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PHENYLALANINE HYDROXYLASE FROM SPINACH LEAVES*

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Abstract—Spinach leaves contain an enzyme system which catalyses the hydroxylation of L-β-phenylalanine to tyrosine. The crude active extract has been partially purified by fractional precipitation with acetone, adsorption on DEAE-cellulose, and with calcium phosphate gel. Such preparations showed a 66-fold increase in specific activity. The optimum pH of the reaction was at 4·2. Only L-phenylalanine and L-*p*-fluorophenylalanine among the compounds tested were substrates of the enzyme system and each was converted to tyrosine. Cinnamic acid was not hydroxylated. No requirement for metal ions could be demonstrated. The partially purified system showed an absolute requirement for electron donors which was satisfied by adding tetrahydrofolic acid and a reduced pyridine nucleotide. The latter could be replaced by ascorbic acid, and the former by an extract of spinach leaves. The active factor in the extract was not obtained pure but behaved as expected for a pteridine derivative during fractionation. The enzyme system was inhibited by concentrations of L-phenylalanine above 10^{-3} M. Aminopterin, cinnamic acid, *p*-chloromercuribenzoate and sulfhydryl-containing compounds also inhibited the reaction. A kinetic study suggested that the enzyme system from spinach which hydroxylates phenylalanine is similar to that isolated from animal liver.

INTRODUCTION

AN EARLIER paper[1] described the ability of crude extracts from spinach leaves to catalyse the hydroxylation of cinnamic acid to *p*-coumaric acid. The enzyme preparation was unstable and could not be purified, but it was shown to require supplementation with two electron donors for maximum activity. The requirement was met by adding tetrahydrofolic acid, and a reduced pyridine nucleotide.

A similarity between this system and the phenylalanine hydroxylase of animal liver was evident. Further examination of the crude spinach extract revealed that it was also able to catalyse the hydroxylation of phenylalanine. Since many aryl hydroxylases appear to be relatively non-specific there was a strong possibility that the hydroxylation of both substrates was catalysed by a single enzyme system. Some support for this was provided by the observation that L-phenylalanine inhibited the hydroxylation of cinnamic acid, suggesting competition for the same active centre.

Because a decision on whether the two types of activity were catalysed by the same or separate enzymes could not be arrived at by further study of unstable cinnamic acid hydroxylase attention was directed to the phenylalanine hydroxylating system. This proved to be more stable and a partial purification, resulting in separation of the two activities, has been achieved.

RESULTS AND DISCUSSION

The ability to catalyse hydroxylation of L-phenylalanine in an assay system containing tetrahydrofolic acid (THFA) and reduced nicotinamide adenine dinucleotide (NADH) was

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[1] P. M. NAIR and L. C. VINING, *Phytochem.* 4, 161 (1964).

present in an aqueous extract of frozen spinach leaves. Precipitation with acetone gave an active residue. When this was re-extracted with water, and the solution fractionated by stepwise addition of acetone, all of the activity was recovered in the material precipitated at concentrations of acetone between 40 and 75 per cent. Additional purification was accomplished by adsorbing the enzyme on to diethylaminoethyl (DEAE)-cellulose and eluting immediately with a buffer of higher ionic strength. Chromatographic procedures gave poor recoveries of the enzyme. Further treatment of the eluate with calcium phosphate gel at pH 7·0 removed a large amount of protein without lowering phenylalanine hydroxylase activity. By these steps a 66-fold increase in specific activity was achieved (Table 1) and this preparation was used in subsequent studies.

The single product formed from L-phenylalanine in the enzyme reaction was identified as tyrosine. Tyrosine was also produced from *p*-fluorophenylalanine and in this respect the spinach enzyme resembles the phenylalanine hydroxylase of animal liver.[2] On the other hand no activity was observed with L-tryptophan as substrate. The animal system has been reported to catalyse the formation of 5-hydroxytryptophan but a high concentration of L-tryptophan

TABLE 1. PURIFICATION OF PHENYLALANINE HYDROXYLASE FROM SPINACH LEAVES

	Volume (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg) protein*	Recovery† (%)	Purification
Crude	30	0·80	7·0	0·11	100	—
45–75% Acetone	28	0·80	1·5	0·53	100	4·8
DEAE-cellulose adsorption	12·5	1·09	0·40	2·7	68	25
Calcium phosphate gel treatment	10	1·09	0·15	7·3	68	66

* One unit is 3 μM tyrosine/hr under the standard assay conditions used.
† Adjusted for the removal of aliquots at each step for assay.

was required.[3] Cinnamic acid, anthranilic acid, acetanilide and phenylpyruvic acid were also ineffective as substrates for the purified enzyme, although the first three compounds yielded phenolic products when tested with the crude preparation.

Maximum activity with L-phenylalanine as substrate was observed at pH 4·2 (Fig. 1). As reported by Kaufman[4] with rat liver phenylalanine hydroxylase high substrate concentrations inhibited the system (Table 2). No change in activity was observed when the reaction mixture was supplemented with $MgSO_4$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, $CdSO_4$ or $Fe_2(SO_4)_3$ at 5×10^{-4} M. However, $HgCl_2$ at this concentration caused a 71 per cent inhibition. Of the metal chelating agents tested α,α'-dipyridyl and *o*-phenanthroline did not inhibit, but cyanide caused a small decrease in activity (Table 3). *p*-Chloromercuribenzoate and a variety of sulfhydryl-containing compounds caused appreciable inhibition. Involvement of a pteridine cofactor is inferred from the inhibition by aminopterin. It is also noteworthy that cinnamic

[2] S. KAUFMAN, in *Oxygenases* (Edited by O. HAYAISHI), p. 129. Academic Press, New York (1962)
[3] J. RENSON, H. WEISSBACH and S. UDENFRIEND, *J. Biol. Chem.* **237**, 2261 (1962).
[4] S. KAUFMAN, in *Methods in Enzymology*, Vol. 5 (Edited by S. P. COLOWICK and N. O. KAPLAN), p. 809, Academic Press, New York (1962).

acid caused some inhibition; inhibition of cinnamic acid hydroxyiase activity by L-phenylalanine was observed previously.[1]

The reaction exhibited an absolute requirement for added electron donors (Table 4). Addition of nicotinamide adenine dinucleotide (NADH) or its 2'-phosphate (NADPH) alone

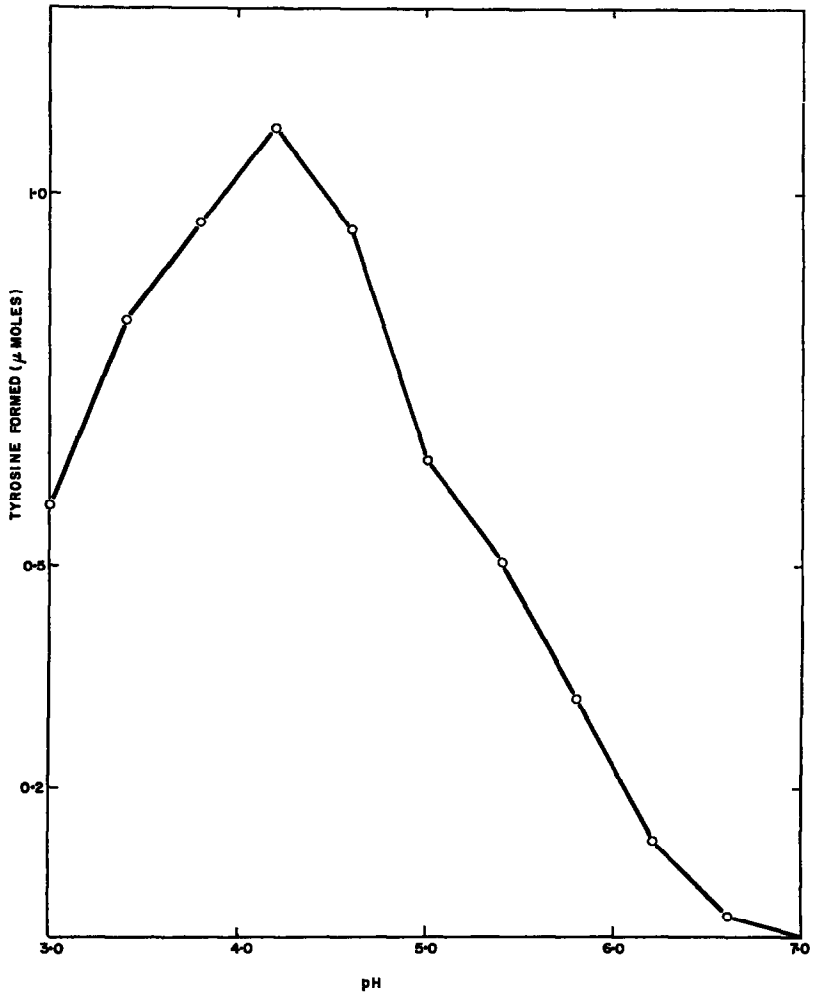

FIG. 1. EFFECT OF PH ON HYDROXYLATION OF L-PHENYLALANINE.
Standard assay conditions were used, except for changes in pH of the 0·1 M citric acid–0·2 M sodium phosphate buffer.

resulted in only a small amount of activity compared with that obtained when tetrahydrofolic acid (THFA) was also added. This property distinguishes the enzyme from the inducible phenylalanine hydroxylase of *Pseudomonas* species,[5] which requires only NADH for maximum activity. THFA alone gave approximately half of the activity which could be obtained by the combined addition of THFA and either NADH or NADPH to the assay system. The

[5] G. GUROFF and T. ITO, *Biochim. Biophys. Acta* 77, 159 (1963).

reduced pyridine nucleotides could be replaced by ascorbic acid. Thus the enzyme system of spinach which hydroxylates L-phenylalanine appears to be less exacting in its requirement for electron donors than that which hydroxylates cinnamic acid, since ascorbic acid did not

TABLE 2. EFFECT OF SUBSTRATE CONCENTRATION ON ENZYME ACTIVITY*

L-Phenylalanine concentration (μmoles)	Tyrosine formed (μmoles)
0·30	0·12
0·60	0·38
1·2	0·52
1·8	0·79
2·4	0·96
3·0	1·10
6·0	0·78
9·0	0·45

* Standard assay conditions were used, except for changes in the amount of L-phenylalanine.

TABLE 3. EFFECT OF INHIBITORS ON ENZYME ACTIVITY

Supplement*	Tyrosine formed (μmoles)	Inhibition (%)
None	1·15	--
p-Chloromercuribenzoate	0·76	34
N-Ethylmaleimide	1·12	3
Glutathione (reduced)	0·83	28
Cysteine	0·64	44
Mercaptoethanol	0·06	95
2,3-Dimercaptopropan-1-ol (BAL)	0	100
α,α′-Dipyridyl	1·15	0
o-Phenanthroline	1·15	0
Cyanide	1·02	11
Aminopterin	0·13	89
Cinnamic acid	0·64	44

* Standard assay conditions were used except for addition of the inhibitor. Aminopterin and cinnamic acid were used at 10^{-4} and 10^{-3} M respectively. Other substances were added to give 5×10^{-4} M in the reaction mixture.

serve as an electron donor in the latter. Moreover, ascorbic acid has not been reported to be active in the phenylalanine hydroxylating system of animals.

With an enzyme prepared from rat liver Kaufman[2, 6] has demonstrated that the cofactor which donates electrons directly to the substrate is a tetrahydropteridine which is oxidised during the reaction to the dihydro-state. For catalytic activity the dihydropteridine must be

[6] S. KAUFMAN, *J. Biol Chem.* **239**, 332 (1964).

immediately reduced again by an auxiliary electron donor, either non-enzymically with certain reducing agents or with a reduced pyridine nucleotide in the presence of a second enzyme. The pyridine nucleotide may be subsequently returned to the reduced state by participation in any suitable oxido-reductase reaction in the metabolism of the organism. A kinetic study with the spinach enzyme system (Fig. 2) has indicated that a similar sequence of events probably occurs. With THFA as the only cofactor in the incubation mixture the reaction rate was slower than when both NADH and THFA were present and levelled off after 15 min at less than half the value attained with the complete system. Adding NADH to the reaction at this time had little effect. The result is similar to that observed by Kaufman,[6,7] who has shown that the quinone form of the oxidized pteridine rearranges rapidly to the 7,8-dihydro derivative which cannot be reduced back to the tetrahydro state. The crude cinnamic acid hydroxylase of spinach did not show this effect.[1]

TABLE 4. EFFECT OF ELECTRON DONATING COFACTORS ON ENZYME ACTIVITY*

Supplement	Tyrosine formed (μmoles)
None	0
NADH	0·05
NADPH	0·05
Ascorbic acid	0·06
THFA	0·48
THFA + NADH (0·2 μmole)	0·80
THFA + NADH (0·5 μmole)	1·09
THFA + NADH (1·0 μmole)	1·15
THFA + NADH (2·0 μmole)	1·15
THFA + NADPH	1·10
THFA + ascorbic acid (0·2 μmole)	0·81
THFA + ascorbic acid (0·5 μmole)	1·11
THFA + ascorbic acid (1·0 μmole)	1·11

* Assay conditions were standard, except for variations in the cofactor supplement. The amount of THFA present was 0·2 μmoles. Unless noted otherwise 0·5 μmoles of the remaining cofactors were used.

A second addition of THFA to a reaction, which had proceeded for 15 min with only THFA present, permitted hydroxylation to continue with little change in rate. When both NADH and THFA were added after 15 min to the reaction which had received only THFA the rate of hydroxylation increased. It was slightly lower than when both cofactors were added at the start of the reaction, presumably because of some inactivation of the enzyme during incubation.

Although THFA is effective as an electron donor in the phenylalanine hydroxylase systems of animals as well as spinach, Kaufman has demonstrated[2] that it is not the natural cofactor of rat liver. He has recently isolated the latter substance[8] and shown it to be dihydrobiopterin. Aqueous extracts of spinach leaves were found to contain a cofactor able to replace THFA as an electron donor in the spinach phenylalanine hydroxylase system. The extract

[7] S. KAUFMAN, *J. Biol. Chem.* **234**, 2677 (1959).
[8] S. KAUFMAN, *Proc. Nat. Acad. Sci. U.S.* **50**, 1085 (1963)

was fractionated by adsorption chromatography on Florisil and then by partition chromatography on a column of silicic acid. The absorption spectrum is recorded in Fig. 3. Paper chromatographic examination of the active product showed it to contain a blue-fluorescent substance which became yellow, with a deep yellow fluorescence, upon treating with alkali.

With the cofactor preparation from spinach in the phenylalanine hydroxylase assay system and L-phenylalanine as substrate tyrosine was again the sole product of the reaction. The rate of hydroxylation was linear for 10 min with no initial lag (Fig. 4). Kaufman[8] has reported that the natural cofactor of rat liver phenylalanine hydroxylase is inactive when

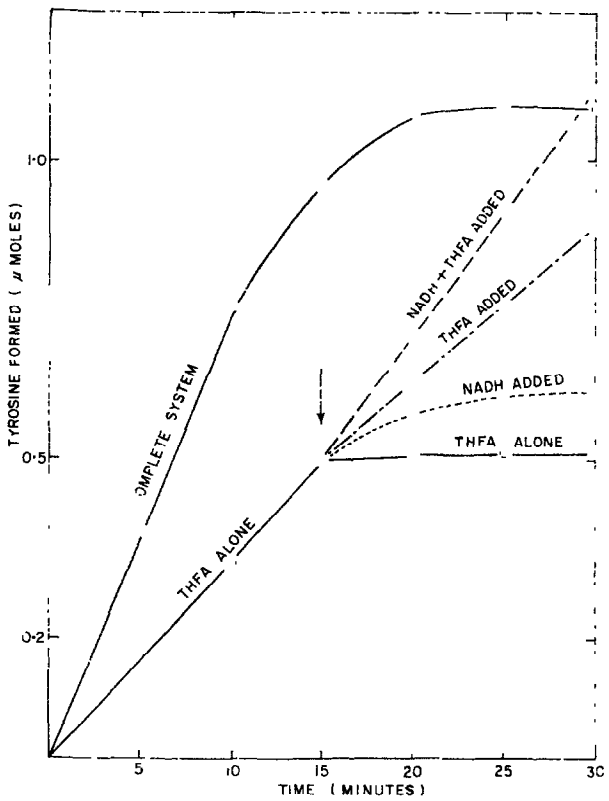

FIG. 2. RATE OF HYDROXYLATION OF L-PHENYLALANINE.

Assay conditions were standard except for variations in the addition of THFA (0·2 μmole) and NADH (0·5 μmole). Delayed additions were made 15 min after the start of the reaction.

prepared in a pure state unless first reduced to the tetrahydro derivative with a dihydrofolate reductase. However, no lag was observed with crude or partially purified preparations.

Although the conversion of phenylalanine to tyrosine in animals has been extensively studied little attention appears to have been given to the occurrence of this reaction in the plant kingdom. Some evidence has suggested that the reaction may be of only minor importance in plants and microorganisms.[9] Nevertheless results obtained in feeding experiments with ^{14}C-labelled phenylalanine have occasionally indicated that hydroxylation to tyrosine

[9] K. BLOCH, *Federation Proc.* **21**, 1058 (1962).

does occur. Values reported by Neish and coworkers[10,11] for the specific activity of tyrosine isolated from *Salvia splendens*, *Triticum vulgare* and *Fagopyrum tataricum* which had been administered radioactive phenylalanine, or compounds easily converted to this amino acid, show that direct conversion by a hydroxylation reaction must have been appreciable. Similar evidence has been obtained with fungi[12,13] and examples of the induction of a phenylalanine hydroxylase in bacteria are known.[14,15]

The isolation and partial purification from spinach leaves of a cell-free system which can catalyse the hydroxylation of phenylalanine strengthens the direct evidence from feeding labelled precursors for the existence of this pathway in plants. The demonstration that a natural cofactor which can substitute for THFA in the test system is also present in spinach

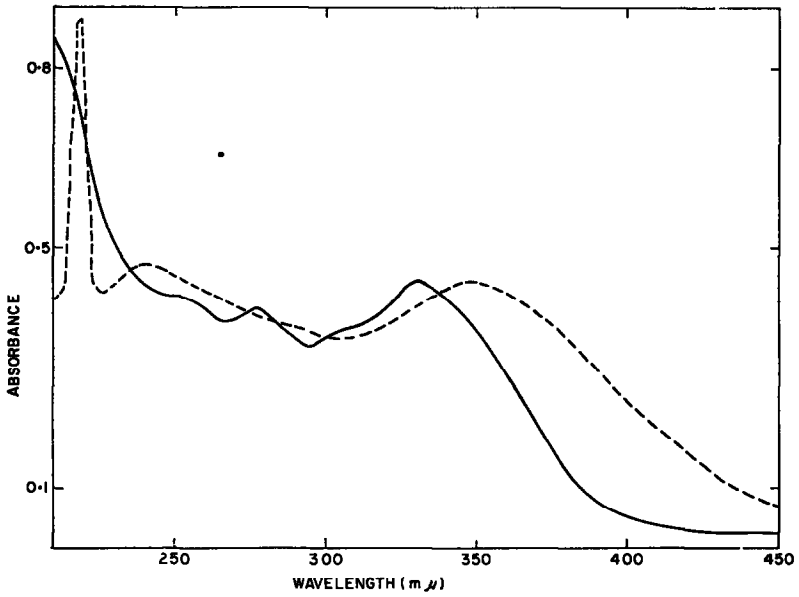

FIG. 3. ULTRAVIOLET ABSORPTION SPECTRA OF A PREPARATION CONTAINING A NATURAL COFACTOR FOR THE PHENYLALANINE HYDROXYLASE OF SPINACH.

The active fractions from a silicic acid partition column were measured in 0·1 N HCl (solid line) and 0·1 N NaOH (hatched line).

leaves increases the likelihood that the enzyme is functional. The enzyme system is spinach is clearly similar in most, though possibly not all, respects to that present in animals. It is also similar to, but distinct from, the system in spinach which hydroxylates cinnamic acid. Spinach phenylalanine hydroxylase is now known to be devoid of activity on cinnamic acid; it has not been shown whether the converse is true.

[10] D. R. MCCALLA and A. C. NEISH, *Canad. J. Biochem. Physiol.* **37**, 531 (1959).
[11] O. L. GAMBORG and A. C. NEISH, *Canad. J. Biochem. Physiol.* **37**, 1277 (1959).
[12] L. C. VINING and W. A. TABER, *Canad. J. Microbiol.* **9**, 291 (1963).
[13] L, C. VINING, Unpublished results.
[14] S. DAGLEY, M. E. FEWSTER and F. C. HAPPOLD, *J. Gen. Microbiol.* **8**, 1 (1953).
[15] S. UDENFRIEND and C. MITOMA, in *Amino Acid Metabolism* (Edited by W. D. MCELROY and H. B. GLASS), p. 876, The Johns Hopkins Press, Baltimore (1955).

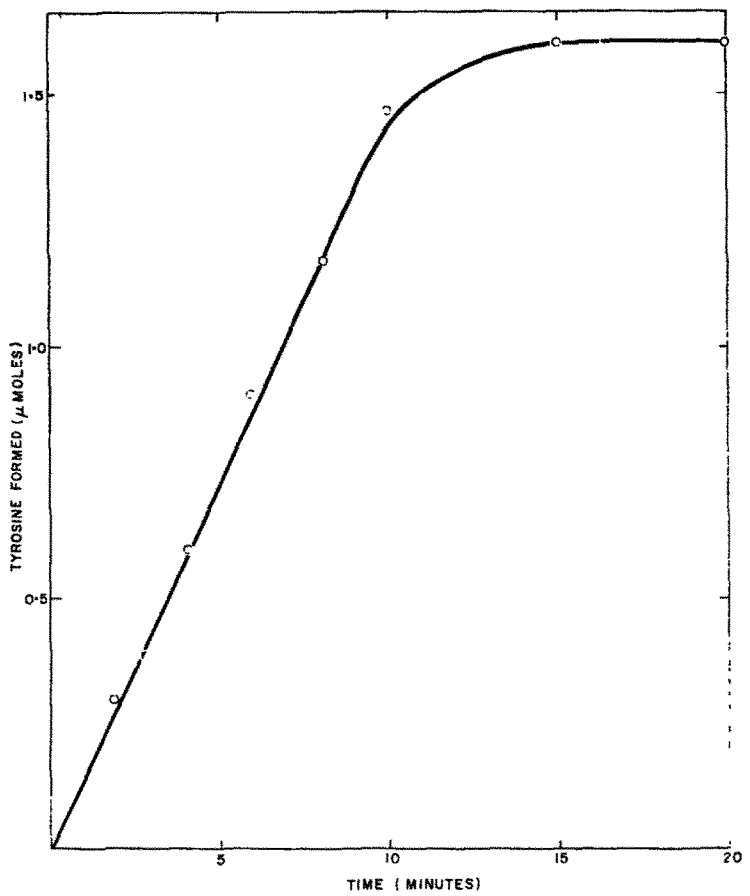

FIG. 4. RATE OF L-PHENYLALANINE HYDROXYLATION WITH A NATURAL COFACTOR PREPARATION IN PLACE OF THFA.

Separate reactions were assayed after different periods of incubation. Other conditions were standard.

EXPERIMENTAL

Assay

The reaction mixture consisted of L-phenylalanine (3 μmole), NADH (0·5 μmole), THFA (0·2 μmole), enzyme solution (1 ml), 0·1 M citric acid–0·2 M sodium phosphate buffer, pH 4·2 (1 ml), and water to a total volume of 3 ml. It was incubated at 30 for 20 min, then trichloroacetic acid (0·5 ml of a 20 % w/v solution) added and the precipitate removed by centrifugation. An aliquot (1 ml) of the supernatant solution was used for the estimation of phenolic substances by a modification of the procedure of Booth and Boyland.[16] Suitable controls and a reagent blank were included in the assay. The response to increasing concentrations of tyrosine was linear. A unit of activity is defined as the amount of enzyme required to form 1 μmole of tyrosine under the standard conditions of assay.

Protein was estimated by the method of Lowry *et al.*[17] with bovine serum albumin as standard.

[16] J. BOOTH and E. BOYLAND, *Biochem. J.* **66**, 73 (1957).
[17] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).

Purification of Enzyme

Except where noted otherwise all manipulations were carried out at 4°.

Crude preparation. Spinach leaves (250 g fresh weight) frozen at −20° were crushed in a mortar with a pestle. The powder was stirred into water (250 ml) and the suspension filtered through cheese cloth. To the filtrate three volumes of acetone at −20° were added. The precipitate was separated by centrifugation, washed three times with cold acetone, and dried thoroughly *in vacuo*.

A solution of the crude enzyme preparation was obtained by dispersing the residue (0·6 g) in water (30 ml) and centrifuging the suspension at 10,000 *g* for 5 min to remove debris.

Acetone fractionation. To the crude enzyme solution (28 ml) acetone (19·6 ml) at −20° was added and the precipitate, removed by centrifugation at 12,000 g for 10 mins, was discarded. An additional portion (64·4 ml) of acetone was mixed with the supernatant. The second precipitate, separated in the same manner as the first, was collected and redissolved in 0·01 M sodium phosphate buffer, pH 7·2 (28 ml).

Adsorption on DEAE-cellulose. A solution (25 ml) of the acetone-fractionated enzyme was stirred for 15 mins with 500 mg of DEAE-cellulose which had been prepared by the procedure of Peterson and Sober[18] and previously equilibrated with 0·01 M sodium phosphate buffer, pH 7·2. The mixture was filtered through a sintered glass funnel; the cellulose derivative, containing the adsorbed enzyme was washed in the funnel with 50 ml of the same buffer and again in a similar manner with 0·01 M sodium phosphate buffer, pH 7·2, containing 0·05 M sodium chloride. The enzyme was finally eluted by stirring the residue with 12·5 ml of the buffer to which sodium chloride at 0·4 M concentration had been added.

Calcium phosphate gel adsorption. The eluate (10 ml) from DEAE-cellulose was adjusted to pH 7·0 with 0·1 N acetic acid. Calcium phosphate gel (35 mg) prepared as described by Keilin and Hartree[19] was stirred into the solution and, after 15 mins, removed by centrifugation. Most of the phenylalanine hydroxylase activity was in the supernatant fraction. The course of purification is summarized in Table 1.

Identification of Product

L-Phenylalanine (3 μmole) uniformly labelled with ^{14}C (0·1 μc) was incubated with a purified enzyme preparation under standard assay conditions. Protein was removed after precipitation with trichloroacetic acid and the solution applied to a column of Dowex-50 × 8 (H^+). Amino acids adsorbed on the resin were eluted with 2 N ammonium hydroxide; the eluate was evaporated *in vacuo* and redissolved in water (1 ml). An aliquot (50 μl) of the solution was chromatographed on a Whatman No. 1 paper strip using the solvent mixture *n*-butanol: acetic acid: water (12:3:5). When scanned for radioactivity the developed chromatogram showed two zones at R_f values of 0·55 and 0·48 corresponding to those of authentic specimens of phenylalanine and tyrosine respectively. Both radioactive areas coincided with purple zones which appeared when the chromatogram was treated with ninhydrin.

To confirm the formation of tyrosine a sample of L-phenylalanine-U-^{14}C-containing 0·5 μc of radioactivity was incubated with the enzyme system under standard conditions. The amino acid fraction obtained from the reaction mixture was diluted with carrier L-tyrosine (250 mg) and the amino acid crystallized four times from water to a constant specific activity of 1·39 mμc/mg.

[18] E. A. PETERSON and H. A. SOBER, in *Methods in Enzymology*, Vol. 5 (Edited by S. P. COLDWICK and N. O. KAPLAN), p. 3, Academic Press, New York (1962).
[19] D. KEILIN and E. F. HARTREE, *Proc. Roy. Soc.* **124** (B), 397 (1937).

Specificity

Under standard assay conditions with the purified enzyme preparation no hydroxylation was detected when L-phenylalanine in the reaction mixture was replaced with phenylpyruvic acid, cinnamic acid, anthranilic acid, acetanilide or L-tryptophan at equivalent concentrations. L-*p*-fluorophenylalanine, on the other hand, was a suitable substrate. The product of the reaction was indistinguishable from tyrosine by co-chromatography in *n*-butanol:acetic acid:water (12:3:5). The rate of the reaction under standard conditions was only slightly less than that with L-phenylalanine (1·02 vs. 1·09 μmole of tyrosine formed, respectively).

Isolation of Natural Cofactor

Frozen spinach leaves (500 g) crushed with a pestle in a mortar were mixed with 1 litre of water and macerated in a Waring blendor for 5 min. The slurry was filtered through cheese cloth and the filtrate boiled for 5 min, then immediately cooled to 0 . After the pH had been adjusted with acetic acid to 4·0 the extract was clarified by filtration through Celite and applied to a column (2·5 × 15 cm) of Florisil (60–100 mesh). Thorough washing with water removed a large quantity of inactive yellow-colored effluent. The active substance was eluted with 20% aqueous acetone. It was concentrated *in vacuo* to remove the acetone and the volume adjusted to 20 ml with water. An inactive gelatinous yellow precipitate which settled on storage at 0° was separated leaving a clear yellow solution.

A portion (15 ml) of this was concentrated *in vacuo* at 30 to a small volume and applied to a partition column (2·5 × 12 cm) prepared from a slurry of silicic acid (21 g), water (11 ml), and a chloroform:*n*-butanol (3:17) mixture as described by Kaufman and Levenberg.[20] The column was developed in the dark under nitrogen with the organic phase of the solvent mixture and 10 ml fractions collected. Cofactor activity was present in fractions 3 to 8. These were combined, concentrated almost to dryness at 40° *in vacuo*, and diluted to 5 ml with water.

The activity of extracts and concentrates was tested by substituting 0·2 ml of the solution for the THFA used in the standard assay of phenylalanine hydroxylase activity. A control with boiled enzyme was included. No attempt was made to develop a quantitative assay, but where the test showed the preparation to be very active the assay was repeated after suitable dilution. A comparison of the activity of preparations at different stages of purification is given in Table 5.

TABLE 5. COFACTOR ACTIVITY OF FRACTIONS FROM SPINACH EXTRACT*

	Volume of extract (ml)	Dilution for assay	Tyrosine formed (μmoles)
Nil	–	–	0
THFA	–	–	1·09
Crude boiled extract of spinach	1000	nil	0·32
Florisil column eluate	20	1·20	1·53
Silicic acid column eluate	5	1·20	0·96

* Standard conditions for assaying phenylalanine hydroxylase activity were used except that THFA was replaced, where indicated by 0·2 ml of the test solution.

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

Evidence that the reaction catalysed by the enzyme in the presence of the natural cofactor was the same as when THFA was used was obtained at each step in the purification by using L-phenylalanine-U-^{14}C as the substrate. In each instance radioactive tyrosine was identified in the incubation mixture by paper chromatography.

Paper Chromatography of Natural Cofactor Preparation

Samples of the purified material were chromatographed on Whatman No. 1 paper strips by the descending technique. Folic acid was used as a reference compound and the chromatograms were examined under u.v. light of 253·7 mμ. The cofactor solution gave a single zone with blue fluorescence, which changed to a deep yellow fluorescence under light of 366 mμ after the paper had been sprayed with 5% aqueous sodium bicarbonate. The fluorescent area coincided with a yellow zone which could be seen under visible light after the alkaline treatment. The R_f values of this zone and the references compound in three solvent systems are given in Table 6.

TABLE 6. R_f VALUES OF FLUORESCENT SUBSTANCE IN NATURAL COFACTOR PREPARATION

Solvent system	R_f values	
	Folic acid	Cofactor solution
n-Butanol:acetic acid:water (12:3:5)	0·38	0·39
Isopropanol:2N ammonia:water (40:1:20)	0·60	0·70
5% Aqueous acetic acid	0	0·35