloth. Three volumes of acetone at −20° were added to the filtrate and the precipitated material separated by centrifugation, washed three times with cold acetone, and dried thoroughly *in vacuo*. The powder was stored at −20°. Immediately before use a portion (200 mg) was dispersed in water (10 ml).

Identification of Reaction Product

Cinnamic acid-α-^{14}C (150 μg) was incubated with the enzyme under the conditions used for assay of hydroxylase activity. After 20 min the reaction was stopped by acidification with 10 N H_2SO_4 and suspended material removed by centrifugation. The supernatant solution was extracted with three portions (5 ml) of ether and the extracts evaporated to dryness leaving a residue which was taken up in ethanol (0·5 ml). An aliquot (0·2 ml) was chromatographed on Whatman No. 1 filter paper using the upper phase of a mixture of benzene: acetic acid: water (2:2:1) as developing solvent. When the paper strip was scanned for radioactivity, two radioactive zones were revealed at R_f values of 0·26 and 0·80. The faster-moving zone corresponded in R_f value with cinnamic acid, and gave no reaction when the chromatogram was sprayed with a solution of Fast Bordeaux Salt B.D.[19] followed by sodium carbonate. The slower-moving radioactive zone corresponded in R_f value with a pale blue fluorescent area and gave a purple colour similar to that obtained with *p*-coumaric acid when sprayed with the reagent used to detect phenols. Co-chromatography with an authentic specimen of *p*-coumaric acid gave a single zone with no separation of radioactivity from the chromogenic spot.

A larger sample of cinnamic acid-α-^{14}C (1·5 mg; 900 mμc) was then incubated with the enzyme extract (10 ml) under the conditions of the assay procedure, using equivalent concentrations of cofactors. The product was extracted into ether and chromatographed, in the solvent system described above, on a large sheet of filter paper. The fluorescent zone suspected to be due to *p*-coumaric acid was cut from the chromatogram and extracted with methanol. To the residue, left upon evaporation of the extract, authentic *p*-coumaric acid (100 mg) was added and the mixture crystallized repeatedly from water. The specific activities of the samples of *p*-coumaric acid obtained from the first three recrystallizations were 0·965, 0·980 and 0·969 mμc per mg respectively.

[19] I. A. PEARL and P. F. MCCOY, *Analyt. Chem.* **32**, 1407 (1960).

Hydroxylation of L-*Phenylalanine*

In the reaction mixture for assaying cinnamic acid hydroxylase activity L-phenylalanine-*U*-^{14}C (3 μmoles containing 100 mμc) was used instead of cinnamic acid. After 30 min. the reaction was stopped by the addition of 0·5 ml of 20% trichloroacetic acid and centrifuged to remove the precipitated proteins. The clear supernatant was passed through a Dowex 50–H^+ column. The resin was washed thoroughly with distilled water and eluted with 2 N NH_4OH. The eluate was evaporated to dryness *in vacuo*, the residue taken up in 1 ml of water and 50 μl of the solution spotted on a strip of Whatman No. 1 paper. The chromatogram was developed with *n*-butanol:acetic acid:water (120:30:50) then scanned for radioactivity and sprayed with ninhydrin. Two ninhydrin-positive radioactive zones at R_f values of 0·55 (phenylalanine) and 0·48 (tyrosine) were detected. The ratio of the peak areas of phenylalanine:tyrosine was 5·75:1, from which the amount of tyrosine formed was calculated to be 0·44 μmoles. With a similar enzyme preparation, and using the standard assay conditions with cinnamic acid the ratio of the peak areas of cinnamic:*p*-coumaric acids was 1·36:1, giving a yield of 1·27 μmoles of *p*-coumaric acid.