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FORMATION OF *m*- AND *p*-COUMARIC ACIDS BY ENZYMATIC DEAMINATION OF THE CORRESPONDING ISOMERS OF TYROSINE*

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Abstract—An enzyme was found which catalyzes the deamination of L-tyrosine, giving equimolar amounts of *trans-p*-coumaric acid and ammonia as the products. This enzyme (tyrase) was readily detected in sorghum, barley, rice, wheat, oat, corn and sugar cane plants; but not in pea, lupine, alfalfa or white sweet clover plants, or in yeast. Tyrase was concentrated in the stems of barley rather than the leaves, and reached its maximum concentration at about the time the heads were emerging. The crude, soluble protein extracted from an acetone powder of barley stems was purified about forty-fold with respect to tyrase. Tyrase preparations from this source were also found to convert DL-*m*-tyrosine to *m*-coumaric acid and ammonia, and have been shown by Koukol and Conn[1,2] to contain an enzyme (phenylalanase) which can catalyze the conversion of L-phenylalanine to cinnamic acid and ammonia. The data suggest that tyrase is distinct from the enzymes (or enzyme) catalyzing the deaminations of phenylalanine and *m*-tyrosine.

INTRODUCTION

IN RECENT years there has been considerable interest in the formation of secondary growth substances in plants, especially substances with an aromatic structure such as flavanoids, coumarins, lignin and related compounds. Tracer studies on the biosynthesis of these compounds have been reviewed.[3] They are probably derived from phenylalanine and, in some species, from tyrosine as well. Cinnamic acid, *p*-coumaric acid and other phenolic cinnamic acids are likely intermediates. In order to account for the formation of

$$R{-}CH{=}CH{-}COOH \text{ from } R{-}CH_2{-}CH(NH_2){-}COOH$$

the following sequence has been suggested:[3-5]

$$R{-}CH_2{-}CH(NH_2){-}COOH \longrightarrow R{-}CH_2{-}CO{-}COOH \longrightarrow R{-}CH_2{-}CH(OH){-}COOH \longrightarrow R{-}CH{=}CH{-}COOH$$

This postulates formation of the ethylenic bond by dehydration, but the tracer work can be explained just as well by postulating a direct deamination of the amino acid side chain as in the aspartase,[6,7] β-methyl aspartase[8] and histidase[9,10] reactions, i.e.

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$$R{-}CH_2{-}\underset{\displaystyle +NH_3}{\overset{\displaystyle |}{CH}}{-}COO^- \longrightarrow R{-}CH{=}CH{-}COO^- + NH^+$$

Koukol and Conn[1,2] found an enzyme in sweet clover that catalyzes the formation of cinnamic acid from phenylalanine. This enzyme (phenylalanine deaminase) was also found in other legumes and in members of the *Gramineae*. The reaction has been established as a direct deamination of the type shown above (R = phenyl).

The present paper describes two other deamination reactions of the same general type: (1) the conversion of L-tyrosine to *p*-coumaric acid (R = *p*-hydroxyphenyl) and (2) the conversion of DL-*m*-tyrosine to *m*-coumaric acid (R = *m*-hydroxyphenyl). The enzyme catalyzing deamination of L-tyrosine has been named tyrase. It was found in substantial amounts in grasses, but not in any of the legumes examined.

EXPERIMENTAL AND RESULTS

Detection of Tyrase in Plants

The first direct evidence for the existence of tyrase was obtained as follows: A sample of acetone powder (0·2 g) prepared from 6-day-old sorghum seedlings was incubated at room temperature (about 22°) with 3 ml of 0·05 M tris(hydroxymethyl) aminomethane-hydrochloric acid buffer (pH 8·8) containing 0·1 per cent of L-tyrosine. After 3 hr the mixture was diluted by 7 ml of water and filtered in a Buchner funnel. The filtrate was acidified with 0·2 ml of 5 N hydrochloric acid, extracted with 10 ml of ether, the ether evaporated and the residue redissolved in 0·2 ml of 95 per cent ethanol. About one-third of this solution was chromatographed on paper using solvent B (Table 1) as the irrigant.

TABLE 1. COMPARISON OF *cis-* AND *trans-* ISOMERS OF *p*-COUMARIC ACID

	Isomer of *p*-coumaric acid*	
	cis-	*trans-*
Corrected m.p., °C	131	219–220
Absorption peak of salt (mμ)†	290	333
Rf in solvent A‡	0·66	0·35
Rf in solvent B‡	0·64	0·64
Rf in solvent C‡	0·40	0·34
Rf in solvent D‡	0·36	0·29

* Both isomers give the same colored spot with diazotized *p*-nitroaniline, i.e. orange, changing to blue when sprayed with alkali.[22,23]

† Measured in 0·05 N sodium hydroxide. The fluorescence of the *cis-* isomer in this solution is less than one-tenth that of the *trans-* isomer.

‡ Solvent A—2 per cent acetic acid in water.
Solvent B—Upper phase of benzene–acetic acid–water (6 : 7 : 3) (Ibrahim and Towers[23]).
Solvent C—*n*-Propanol–conc. NH_4OH (7 : 3).
Solvent D—Isopropanol–conc. NH_4OH–water (8 : 1 : 1).

A component with the properties of *p*-coumaric acid was observed; it had the correct mobility and gave the same color reactions with diazotized *p*-nitroaniline (i.e. orange changing to blue in alkali). This component was not detected in parallel experiments where

tyrosine was omitted, even if *p*-hydroxyphenyl pyruvic acid or *p*-hydroxyphenyl lactic acid was added.

Acetone powders from other sources were tested in the same way (Table 2). Evidence for an enzyme converting tyrosine to *p*-coumaric acid was obtained for sorghum, wheat, rice, barley, oats, corn and sugar cane. In some of these tests *p*-coumaric acid was detected when tyrosine was omitted, but the parallel experiment with tyrosine always gave a more intense spot. Sorghum seeds did not contain a detectable amount of tyrase. Negative tests were also obtained with acetone powders of yeast and three species of legumes. No *p*-coumaric acid was observed in the negative tests, whether tyrosine was added or not.

TABLE 2. DETECTION OF TYRASE IN ACETONE-DRIED PLANT MATERIALS

Source of plant material	Presence of tyrase
Sorghum vulgare Pers. (sorghum) var. Honey Drip.	
seeds soaked overnight	−
4-day old, etiolated seedlings	+
6–12-day old, non-etiolated seedlings	+
Triticum vulgare Vill. (wheat) var. Ramona.	
4-day old, etiolated seedlings	+
4-day old, non-etiolated seedlings	+
Zea mays L. (corn) var. Minnesota B164.	
6-day old, non-etiolated seedlings	+
Hordeum vulgare L. (barley) var. Mariout.	
4-day old, etiolated seedlings	+
4-day old, non-etiolated seedlings	+
Avena sativa L. (oats) var. Kanota.	
6-day old, non-etiolated seedlings	+
Oryza sativa L. (rice) var. Caloro.	
10-day old, non-etiolated seedlings	+
**Saccharum officinarum* L. (sugar cane).	
stalk from plant about 150 cm tall	+
†*Pisum sativum* L. (peas) var. Alaska.	
6-day old, etiolated seedlings	−
†*Lupinus albus* L. (white lupin).	
6-day old, etiolated seedlings	−
†*Melilotus alba* Desr. (white sweet clover).	
Shoots, 30–40 days old	−
Fleischman's dried bakers' yeast	−

* Obtained from the Department of Agronomy, University of California, at Davis by courtesy of Dr. D. S. Mikkelsen.
† These acetone powders were prepared by Dr. J. Koukol.

A positive test for tyrase was also obtained with soluble protein fractions from sorghum or barley seedlings. These fractions were extracted by 0·05 M tris(hydroxymethyl)-aminomethane-hydrochloric acid (pH 8·8), precipitated by ammonium sulfate at 70 per cent saturation, then dissolved and dialyzed overnight at 0°, using buffer of the same concentration. They lost the tyrase activity on heating at 100° for 3 min. It was concluded that

certain plants, especially members of the *Gramineae*, contain an enzyme that can catalyze the conversion of L-tyrosine to *p*-coumaric acid.

Quantitative Assays for Tyrase

Tyrase was estimated by measuring the rate of formation of *p*-coumaric acid in the presence of excess L-tyrosine. The *p*-coumaric acid was isolated from the reaction mixture by extraction with ether and assayed either spectrophotometrically or fluorometrically in sodium hydroxide solution (Figs. 1 and 2). In most experiments a temperature of 40° was used (see Fig. 3) and the reaction mixture was buffered at pH 8·8 (Fig. 4) by 0·05–0·10 M sodium borate containing 0·1 per cent of L-tyrosine. One unit of tyrase is defined as the amount of enzyme which catalyzes formation of 0·1 μmole of *p*-coumaric acid per hour,

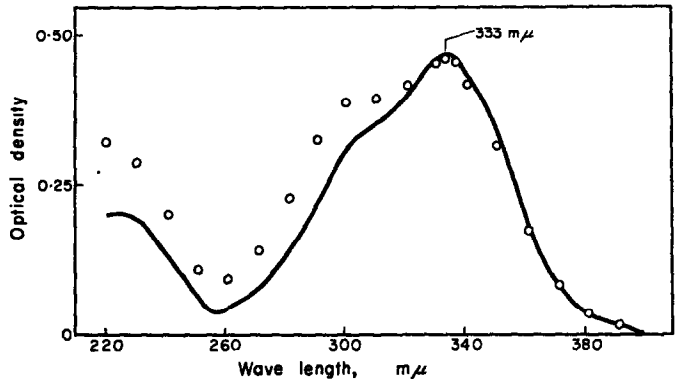

FIG. 1. ULTRAVIOLET ABSORPTION SPECTRUM OF *p*-COUMARIC ACID.
The solid line is for purified *trans-p*-coumaric acid (3·33 μg per ml of 0·05 N sodium hydroxide); the circles are for an ether-extractable acid fraction obtained following treatment of L-tyrosine with barley-stem tyrase (preparation C, Table 6).

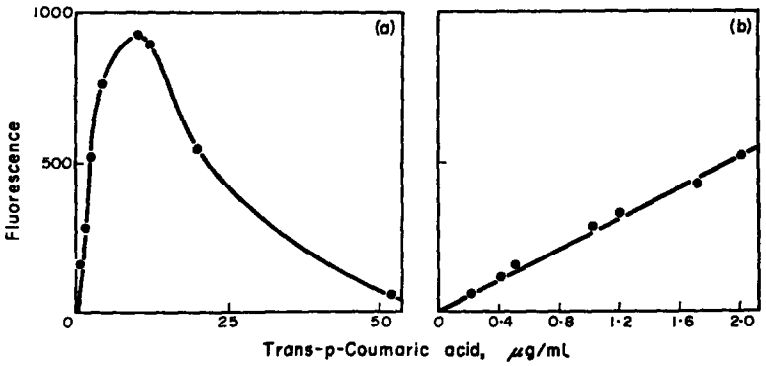

FIG. 2. RELATION BETWEEN CONCENTRATION AND FLUORESCENCE FOR *trans-p*-COUMARIC ACID IN ALKALI.
The acid was dissolved in 0·05 N sodium hydroxide, the solution put in a round quartz cuvette and fluorescence measured with an Aminco–Bowman spectrophotofluorometer using the 350 mμ setting for activation and measuring the output at 440 mμ. Fluorescence is reported in arbitrary units; a reading of unity on the per cent transmission scale at the 0·01 sensitivity setting was defined as one unit of fluorescence. (a)—Curve when concentration was varied over a wide range. (b)—Linear relation obtained for dilute solutions.

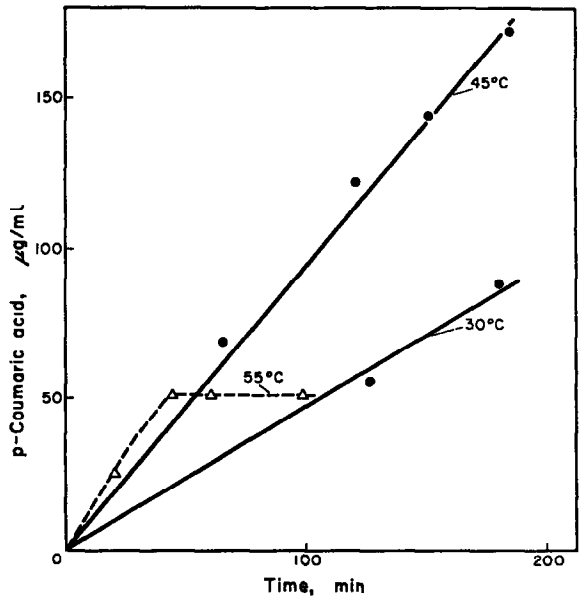

FIG. 3. RATE OF FORMATION OF *p*-COUMARIC ACID BY TYRASE AS A FUNCTION OF TEMPERATURE. The tyrase preparation was a rice seedling protein obtained between 35–70 per cent saturation on the first ammonium sulfate fractionation. Each ml of reaction mixture contained 4·3 mg of protein, 3·1 units of tyrase, 1·0 mg of L-tyrosine and 100 μmoles of sodium borate at pH 8·9.

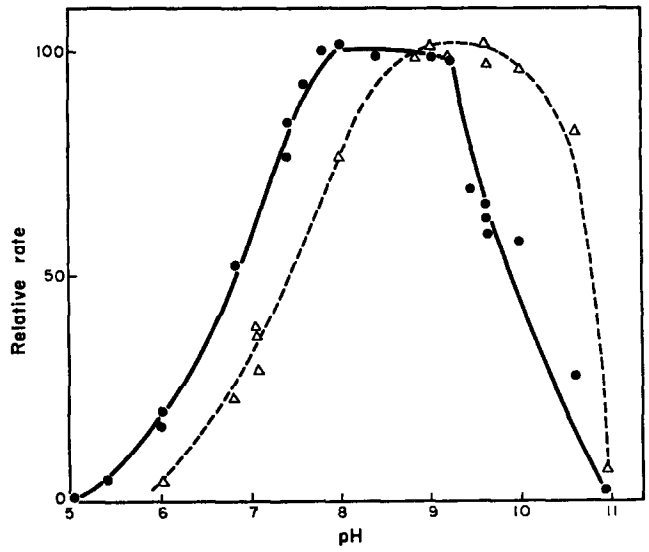

FIG. 4. EFFECT OF pH ON THE ENZYMATIC FORMATION OF *m*- AND *p*-COUMARIC ACIDS FROM THE CORRESPONDING ISOMERS OF TYROSINE.

Barley-stem protein purified through step 4 (Table 5) was used as the enzyme. The solid line (filled circles) is for formation of *p*-coumaric acid from L-tyrosine; the broken line (triangles) is for formation of *m*-coumaric acid from DL-*m*-tyrosine. The rate at any given pH is expressed as the percentage of the rate at pH 8·8 (0·1 M sodium borate) as determined in a parallel experiment. Sodium acetate buffer (0·1 M sodium borate) as determined in a parallel experiment. Sodium acetate buffer (0·1 M) was used for pH 5·0 and 5·4; potassium phosphate buffer (0·1 M) was used for pH 6·0–8·0; sodium borate buffer (0·1 M) was used for pH 8·4–9·6; ethanolamine hydrochloride buffer (0·2 M) was used for pH 10·0 and 10·6 and, methylamine hydrochloride (0·2 M) for pH 10·9.

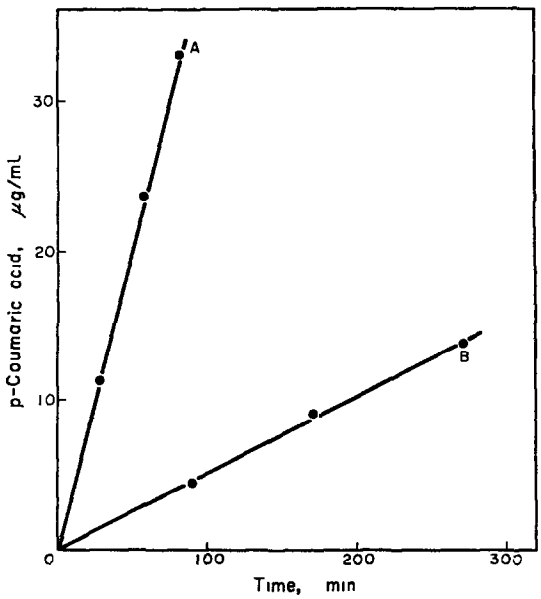

FIG. 5. TIME COURSE OF THE ENZYMATIC FORMATION OF *p*-COUMARIC ACID FROM EXCESS L-TYROSINE. Line A is for preparation C (Table 6) under the usual conditions of assay (i.e. pH 8·8, 40°), using 0·08 mg of protein per ml. Line B is for a crude protein from etiolated barley seedlings obtained between 40–60 per cent saturation on the first ammonium sulfate fractionation. This experiment was run at pH 8·8 and 31° using 2·8 mg of protein per ml.

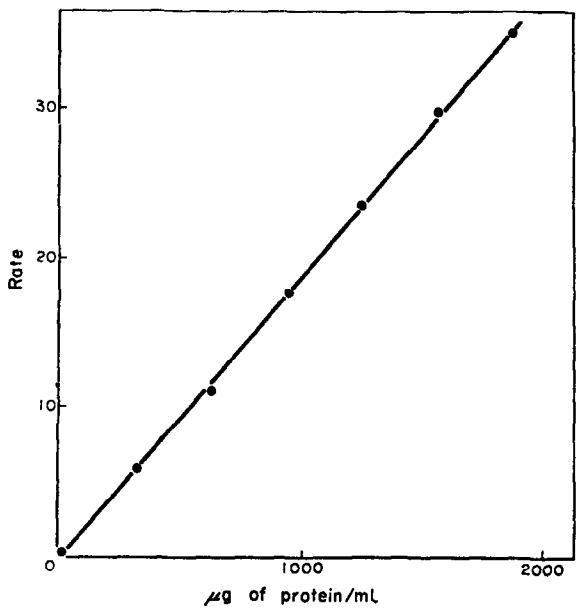

FIG. 6. RATE OF FORMATION OF *p*-COUMARIC ACID AS A FUNCTION OF ENZYME CONCENTRATION. The rate is expressed as μg of *p*-coumaric acid formed in 45 min. Each ml of reaction mixture contained 1·0 mg of L-tyrosine, 100 μmoles of sodium borate (pH 8·8) and enzyme as indicated. The reaction was run at 40°. The enzyme used was a barley-stem protein fraction precipitated at 40–60 per cent saturation on the second ammonium sulfate fractionation.

under these conditions. The rate of formation of *p*-coumaric acid was constant (Fig. 5) and directly proportional to the enzyme concentration (Fig. 6).

Comparison of Sources of Tyrase

Table 3 lists the tyrase contents of acetone powders prepared from the seedlings of several species belonging to the *Gramineae*. Rice seedlings were a good source, particularly the roots. The relatively low tyrase content of acetone-dried whole seedlings is due to dilution by starch and other inert substances in the seed residues. Wheat germ acetone powder was practically free from tyrase. It seems likely that there is little, if any, tyrase in seeds (compare with sorghum seeds, Table 2), but the enzyme is readily formed on germination.

TABLE 3. TYRASE CONTENT OF VARIOUS ACETONE POWDERS

Source*	Age (days)	Yield of acetone powder (% of fresh wt.)	Units of tyrase per g of acetone powder
Rice			
Whole seedlings	12	24	27
" "	11	28	21
Shoots of seedlings	11	12·6	52
Roots of seedlings	11	5·0	95
" " "	11	5·0	138
" " "	12	6·9	104
Barley			
Etiolated whole seedlings	4	11·0	13
Etiolated seedling shoots	5	5·6	36
Non-etiolated seedling shoots	7	6·5	25
" " " roots	7	3·0	31
Wheat			
Shoots of seedlings	7	10·4	43
Sperry wheat germ	—	86	nil

* Seedlings were grown in the laboratory from Caloro rice, Mariout barley and Ramona wheat.

The tyrase content of barley seedlings grown in a field was relatively low, but it increased as the plants developed and reached a peak at about the time the heads were emerging (Table 4). The tyrase content of barley stems was considerably higher than that of the leaves.

Extraction and Partial Purification of Tyrase

(a) *General.* Tyrase was extracted and concentrated from several sources. Acetone powders of wheat seedling shoots, barley seedlings, rice seedlings and roots of rice seedlings were tried, but the best results were obtained with acetone powders from barley stems. Table 5 summarizes the results obtained with barley stems. Each step in the fractionation is described below, with observations on results obtained with other sources. Table 6 describes preparations that were used in a study of the properties of tyrase.

Although rice root acetone powders were rich in tyrase (Table 3), attempts to concentrate the enzyme from this source met with little success because of the instability of the extracts. Sometimes more than half the activity was lost during dialysis overnight at 0·5°. A sample purified through step 3 (Table 5), lyophilized, and stored 83 days at −20°, lost about half of its activity. Tyrase prepared from barley stems was much more stable; a

TABLE 4. CHANGES IN TYRASE CONTENT DURING DEVELOPMENT OF BARLEY*

Date	Stage of development	Yield of acetone powder (% of fresh wt.)	Units of tyrase per g of acetone powder from				Units of tyrase found	
			Whole shoot	Leaves	Stems	Heads	per 100 g† of fresh wt.	per mg of extracted protein‡
Jan. 30	Plants up to 12 cm tall; 2–3 leaves	11·6	5·1	—	—	—	59	0·02
Feb. 26	Plants up to 30 cm tall; 3–4 leaves	10·6	6·0	—	—	—	63	0·04
March 11	Plants up to 40 cm tall; 4 leaves	9·0	17·0	—	—	—	150	0·06
March 18	Plants up to 45 cm tall	9·0	22·0	11	39	—	200	0·46
March 26	Plants up to 65 cm tall	11·8	37·0	9·6	72	—	430	0·67
April 3	Plants up to 80 cm tall; heads ready to emerge	14·7	23·0	7·7	35	—	340	0·47
April 16	Plants up to 90 cm tall; heads just emerged	25·4	32·0	2·8	44	47	810	0·44
April 30	Heads in milk stage	32·1	12·0	4·2	15	12	380	—

* *Hordeum vulgare* L. (var. Aravat) growing in field, University of California at Davis, 1960.
† Calculated for the whole shoot by multiplying the units of tyrase per g of acetone powder by the percentage yield of acetone powder.
‡ The first three figures apply to protein extracted from the whole shoot acetone powder, the others to protein from stem acetone powders. The protein was extracted by 0·10 M sodium borate (at pH 8·8), precipitated by ammonium sulfate (0–70 per cent saturation) and dialyzed overnight against 0·05 M sodium borate (pH 8·8).

TABLE 5. PURIFICATION OF TYRASE FROM AN EXTRACT OF BARLEY-STEM ACETONE POWDER*

Step No.	Description of step	Volume (ml)	Total† protein (mg)	Tyrase (units)	Specific activity (units/mg of protein)	% Recovery‡
1	First ammonium sulfate precipitate, 0–70 per cent	195	3100	1340	0·43	—
2	Supernatant after titrating to pH 6	190	1690	1330	0·80	99
3	Fraction precipitated between 50–60 per cent saturation with ammonium sulfate	21·2	218	725	3·3	54§
4	Chromatographed on DEAE-cellulose	99	33	376	11·4	28
5	Fraction from step 3 purified by calcium phosphate gel	33	49	388	7·9	38
6	Fraction from step 5 chromatographed on DEAE-cellulose	30	14	258	18·4	19

* Barley stem acetone powder (171 g, 5400 units of tyrase) from April 16 (Table 4) was extracted by 2400 ml of 0·10 M sodium borate at pH 8·8. Half of this was purified by steps 1, 2, 3 and 4 and the other half by steps 1, 2, 3, 5 and 6. The results are calculated back to the total.
† The ratio of optical density at 280 mμ to that at 260 mμ was 0·9 after the first ammonium sulfate precipitation, 1·4 after the second ammonium precipitation and 1·6 for the most highly purified sample of tyrase.
‡ Recoveries are based on the tyrase found in the first ammonium sulfate precipitation. This was only 25 per cent of the total tyrase in the acetone powder.
§ The fraction precipitated between 40–50 per cent saturation with ammonium sulfate contained an additional 377 units of tyrase (28 per cent) with a specific activity of 1·14.

sample purified through step 3 and stored 70 days at −20° lost only about 10 per cent of its activity, and when it was lyophilized there was no detectable loss of activity during 70 days. The more dilute solutions obtained from DEAE-cellulose (diethylaminoethyl cellulose) columns (Table 6) lost about half of their activity in the 2 weeks, but during this period they were thawed 8–10 times for sampling. Loss of activity of tyrase preparations may be due to proteolysis, since free amino-acids have been detected, after storage, in preparations purified through step 3.

TABLE 6. DESCRIPTION OF SOME TYRASE PREPARATIONS

Preparation	Source	Steps used in purification*	Composition of solution	Specific activity
A	Rice seedlings, 12 days old, acetone-dried	35–70 fraction of step 1, purified by steps 2, 5 and 6	0·35 mg of protein per ml in 0·025 M potassium phosphate at pH 6·8 and 0·2 M potassium chloride	4·0
B	Barley stems, pre-head stage, acetone-dried	1, 2, 3 and 4	0·31 mg of protein per ml in 0·035 M potassium phosphate at pH 6·8 and 0·2 M potassium chloride	8·0
C	Barley stems just after emergence of heads, acetone-dried	1, 2, 3 and 4	0·34 mg of protein per ml, otherwise as B	11·4
D	Same as C	1, 2, 3, 5 and 6	0·40 mg of protein per ml, otherwise as B	18·4

* The steps are numbered as in Table 5; see Table 5 and text for description.

(b) *Preparation and extraction of acetone powders.* A Waring blendor was used. Fresh plant material was cut into convenient lengths (about 1 in.) by scissors, barely covered with acetone at −20°, blended for 1–1·5 min at full speed, filtered by suction and washed three times with acetone at −20°. After drying for about 5 min on the Buchner funnel, the powder was spread on paper, air-dried in a fume hood for about 15 min and finally dried *in vacuo* at room temperature for at least 1 hr. The powders were stored at 4–6° in containers with tight caps. Although referred to as "powders", some of the acetone-dried materials were rather fibrous, particularly those made from stems of cereals.

The acetone powder was mixed with about 15 times its weight of 0·10 M sodium borate, pH 8·8, at room temperature. The mixture was cooled in an ice-bath and stirred manually from time to time during 30 min, then filtered through a double layer of cheese cloth. The filtrate was centrifuged at 8000 × *g* and 0° for 15 min. The supernatant fluid contained much of the tyrase; the sediment was discarded. Usually only 20–40 per cent of the tyrase present in a sample of acetone powder was obtained in solution. All subsequent steps were carried out at 0–4°.

(c) *First ammonium sulfate precipitation (step 1, Table 5).* The supernatant from the preceding step was cooled in ice, and solid ammonium sulfate, to give 70 per cent saturation, was added gradually, with stirring, during 5–10 min, then the precipitate was collected by centrifugation at 8000 × *g* for 15 min. The precipitate was taken up in 0·05 M sodium borate at pH 8·8, using a volume about one-twentieth the original extract. This was dialyzed overnight against 50 volumes of the same buffer. The precipitate nearly all dissolved giving a slightly turbid solution.

In early experiments the fractions precipitating between 0–35 and 35–70 per cent

saturation were collected separately; tyrase was found chiefly in the 35–70 fraction. Extracts from rice seedlings showed unique behavior; the 35–70 fraction rose to the surface. After allowing about 10 min for separation most of the clear solution was siphoned out from under the surface layer of insoluble material. Centrifugation of this insoluble fraction gave a pellicle containing the enzyme; the clear solution was poured out and the pellicle taken up in 0·05 M sodium borate (pH 8·8) and dialyzed as above.

(d) *Precipitation of inactive protein at pH 6 (step 2, Table 5).* The slightly turbid solution from the preceding step was titrated to pH 5·9–6·1 (glass electrode) using M acetic acid at 0°. A heavy precipitate formed; it was removed by centrifugation at $11{,}000 \times g$. The supernatant fluid contained practically all of the tyrase; it was titrated to pH 8·6 by N sodium hydroxide. This clear solution was used at once for the next step.

(e) *Second ammonium sulfate precipitation (step 3, Table 5).* Solid ammonium sulfate was added carefully to the tyrase solution at 0°; fractions precipitated at 0–40, 40–50, 50–60 and 60–75 per cent saturation were collected separately by centrifugation at $8000 \times g$. Tyrase was always concentrated in the fractions at 40–60 per cent saturation, sometimes chiefly in the 40–50 fraction and other times in the 50–60 cut. The precipitates were dissolved in 0·02 M potassium phosphate at pH 6·8, to give a protein concentration between 1 and 2 per cent. They were then dialyzed overnight against 0–100 volumes of the same buffer at 0°. The dialyzed solution was ready for chromatography on DEAE-cellulose.

(f) *Chromatography of tyrase on DEAE-cellulose (steps 4 and 6, Table 5).* The DEAE-cellulose was a commercial preparation; i.e. Biorad Cellex-D. It was washed with 0·1 N sodium hydroxide, suspended in deionized water, titrated to pH 6·8 by M phosphoric acid, filtered on a Buchner funnel and washed with 0·02 M potassium phosphate at pH 6·8. Only this purified material was used for chromatography.

Two sizes of columns were employed. The small size ($1{\cdot}1 \times 10$ cm) was loaded with about 20 mg of protein; the large size ($2{\cdot}2 \times 17$ cm) was loaded with about 100 mg. The small column was operated at 0°, in an ice bucket, using a flow rate of about 30 ml per hr; the fractions (3 ml) were collected manually. The large column was operated at 4°, in a cold room, using a flow rate of about 90 ml/hr; the fractions (4 ml) were collected automatically. Columns were packed in Pyrex tubes with retaining plugs of fine glass wool. After washing the packing thoroughly with 0·02 M potassium phosphate (pH 6·8) the enzyme solution (see above) was put on and washed in. The column was developed using a linear gradient[11] between equal volumes of 0·02 M potassium phosphate at pH 6·8 and 0·05 M potassium phosphate at the same pH but containing 0·4 M potassium chloride. The total volume in the gradient elution apparatus was 80 ml for the small column and 400 ml for the large one.

Tyrase was detected in the eluate by the direct fluorometric method described in the next paragraph. It emerged from the column after about one-half the contents of the elution apparatus had been used. The enzyme was thus obtained in about 0·035 M potassium phosphate at pH 6·8, 0·2 M with respect to potassium chloride. The protein concentration was usually 0·3–0·4 mg/ml. The active fractions were stored at −20° (Table 6).

The tyrase content of fractions eluted from DEAE-cellulose columns was determined by a rapid fluorometric procedure which omitted ether extraction and zero-time controls. An aliquot (0·1–0·2 ml) of the eluate was mixed with 0·5 ml of 0·1 per cent tyrosine in 0·15 M sodium borate (pH 9·0). This mixture was incubated in a 13×100 mm culture

[11] R. M. BOCK and N. LING, *Anal. Chem.* **26**, 1543 (1954).

tube for 30 min at 40°, then 1·0 ml of 0·13 N sodium hydroxide was added and the mixture poured into a round quartz cuvette for measurement of its fluorescence. All fractions had a fluorescence, but this was augmented 3–5 times in tubes containing appreciable amounts of tyrase. This simple, rapid method showed which fractions could be recombined. The composite fraction was then analyzed for tyrase by one of the more accurate methods outlined above.

(g) *Purification by calcium phosphate gel (step 5, Table 5).* Fractionation by positive adsorption on a calcium phosphate gel was used in some instances. This fractionation was applied to fractions after removal of inert protein at pH 6 or after the second ammonium sulfate precipitation. The calcium phosphate gel[12] contained 37·8 mg of solids per ml. The enzyme solution was dialyzed for at least 4 hr against 100 volumes of 0·01 M potassium phosphate at pH 6·0. The volume was then adjusted to give a protein concentration of 1 per cent; 0·1 volume of the gel was added, the mixture stirred for about 5 min then centrifuged at $6000 \times g$ for 10 min. The supernatant solution was treated with 0·3 volumes of gel, stirred 10 min and centrifuged as before. Most of the tyrase was absorbed on the second portion of gel; this precipitate was washed with 0·01 M potassium phosphate at pH 6·0, and the enzyme eluted by mixing for 10 min with 0·02 M potassium phosphate at pH 7·4, then with 0·05 M potassium phosphate at pH 8·0. These eluates were combined. The enzyme was recovered in a volume about 1·5 times as large as the original volume (Table 5).

(h) *Other fractionation procedures.* A number of other standard fractionation methods were tried in addition to those described above. These procedures were applied to a rice seedling protein fraction obtained between 35–70 per cent saturation with ammonium sulfate (first precipitation). Fractional precipitation by acetone resulted in considerable loss of tyrase with no increase in specific activity. Protamine sulfate (0·01 M potassium phosphate, pH 6·0) was found to remove tyrase from solution; no attempt was made to recover the enzyme from the precipitate. A 2·6 fold purification of tyrase was obtained using alumina C_γ[12] for positive adsorption in the same manner described above for calcium phosphate. Most of the rice seedling proteins, including tyrase, passed through a carboxymethyl-cellulose column (Biorad Cellex-CM) at pH 6·0 (0·02 M potassium phosphate).

Specificity of a Tyrase Preparation

Chromatography on DEAE-cellulose removed practically all the free ammonia from tyrase preparations. It was found that preparations purified in this way formed ammonia and *p*-coumaric acid in equimolar amounts when incubated with tyrosine (see section on stoichiometry below). Other amino-acids were substituted for tyrosine, and ammonia formation was measured, in order to obtain information on the specificity of tyrase (Table 7). Two amino-acids, L-phenylalanine and DL-*m*-tyrosine (i.e. *m*-hydroxy-phenylalanine), induced a more rapid formation of ammonia than did L-tyrosine. The formation of ammonia from L-phenylalanine was expected since Koukol and Conn[1,2] had already demonstrated that barley and other plants contain an enzyme (phenylalanine deaminase) which forms cinnamic acid and ammonia from L-phenylalanine. The deamination of *m*-tyrosine was unexpected; it was investigated further and *m*-coumaric acid identified as the product (see below). There was little, if any, deamination of the other amino-acids. Histidase[9,10] and aspartase[6,7] were either absent or present in very low concentrations.

[12] S. P. COLOWICK, *Methods in Enzymology*, Vol. I, pp. 97, 98. Academic Press, New York (1955).

TABLE 7. DEAMINATION OF VARIOUS AMINO-ACIDS BY A TYRASE PREPARATION*

Amino-acid added	μg of NH_3 found		μmoles of NH_3 formed
	at start	after 3 hr	
L-Tyrosine	4·4	16·6	0·79
DL-*o*-Tyrosine	5·3	6·1	0·05
DL-*m*-Tyrosine	3·0	24·6	1·40
DL-DOPA†	4·7	7·2	0·16
L-Phenylalanine	8·2	72	4·00
L-Aspartic acid	4·1	6·2	0·13
L-Alanine	5·2	6·0	0·05
L-Histidine	4·2	6·2	0·13
L-Tryptophan	4·5	6·5	0·13

* Preparation B (Table 6). The reactions were run in air-filled sealed Thunberg tubes at 40° for 3 hr. Each tube contained 0·61 mg of protein, 150 μmoles of sodium borate (pH 9·0), and 10 μmoles of L-amino acid (or 20 μmole of DL-form) in a total volume of 3·0 ml. The cap of the tube contained 0·3 ml of N sulfuric acid. At the end of the reaction the tubes were cooled in ice, the acid mixed in and a 2 ml aliquot of the mixture removed for estimation of ammonia.

† 3,4-Dihydroxyphenylalanine.

Identification of p-*Coumaric Acid as the Product of the Enzymatic Deamination of Tyrosine*

The product of the tyrase reaction had the same R_f as *trans-p*-coumaric acid in the solvents listed at the foot of Table 1. Tests with the diazotized *p*-nitroaniline spray reagent followed by alkali gave the same colors (orange changing to blue) as were exhibited by an authentic sample. The fluorescence was also the same for both samples; a blue fluorescence was noted on the paper chromatograms when viewed under ultraviolet light (366 mμ) only after the sheet had been sprayed with alkali. The maximum settings of the spectrophotofluorometer were the same, both for activation (350 mμ) and emission (440 mμ), as for *trans-p*-coumaric acid. It is unlikely that *cis-p*-coumaric acid was formed, even transitorally, since it was not observed on paper chromatograms irrigated with solvent A (Table 1) and since *cis-p*-coumarate was not converted to the *trans-* isomer by an active tyrase preparation.

The product of the tyrase reaction was rigorously indentified as *trans-p*-coumaric acid by isolating enough crystalline material for a mixed melting point determination. This was done both with rice and barley preparations. Ten ml of barley stem tyrase purified through step 3 (Table 5), containing 178 units of enzyme and 150 mg of protein, was mixed with 20 ml of 0·15 M sodium borate (pH 9·0) containing 30 mg of L-tyrosine. This mixture was incubated at 40° for 4½ hr, cooled in ice, acidified with 2 ml of 5 N hydrochloric acid, then shaken with 80 ml of ether. The ether phase was separated and back-extracted with 12 ml of 0·1 M sodium bicarbonate. The bicarbonate extract was acidified with 1 ml of 5 N hydrochloric acid, shaken with 15 ml of ether and the ether separated and allowed to evaporate at room temperature. The crystalline residue (9 mg) was recrystallized from water (using charcoal) and the colorless product collected on a glass filter, washed with ice–water and air dried. This gave 7 mg of a crystalline solid, m.p. 219–220°. The melting point was not depressed when the sample was mixed with authentic *trans-p*-coumaric acid, m.p. 219–220°. Both samples evolved gas on melting.

Another crystalline sample was obtained using a rice seedling protein fraction purified only through steps 1 and 2 (Table 5). This tyrase solution (91 ml) contained 100 units of enzyme and 700 mg of protein. The reaction was run essentially as described above. The

crude *p*-coumaric acid, obtained by evaporation of the ether extract, was digested with hot toluene (2 ml), then cooled and 2 ml of 30–60° petroleum ether was added. The solid was filtered out, dried at room temperature, then recrystallized from water as described above. This gave about 4 mg of a colorless crystalline solid m.p. 218–219°. The m.p. was not depressed by admixture with authentic *trans*-*p*-coumaric acid.

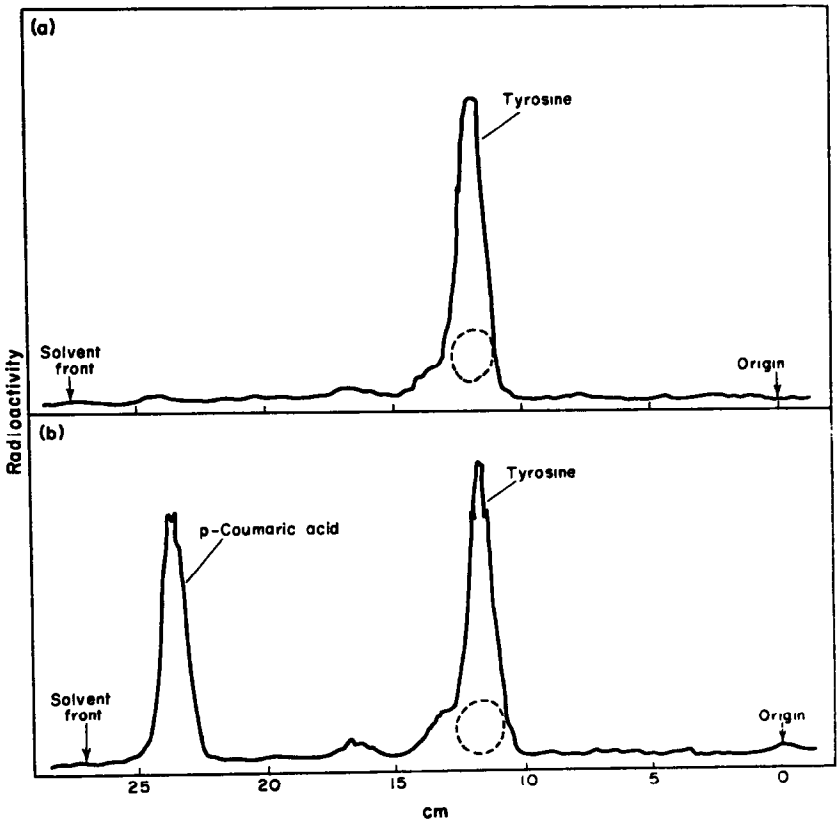

FIG. 7. ENZYMATIC SYNTHESIS OF *p*-COUMARIC ACID-C^{14} FROM L-TYROSINE-C^{14}.

The enzyme was a barley-stem protein fraction precipitated at 40–60 per cent saturation on the second ammonium sulfate fractionation. Each ml of reaction mixture contained 320 μg (1·6 μc) of L-tyrosine-C^{14} (uniformly labelled), 70 μmoles of sodium borate (pH 8·8), 4·4 mg of protein and 7·9 units of tyrase. Samples were chromatographed on Whatman No. 1 paper using *n*-butanol-acetic acid-water (4 : 1 : 1·8) as the irrigant. The chromatograms were scanned by a Nuclear-Chicago Actigraph II. (a)—Tracing of scanner record of 8 μl of reaction mixture, sampled immediately after mixing. (b)—Tracing of scanner record of 15 μl of reaction mixture, sampled after 3 hr incubation at 40°. The broken circles show position of tyrosine as revealed by ninhydrin.

Experiments with L-tyrosine-C^{14} (uniformly labelled) showed that C^{14}-labelled *p*-coumaric acid was formed as the major product and with practically the same specific activity as the tyrosine. The first experiment (Fig. 7) showed one major product with the mobility of *p*-coumaric acid in the irrigant employed. A possible minor component may be an impurity in the tyrosine. The bulk of the reaction mixture was acidified with hydrochloric acid, extracted by ether, and aliquots of the extract were plated and counted under a Nuclear-Chicago gas-flow counter fitted with a micromil window. This showed 42 per cent of the

C^{14} had been converted to an ether-extractable form (i.e. *p*-coumaric acid). Another aliquot of the ether extract was analyzed for *p*-coumaric acid by ultraviolet absorptiometry The specific activity was calculated to be 92 per cent of that expected assuming all the C^{14} to be in *p*-coumaric acid. A similar experiment was run using enzyme preparation *D* (Table 6). The conversion of substrate was 23 per cent and the specific activity of the *p*-coumaric acid formed was 96 per cent of the expected value. Crude preparations probably contain or generate some endogenous tyrosine.

Identification of m-*Coumaric Acid as the Product of the Enzymatic Deamination of* m-*Tyrosine*

The ether-soluble acid formed by incubating tyrase preparations with DL-*m*-tyrosine had the same mobility in solvent D (Table 1) as authentic *m*-coumaric acid, and it gave the same color changes when the paper was sprayed with diazotized *p*-nitroaniline followed by alkali (yellow changing to red). The ultraviolet absorption spectrum in alkali coincided with that of authentic *trans-m*-coumaric acid (Fig. 8). The identity was firmly established by isolation of enough crystalline product for a mixed melting point test. Five ml of barley stem tyrase purified through step 3 (Table 5), containing 87 units of tyrase and 78 mg of protein, was mixed with 10 ml of 0·15 M sodium borate (pH 9·0) containing 100 mg of DL-*m*-tyrosine. This mixture was incubated at 40° for 4½ hr then worked up as described above for isolation of *p*-coumaric acid. The crystalline residue (10 mg) from evaporation of the ether extract was recrystallized from water. This gave 6 mg of colorless crystals m.p. 194–196°; the m.p. was not changed by mixing with authentic *m*-coumaric acid (m.p. 194–196°).

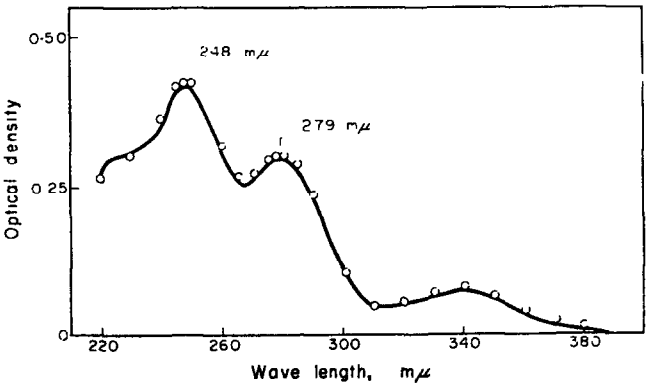

FIG. 8. ULTRAVIOLET ABSORPTION SPECTRUM OF *m*-COUMARIC ACID.
The solid line is for the synthetic material (3·3 μg per ml of 0·05 N sodium hydroxide); the circles are for an ether-extractable acid fraction formed by the action of a barley-stem tyrase on DL-*m*-TYROSINE.

Stoichiometry of the Enzymatic Deamination of Tyrosine and m-*Tyrosine*

Both reactions were found to form one mole each of ammonia and the corresponding coumaric acid for each mole of the amino acid used. The data obtained for the deamination of L-tyrosine by three different enzyme preparations are collected in Table 8. The disappearance of tyrosine was measured only when the amount present initially was low enough to permit utilization of a reasonably large fraction. Enzymes from rice or barley formed *p*-coumaric acid and ammonia in equimolar amounts ±10 per cent. This was also true for the formation of *m*-coumaric acid and ammonia from DL-*m*-tyrosine by a barley-stem preparation (Table 9).

TABLE 8. STOICHIOMETRY OF TYROSINE DEAMINATION

Enzyme*	Protein added (mg)	Incubation time (hr)	μmoles of L-tyrosine		μmoles change on incubation		
			at start	at finish	tyrosine	*p*-coumaric acid	NH_3
A	0·72	5	16·5	—	—	+0·79	−0·77
A	0·72	5	nil	—	—	nil	+0·01
B	0·60	3	1·66	1·01	−0·65	+0·61	+0·54
B	0·60	3	1·53	—	—	+0·97	+0·84
C	0·51	3	1·65	1·05	−0·60	+0·78	+0·87
C	0·51	3	3·31	2·39	−0·92	+1·04	−1·18
C	0·51	3	13·2	—	—	+1·41	−1·50

* The enzyme preparations are described in Table 4. The reactions were run in Thunberg tubes as described in footnote to Table 7. After cooling and acidification one aliquot was analysed for ammonia and another fractionated by ether extraction; *p*-coumaric acid was measured in the ether extract and L-tyrosine in the aqueous residue (see text for further details).

Effect of Inhibitors and Activators on Tyrase

Table 10 summarizes the results of a series of experiments where sulfhydryl compounds or sulfhydryl reagents were added to tyrase reactions. The reactions were run about 1 hr at 40° then the amount of *p*-coumaric acid formed was measured. The relative rate is the amount of *p*-coumaric acid formed, expressed as the percent of the amount formed in a control test where no sulfhydryl compound or inhibitor was added. The sulfhydryl compounds caused a marked decrease in formation of *p*-coumaric acid by crude enzyme preparations, but as the enzyme was purified this effect disappeared. Tyrase was strongly inhibited by *p*-chloromercuribenzoate, but not by iodoacetamide.

Various salts were tested at 0·05 and 1·1 mM concentration, especially those of divalent metals, such as Ca, Mg, Mn, Cu, Co and Fe. None of these gave a marked stimulation; cupric sulfate was rather toxic. Quite large concentrations of ammonium sulfate (0·11 M) had no effect. No evidence could be obtained for participation of a divalent cation in the tyrase reaction. It was not inhibited by EDTA (ethylenediamine tetra-acetate). There is a possibility that a firmly bound cation is involved. The role of monovalent ions was not investigated. The reaction mixtures always contained fairly high concentrations of sodium and potassium ions.

TABLE 9. STOICHIOMETRY OF *m*-TYROSINE DEAMINATION

μmoles of DL-*m*-tyrosine		μmoles change on incubation		
at start	after incubation	*m*-tyrosine	*m*-coumaric acid	NH_3
2·76	2·08	−0·68	+0·72	+0·76
5·52	4·45	−1·07	+0·98	+0·96
22·1	—	—	+1·97	+1·90

Enzyme preparation C (Table 6) was used. The reaction mixture (2 ml) contained 0·34 mg of protein and 150 μmoles of sodium borate (pH 9·0). The reactions were run for 3 hr at 40° in sealed Thunberg tubes with 0·2 ml of N sulfuric acid in the cap. The reaction was terminated by cooling the tubes in ice and mixing in the acid. One aliquot was analysed for ammonia and another was fractionated by ether; *m*-coumaric acid was measured in the ether extract and *m*-tyrosine in the aqueous residue (see text for further details).

TABLE 10. EFFECT OF SULFHYDRYL COMPOUNDS AND INHIBITORS ON BARLEY TYRASE PREPARATIONS AT DIFFERENT STAGES OF PURIFICATION*

Specific activity of enzyme preparation	Compound added	μmoles added per ml	Relative rate (% of control)
0·13	Reduced glutathione	10	20
	$CH_2SH.CH_2OH$	10	41
	$CH_2SH.COONa$	10	18
	Iodoacetamide	7	101
	p-Cl-Hg-Benzoate	0·1	57
	" "	0·01	91
1·8	Reduced glutathione	10	49
	" "	1	94
	Cysteine	10	58
	"	1	82
	"	0·1	91
10·7	Reduced glutathione	10	120
	" "	1	98
	Cysteine	10	104
	"	1	116
	Iodoacetamide	70	89
	"	7	99
18·4	Cysteine	10	90
	"	1	121
	p-Cl-Hg Benzoate	0·1	11
	" "	0·001	93

* The preparation with the lowest specific activity is from etiolated barley seedlings purified through steps 1 and 2, the other preparations are from stems of field grown barley purified through steps 3, 4 and 6 respectively (Table 5).

Tyrase was not inhibited by fluoride, but it was inhibited fairly readily by cyanide (Table 11). Pyridoxal phosphate had no effect on the tyrase reaction whether α-keto acids were added or not. Other cofactors that were without effect include folic acid, adenosine triphosphate and coenzyme A.

Attempts to Reverse the Tyrase Reaction

Two attempts to demonstrate reversal of the tyrase reaction gave negative results. In these experiments *p*-coumarate was incubated with a large excess of ammonium sulfate in the presence of tyrase. In the first experiment the *p*-coumarate concentration was measured;

TABLE 11. INHIBITION OF TYRASE BY CYANIDE*

Enzyme preparation	Specific activity	μmoles of KCN/ml	Relative rate (% of control)
Rice seedling protein purified by steps 1 and 2.	0·58	30	27
		2	91
Preparation D, Table 6.	18·2	10	18
		1	47
		0·1	74

* Reactions run one hour at 40° in 0·07 M sodium borate at pH 8·8.

there was no change even after incubation with 8000 times the theoretical amount of ammonia for one hour either at pH 7·4 or at pH 9·0. In the second experiment *p*-coumaric acid-α-C^{14} was used and measurements were made of the C^{14} fixed in a form not readily extracted into ether. This gave a fairly sensitive method of detecting tyrosine formation. The molar ratio of ammonia to *p*-coumarate was 38 : 1 and the reaction was run at pH 8·8 with a highly purified sample of barley stem tyrase (preparation D, Table 6). No formation of tyrosine could be detected although the counting method was sensitive enough to measure conversion of 0·0005 per cent of the *p*-coumarate.

Comparative Studies on Formation of p-*Coumaric Acid,* m-*Coumaric Acid and Cinnamic Acid by Tyrase Preparations*

Partially purified preparations of tyrase from barley stems have been shown to catalyze the deamination of three aromatic amino-acids, i.e. L-tyrosine, DL-*m*-tyrosine and L-phenylalanine (see above; Koukol and Conn[1,2]). These three activities were found at all stages of purification tested so far. However, the studies reported in this section suggest that tyrase is distinct from the enzymes (or enzyme) catalyzing the deamination of *m*-tyrosine or phenylalanine, although this has not been established beyond doubt.

TABLE 12. VARIABILITY IN RELATIVE RATES OF FORMATION OF *m*- AND *p*-COUMARIC ACIDS

Enzyme preparation*	Buffer	pH	μmoles/mg protein/hr		*meta para* ratio†
			p-coumaric acid	*m*-coumaric acid	
B	Potassium phosphate	7·4	0·27	0·52	1·9
B	Sodium borate	8·8	0·43	1·35	3·1
B	„ „	9·6	0·22	1·30	5·9
C	Potassium phosphate	7·4	0·64	1·73	2·7
C	Sodium borate	8·8	0·84	6·00	7·1
C	„ „	9·6	0·54	4·75	8·8

* Enzyme preparations described in Table 6. Each 1·4 ml of reaction mixture contained 100 μmoles of buffer, 12 μ moles of sodium hydroxide, 1·0 mg of L-tyrosine (or 2·0 mg of DL-*m*-tyrosine) and 65 μg of protein.

† In another experiment barley stem protein fractions, purified through step 3, from plants harvested on March 19, April 4 and April 16 (see Table 4), were tested at pH 8·8 and 40° with 0·1 per cent L-tyrosine or 2·0 per cent DL-*m*-tyrosine. The *meta*/*para* ratios found were 3·4, 4·2 and 6·0 respectively.

The pH curve for the formation of *p*-coumaric acid from tyrosine is displaced about one pH unit towards the acid side relative to the curve for formation of *m*-coumaric acid from *m*-tyrosine (Fig. 4). Because of their rather broad peaks these curves overlap and the optima coincide at pH 8·8–9·0. However, the ratio of these reactions varies with the pH. The data in Table 12 show the variation for two different enzyme preparations. It is evident that the ratio of the two activities varies with the pH and also with the enzyme preparation. It seems likely two enzymes are involved and their relative concentration changes as the plant matures (see footnote to Table 12).

The Michaelis–Menten constants (Fig. 9) show that the affinity of tyrase for tyrosine is about 25 times as great as the affinity of the other enzyme for *m*-tyrosine. Koukol and Conn[1,2] have obtained a Km (Michaelis–Menten constant) for phenylalanine deaminase approximately twice that reported here for tyrase. They have observed a strong inhibition

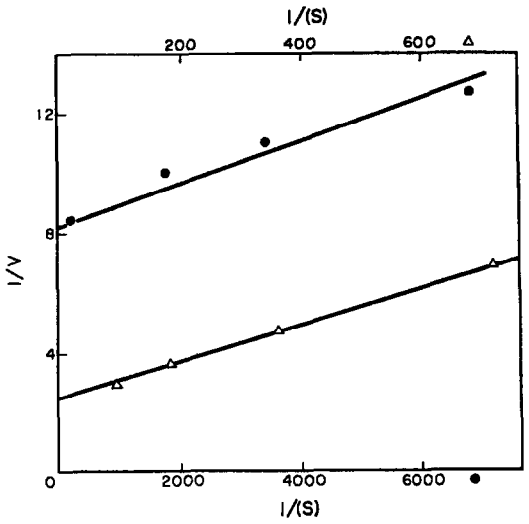

FIG. 9. EFFECT OF SUBSTRATE CONCENTRATION ON THE RATE OF FORMATION OF *m*- AND *p*-COUMARIC ACIDS FROM THE CORRESPONDING ISOMERS OF TYROSINE.

Preparation C (Table 6) was incubated with the substrates 40° and pH 9·0. V = relative rate. (S) = substrate concentration in mole/l. The upper line (filled circles) is for formation of *p*-coumaric acid from L-tyrosine, the lower line (triangles) for formation of *m*-coumaric acid from DL-*m*-tyrosine. Concentrations for *m*-tyrosine were calculated on the assumption that only one enantiomer was active. The Michaelis–Menten constant (Km) for L-tyrosine was found to be $0{\cdot}84\times10^{-4}$ from this data; other determinations gave values of $0{\cdot}74\times10^{-4}$ and $1{\cdot}0\times10^{-4}$. *Km* for *m*-tyrosine was calculated to be $2{\cdot}3\times10^{-3}$; another determination gave a value of $1{\cdot}3\times10^{-3}$.

of phenylalanine deaminase by L-tyrosine. Tyrosine also inhibits the formation of *m*-coumaric acid from *m*-tyrosine (Table 13). On the other hand, the formation of *p*-coumaric acid from L-tyrosine was not inhibited by either L-phenylalanine or DL-*m*-tyrosine (Table 14) even when these substrates were present in a concentration high enough to give an appreciable inhibition, assuming they were competing with tyrosine for the same active center. This supports the view that tyrase is distinct from the enzymes (or enzyme) acting on phenylalanine or *m*-tyrosine.

TABLE 13. EFFECT OF L-TYROSINE ON THE FORMATION OF *m*-COUMARIC ACID FROM DL-*m*-TYROSINE*

Enzyme	μmoles/ml		Relative rate†
	DL-*m*-tyrosine	L-tyrosine	
C (Table 6)	28	5·5	10
D (Table 6)	46	4·6	32

* Run at 40° and pH 8·8 on 0·07 M sodium borate.

† Rate of formation of *m*-coumaric acid as percentage of the control containing no L-tyrosine. The *m*-coumaric acid was extracted by ether and measured at 248 mμ and a correction applied for the absorption by *p*-coumaric acid. This correction was determined in a parallel experiment in which L-tyrosine (but not *m*-tyrosine) was added. This is possible since *m*-tyrosine does not inhibit formation of *p*-coumaric acid (see Table 14).

TABLE 14. EFFECT OF OTHER AROMATIC AMINO-ACIDS ON FORMATION OF *p*-COUMARIC ACID FROM TYROSINE*

Enzyme preparation	Aromatic amino-acid added	μmoles/ml		Relative rate†
		L-tyrosine	Other aromatic amino-acid	
Barley stem protein purified through step 3 Specific activity = 1·8	L-Phenylalanine	3·2	7·2	98
	,,	3·2	1·4	100
Preparation C, Table 6 Specific activity = 11·4	L-Phenylalanine	5·5	11·4	103
	,,	5·5	5·7	102
	DL-*m*-Tyrosine	5·5	28	102
Preparation D, Table 6 Specific activity = 18·4	L-Phenylalanine	4·6	10·1	100
	DL-*m*-Tyrosine	4·6	46	95
	DL-DOPA	4·6	8·1	106

* Run at 40° and pH 8·8 on 0·07 M sodium borate.
† Rate as percentage of the control tube containing L-tyrosine as the only aromatic amino-acid.

Table 15 shows the results of an experiment done in collaboration with Dr. Jane Koukol. Acetone powders from several species were assayed at the same time and under the same conditions for both phenylalanine deaminase and tyrase. The acetone powders from legumes have quite a low tyrase content compared to phenylalanine deaminase; this ratio was not nearly so wide for grasses. It is not proved that legumes contain any tyrase. The figures in Table 15 are maximum values since the ether-soluble C^{14}-labelled acid obtained, in the tyrase assay for acetone powders of legumes (see footnote to Table 15), has not been rigorously identified as *p*-coumaric acid. The ratio of phenylalanine diaminase to tyrase may be even wider than that reported for the legumes.

TABLE 15. COMPARISON OF THE PHENYLALANINE DEAMINASE AND TYRASE ACTIVITIES OF ACETONE POWDERS FROM SEVERAL SPECIES

Source of acetone powder*	μmoles/hr/g†		cinnamic/*p*-coumaric ratio
	cinnamic acid	*p*-coumaric acid	
Barley seedlings, shoots	8·0	2·3	3·5
,, ,, roots	12·2	3·4	3·6
Wheat seedlings, shoots	9·0	2·5	3·6
Rice seedlings, whole	6·5	1·9	3·4
,, ,, roots	31·4	10·4	3·0
White sweet clover, tops	4·5	0·14	32·0
Alfalfa stems	11·0	0·23	48·0

* The alfalfa powder was prepared from *Medicago sativa* L. var. Caliverde, using defoliated stems of shoots about 36 cm long. The other powders were made from the the varieties listed in Table 2.
† Cinnamic acid formation was measured by Dr. J. Koukol using a radiotracer assay method.[1,2] *p*-Coumaric acid formation was measured fluorometrically for barley, rice and wheat, and by a radiotracer method for sweet clover and alfalfa. The tracer assay was based on measurement of the total C^{14}-labelled, ether-extractable acids formed on incubation of L-tyrosine-C^{14} with the acetone powder. All reactions were run at pH 8·8 and 40°.

DISCUSSION

The results of this investigation support the view that lignin and related phenylpropanoid compounds are formed from the aromatic amino acids via the phenolic cinnamic acids,[3] especially when the findings of Koukol and Conn[1,2] are also considered. In view of these new results the scheme suggested previously[3,5,13] for formation of lignin and related compounds from shikimic acid has been revised (Fig. 10). The acids with the

$$-CH=CH-COOH$$

side chain are now shown as originating by direct deamination of the corresponding amino-acid rather than by dehydration of the corresponding α-hydroxy acid. The conversion of the α-hydroxy acids to lignin and related compounds by living plants can be explained by their ready conversion to the amino-acids.[14] There is no longer any reason to postulate these hydroxy acids as having physiological significance in lignification, although it is still possible. All tracer experiments in this field, known to the author, can be explained by Fig. 10, where the α-hydroxy acids are involved in side reactions, if at all.

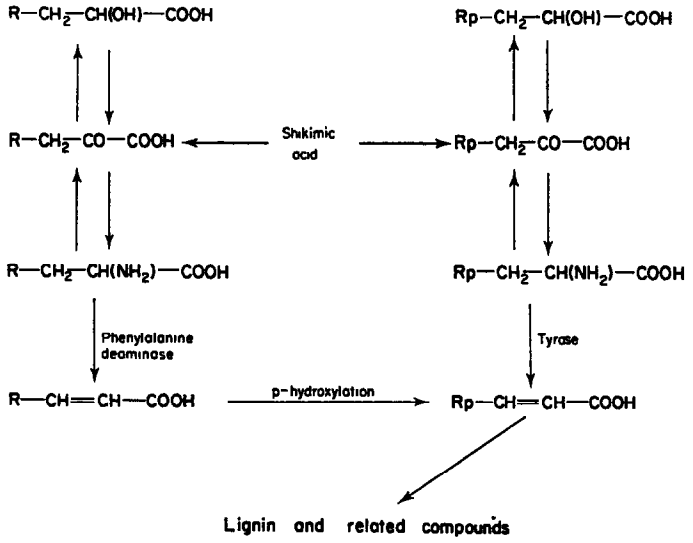

FIG. 10. REVISED SCHEME FOR FORMATION OF LIGNIN AND RELATED COMPOUNDS FROM SHIKIMIC ACID.
R = phenyl; Rp = *p*-hydroxyphenyl.

Conversion of tyrosine-C^{14} to lignin occurs readily only in certain species.[3,15,16] For example, grasses can easily convert tyrosine to lignin while legumes cannot. It is possible that tyrase is necessary for conversion of tyrosine to lignin, since it is found in substantial amounts in grasses with much less, if any, in legumes. This fact agrees with the findings of tracer experiments on living plants, but a more thorough study should be made of the correlation between the ability of a given plant to convert tyrosine to lignin and its tyrase

[13] D. R. MCCALLA and A. C. NEISH, *Can. J. Biochem. and Physiol.* 37, 537 (1959).
[14] O. L. GAMBORG and A. C. NEISH, *Can. J. Biochem. and Physiol.* 37, 1277 (1959).
[15] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. and Physiol.* 34, 769 (1956).
[16] S. A. BROWN, *Can. J. Botany* (in press).

content. At present the correlation is good enough to suggest that *p*-coumaric acid is an obligate intermediate between tyrosine and lignin.

Acerbo *et al.*[17] found that C^{14}-labelled *p*-hydroxyphenyl pyruvic acid was converted to lignin by sugar cane. Its effectiveness was not compared with that of tyrosine or any other potential lignin precursor. However, since sugar cane contains tyrase (Table 2) this result might be explained by the following sequence of reactions:

p-hydroxyphenyl pyruvic acid ⟶ L-tyrosine ⟶ *trans-p*-coumaric acid ⟶ lignin.

The keto-acid fed would be expected to form C^{14}-labelled L-tyrosine.[14] This is presumably accomplished by transamination reactions such as are of general occurrence in all organisms, not merely in lignifying tissues. There is no reason to select *p*-hydroxyphenyl pyruvic acid as an especially important lignin precursor, as has been done by Nord and Schubert,[18] since it is ineffective in many plants.[3,5,19] *p*-Hydroxyphenyl pyruvic acid is probably just an intermediate in tyrosine synthesis and thus convertible to lignin only by plants which can convert tyrosine to lignin. If one is to select an important lignin precursor, *trans-p*-coumaric acid is a much better choice since it is a central intermediate in the formation of lignin and related compounds[3] but is not involved in protein synthesis.[14]

It is difficult to assign a definite physiological role to the deamination of *m*-tyrosine. Neither *m*-tyrosine nor *m*-coumaric acid are of general occurrence in plants. However, it has been recently shown by Winstead and Suhadolnik[20] that *m*-tyrosine may be formed from phenylalanine as a step in the biosynthesis of gliotoxin by *Trichoderma viride*. If this meta-hydroxylation occurred in higher plants, *m*-coumaric acid might be formed and rapidly metabolized to other products. For example, further hydroxylation might give caffeic acid and thus, eventually, lignin.

MATERIALS AND METHODS

Chemicals

L-Tyrosine was obtained from Nutritional Biochemicals Corp. DL-*o*-Tyrosine and DL-*m*-tyrosine were purchased from the H. M. Chemical Co. An additional sample of DL-*m*-tyrosine, synthesized by the procedure of Sealock *et al.*[21] was purified by three recrystallizations from dilute acetic acid–ethanol. These tyrosines all gave a single spot after paper chromatography with *n*-butanol-acetic acid-water (4 : 1 : 1·8) as the irrigant. The sheets were sprayed with ninhydrin and with diazotized *p*-nitroaniline.[22,23] The commercial sample of *m*-tyrosine had an ultraviolet absorption spectrum which agreed closely with that of the synthetic sample.

Some *trans-m*-coumaric acid was prepared from *m*-hydroxybenzaldehyde[24] and purified by two recrystallizations (charcoal) from water; this gave a colorless crystalline solid, m.p. 194–196°. *trans-p*-Coumaric acid (Aldrich Chemical Co.) was purified by recrystallization from water; the final m.p. was 219–220°. A sample of *cis-p*-coumaric acid was prepared by ultraviolet irradiation of a solution of the sodium salt of *trans-p*-coumaric

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acid[25] as follows. A sample (1·87 mM) of *p*-coumaric acid was dissolved in a slight excess of 0·5 N sodium hydroxide and the volume adjusted to about 10 ml. This solution (pH 9) was put in a 50 ml beaker and irradiated for 18 hr by a 100 W, 366 mμ Hg source shining directly on the surface of the solution, from a distance of about 6 in. When the irradiation was finished an excess of hydrochloric acid was added, the product extracted by shaking with ether, the extract evaporated and the residue recrystallized from toluene. This gave 110 mg (36 per cent) of *cis-p*-coumaric acid. It was a colorless solid, m.p. 128–129°. A portion (42 mg) of this was purified further by chromatography on a 2·2 × 29 cm column of Whatman cellulose powder using 2 per cent acetic acid (Solvent A, Table 1) for elution. *p*-Coumaric acid was detected in the eluate by spotting it on paper and spraying with diazotized *p*-nitroaniline[22,23]. The fractions containing the fast moving band were combined and the ether-soluble acids recovered by extraction, and recrystallized from toluene as described above; 20 mg of purified *cis-p*-coumaric acid were obtained, m.p. 131°. This sample was found to be practically free of the *trans-* isomer by chromatographing with solvent A (Table 1).

L-Tyrosine-C^{14} (uniformly labelled) was purchased from the Volk Radiochemical Co.; *p*-coumaric acid-α-C^{14} was prepared by the malonic acid condensation; *p*-hydroxyphenyl pyruvic acid was prepared by the procedure of Billek and Hermann[26] and reduced to *p*-hydroxyphenyl lactic acid by sodium amalgam.[5] The DL-*p*-hydroxyphenyl lactic acid (m.p. 144–145°) was isolated by ether extraction and recrystallized, first from water, then from toluene–acetic acid.

Other chemicals were of reagent grade, or the best grade commercially available. Deionized distilled water was employed for preparation of solutions used in enzyme investigations.

Culture of Plants

Seeds were soaked overnight in four volumes of tap water enriched by addition of calcium nitrate, magnesium sulfate and potassium dihydrogen phosphate—each at 0·2 mM concentration. Air was bubbled through the mixture. The soaked seeds were washed with tap water by decantation, then drained and spread on cheese cloth for germination. The cheese cloth was laid on a perforated nichrome support inside a plastic dishpan. Tap water, enriched as above, was added until the level was just up to the seeds. The pan was covered with a sheet of glass; this was removed after 2–4 days when germination was general. Etiolated seedlings were obtained in a dark cabinet, non-etiolated seedlings by placing the pans underneath a bank of four 40 W "cool-white" fluorescent light tubes. The lights were about 17 in. from the seeds and 12 hr of illumination were given daily.

In addition to seedlings, plants collected from outdoors were used. A field of barley near the laboratory was particularly useful as a source of tyrase.

Analytical Methods

Ultraviolet absorption measurements were made with a Beckman Model DU spectrophotometer. Fluorescence was measured with an Aminco–Bowman spectrophotofluorometer adapted to use round silica cuvettes (Aloe Scientific Co.). These relatively cheap round cuvettes were as good as the square cuvettes for the fluorometric determination of

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p-coumaric acid. Protein was measured, by absorption at 280 mμ,[27] on extracts that had been purified by dialysis. Ammonia was determined colorimetrically after steam distillation from sodium tetraborate; the ammonia was caught in dilute boric acid (0·1 m moles), the distillate diluted to 9 ml and 1·0 ml of Nessler's reagent[28] added. The optical density at (450 mμ) was then determined using a Coleman junior spectrophotometer.

In studies on stoichiometry (Tables 8 and 9), tyrosine and coumaric acid were separated from each other by ether extraction, then measured by ultraviolet absorption, as follows. The acidified reaction mixture was extracted with three successive half-volume portions of ether, the combined ether extracts were evaporated to dryness by an air stream at room temperature, the residue was dissolved in 0·05 N sodium hydroxide and the optical density determined at 248 mμ, for *m*-coumaric acid, or at 333 mμ, for *p*-coumaric acid (Figs. 1 and 8). The aqueous residue from the ether extraction was neutralized, partially evaporated by an air stream to remove dissolved ether, then adjusted to a known volume with enough sodium hydroxide to give a final concentration of 0·05 N and the optical density determined at 240 mμ for tyrosine or at 238 mμ for *m*-tyrosine. The molar extinction coefficients in 0·05 N sodium hydroxide at these peaks were 22,500 for *p*-coumaric acid, 20,300 for *m*-coumaric acid, 9900 for tyrosine and 6850 for *m*-tyrosine.

Quantitative Assay for Tyrase

When acetone powders were assayed, *p*-coumaric acid was measured fluorometrically. A sample (0·05–0·20 g) of the acetone powder was suspended in 3 ml of a 0·1 per cent solution of L-tyrosine in 0·10 M sodium borate (pH 8·8–9·0) in an 18 × 150 mm culture tube and incubated for 30 to 60 min at 40°. Water (7 ml) was added and the debris removed by filtration with suction through a 7 cm disc of Whatman No. 1 paper. The residue was washed with 2–3 ml of water, the filtrate acidified by 0·2 ml of 5 N hydrochloric acid, then shaken with diethyl ether (10 ml) in a glass stoppered tube with a conical bottom. The aqueous phase was removed with a pipette and discarded. The ether extract was poured into a 20 ml beaker, evaporated to dryness by an air stream at room temperature, the residue dissolved in a suitable volume (5–50 ml) of 0·05 N sodium hydroxide and the *p*-coumarate in this solution estimated fluorometrically as described in the next paragraph. A "zero-time" control was run in parallel; this was exactly as described except the incubation period was omitted. The difference between the fluorescent readings of this control and the sample was proportional to the *p*-coumarate formed during the incubation, and thus to the tyrase present.

p-Coumaric acid is known to fluoresce in dilute aqueous alkali; the maximum instrument setting for activation is 350 mμ and the maximum emission is at 440 mμ.[29] A linear relation between fluorescence and *p*-coumarate concentration was found up to about 2 μg/ml but at higher concentrations there was strong quenching (see Fig. 2). For most fluorescence readings two concentrations are possible. It was necessary to dilute the solutions until they contained less than 2 μg of *p*-coumaric acid per ml. If there was any doubt, readings were made at several concentrations to be sure this condition was met. As the instrument was rather unstable, frequent readings of a standard were made during a set of determinations. A stock solution of *p*-coumaric acid (100 μg/ml) in dilute sodium bicarbonate (1 mM) was stored in a refrigerator. Aliquots of this were diluted 100-fold with 0·05 N

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sodium hydroxide to give the working standard; this was freshly prepared for each set of analyses.

The *p*-coumaric acid formed by solutions of crude or partially purified tyrase was measured either fluorometrically or by absorption at 333 mμ (Fig. 1). Usually 1·4 ml of reaction mixture contained 1·4 mg of L-tyrosine, 100 μmoles of sodium borate at pH 8·8, and the enzyme being tested. This mixture was incubated in a glass stoppered tube at 40° for 30–60 min, then acidified with 2·3 ml of 0·15 M hydrochloric acid, cooled in an ice bath and shaken manually with 2·0 ml of ice-cold ether for 1 min. The phases were allowed to separate at 0°, 1·0 ml of the ether layer pipetted into an 18 × 150 mm culture tube and the ether evaporated by an air stream at room temperature. The dry residue was dissolved in a suitable volume of 0·05 N sodium hydroxide. *p*-Coumarate was measured in this solution fluorometrically as described in the preceding paragraph or, by its absorption at 333 mμ. A solution of 1·00 μg of *p*-coumaric acid per ml, in 0·05 N sodium hydroxide had an optical density of 0·137 at 333 mμ. A linear relation between optical density and concentration was found up to the highest optical density measured (i.e. 1·50).

The recovery of *p*-coumaric acid in the 1·0 ml of ether layer taken for analysis was 70 ± 3 per cent. The fraction recovered was independent of the concentration of *p*-coumaric acid but depended on the relative volumes of ether and water. An appreciable fraction of the ether dissolves in the aqueous phase, so the ether layer had a volume of only about 1·4 ml. In tyrase assays a correction was applied for the *p*-coumaric acid not recovered by the extraction; zero-time controls were also run.

The fluorometric and absorptiometric methods gave concordant results with the partially purified enzyme preparations listed in Table 6. The fluorometric method was used in early work with crude preparations and found to agree ±10 per cent with a colorimetric method based on diazotized *p*-nitroaniline.[30] The more precise absorptiometric method was used with preparations purified through the second ammonium sulfate precipitation (Table 6).

An assay based on measurement of the rate of ammonia formation could be used with tyrase solutions purified by chromatography on DEAE-cellulose columns although it failed with crude preparations because of the high blanks. The assays based on measurement of *p*-coumaric acid were about 20 times as sensitive.

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