

The *in Vitro* Biosynthesis of Taxiphyllin and the Channeling of Intermediates in *Triglochin maritima**

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Adrian J. Cutler, Wolfgang Hösel†, Margarete Sternberg§, and Eric E. Conn¶

From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

The *in vitro* biosynthesis of the cyanogenic glucoside taxiphyllin has recently been demonstrated in *Triglochin maritima* (Hösel, W., and Nahrstedt, A. (1980) *Arch. Biochem. Biophys.* 203, 753-757). We have now studied in more detail the multistep conversion of tyrosine into *p*-hydroxymandelonitrile, the immediate precursor of taxiphyllin, catalyzed by microsomes isolated from dark-grown seedlings. The biosynthetic pathway involves *N*-hydroxytyrosine, *p*-hydroxyphenylacetaldoxime, and *p*-hydroxyphenylacetoneitrile. In marked contrast to an analogous pathway in *Sorghum bicolor*, *p*-hydroxyphenylacetoneitrile is the best substrate for cyanide production ($V_{\max} = 224$ nmol/h/g, fresh wt) and the physiological substrate tyrosine is the poorest ($V_{\max} = 18.8$ nmol/h/g, fresh wt). The substrates exhibit alkaline pH optima between 7.5 and 9, and all except tyrosine show pronounced substrate inhibition. We have found that *p*-hydroxyphenylacetoneitrile generated *in situ* from tyrosine is free to equilibrate by diffusion with exogenous material. On the other hand, neither *N*-hydroxytyrosine nor *p*-hydroxyphenylacetaldoxime will readily exchange with exogenous intermediates. We consider both *N*-hydroxytyrosine and *p*-hydroxyphenylacetaldoxime to be channeled in *T. maritima*, whereas in *S. bicolor* *N*-hydroxytyrosine and *p*-hydroxyphenylacetoneitrile are channeled and the aldoxime is freely exchangeable.

The *in vitro* biosynthesis of the aromatic cyanogenic glucoside taxiphyllin in *Triglochin maritima* has recently been demonstrated by Hösel and Nahrstedt (1). *T. maritima* is a salt-tolerant grass found near seacoasts and inland marshes where the soil is alkaline. Taxiphyllin is the epimer of dhurrin, the cyanogenic glucoside of *Sorghum bicolor*. The *in vitro* biosynthesis of dhurrin in etiolated sorghum tissue has been extensively studied (2-4) and one of its most notable features was a marked channeling of pathway intermediates (5). A variety of enzymatic systems has been found to exhibit this phenomenon, including the conversion of L-phenylalanine to hydroxycinnamic acids in cucumber cotyledons (6), the conversion of orotic acid to uridine monophosphate in mouse Ehrlich ascites cells (7), and the conversion of carbon dioxide to carbamylaspartate in *Saccharomyces cerevisiae* (8). The

subject of channeling and other functional roles of multienzyme complexes in metabolism have been discussed by Welch (9).

We present here a general characterization of taxiphyllin biosynthesis in *T. maritima* (see Fig. 1) and a study of the system's channeling properties.

MATERIALS AND METHODS

Chemicals—L-[U-¹⁴C]Tyrosine (specific activity, 530 mCi/mmol) and L-[3,5-³H]tyrosine (42 Ci/mmol) were purchased from Amersham. Aliquots of L-[3,5-³H]tyrosine were dried under nitrogen before stock solutions were prepared for enzyme assays. *N*-Hydroxytyrosine was obtained as described previously (10). NADPH was obtained from Sigma. All other chemicals were of reagent grade or better.

Chemical Synthesis—Radiolabeled intermediates were chemically synthesized from L-[U-¹⁴C]tyrosine.

The synthesis of *p*-hydroxy[U-¹⁴C]phenylacetaldoxime involved dissolving 5 mg of ninhydrin in 300 μ l (28 nmol, 15 μ Ci) of L-[U-¹⁴C]tyrosine and heating this mixture for 20 min at 100 °C. The product *p*-hydroxy[U-¹⁴C]phenylacetaldehyde was immediately purified by silicic acid thin layer chromatography using toluene:ethyl acetate (4:1, v/v) as the developing solvent. After elution of the radioactive product from the silica gel with ethyl acetate, the dry aldehyde was immediately treated with 10 ml of 1 mg/ml of hydroxylamine in 1 M K₂HPO₄ solution. After 10 min the product was extracted into ethyl acetate. *p*-Hydroxy[U-¹⁴C]phenylacetaldoxime was purified in the silicic acid system used above and also on pre-coated 0.1-mm cellulose TLC¹ sheets (E. Merck) with butanol:ammonia, 29% (4:1, v/v) as the developing solvent. In all cases radioactive materials were located by radioscanning. The overall yield of this synthesis was 15%.

p-Hydroxy[U-¹⁴C]phenylacetoneitrile was obtained by the decarboxylation of *p*-hydroxy[U-¹⁴C]phenylpyruvic acid oxime. *p*-Hydroxy[U-¹⁴C]phenylpyruvic acid was synthesized as described previously (11) from 100 μ Ci of L-[U-¹⁴C]tyrosine except that no cold carrier was added. The radioactive product was diluted with 6.6 μ mol of unlabeled *p*-hydroxyphenylpyruvic acid and reacted with 48 μ mol of hydroxylamine hydrochloride at room temperature for 30 min. The aqueous solution also contained 50 μ mol of K₂HPO₄ in a total volume of 2.2 ml. The oxime solution was then refluxed under nitrogen for 2.5 h. The product *p*-hydroxy[U-¹⁴C]phenylacetoneitrile was purified by preparative TLC on silicic acid with toluene:ethyl acetate (4:1, v/v) as the developing solvent and with paper chromatography (Whatman No. 3MM) employing butanol:ammonia, 29% (4:1, v/v) as the developing solvent. The overall yield was 23%.

Unlabeled *p*-hydroxyphenylacetaldoxime was prepared from *p*-hydroxyphenylacetaldehyde (12) and hydroxylamine hydrochloride.

Growth of Plants—Seeds of *T. maritima* were soaked for 2 days in aerated tap water. After being thoroughly washed with water the seeds were spread over a moistened glass tray and covered with a transparent plastic film (Saran wrap). The seedlings were allowed to germinate in an illuminated growth chamber for 12 h (at about 27 °C), after which they were covered with aluminum foil and grown in the dark for 3 or 4 days. Seedlings grown entirely in the dark germinated poorly.

Preparation of Microsomes—After removal of all seed coats, 5-10 g of the seedlings (0.5-1 cm in length) were thoroughly homogenized

¹ The abbreviations used are: TLC, thin layer chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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† Supported by a Fellowship from the Deutsches Forschungsgemeinschaft. Present address, Lehrstuhl für Biochemie der Pflanzen, Hindenburgplatz 55, D-4400 Münster, Germany.

§ Supported by the Studienstiftung des Deutschen Volks.

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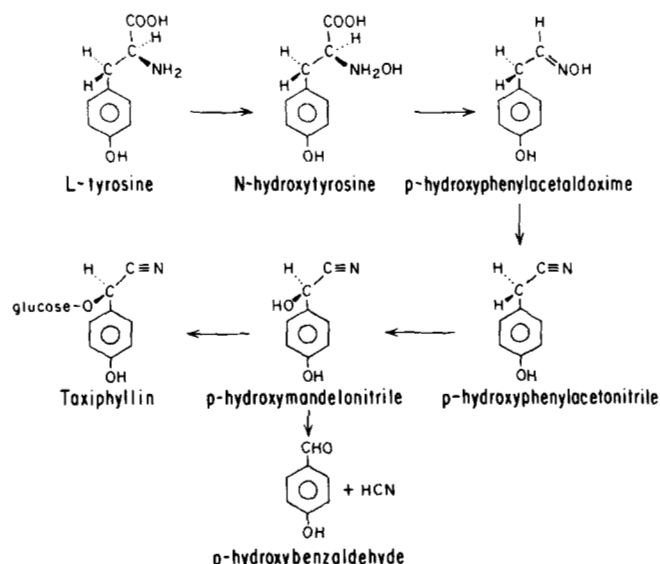


FIG. 1. The biosynthetic pathway for taxiphyllin in *T. maritima*. All the pathway intermediates are shown plus the nonenzymic decomposition of *p*-hydroxymandelonitrile to *p*-hydroxybenzaldehyde and hydrogen cyanide.

in a mortar and pestle with 2 volumes of 0.15 M potassium phosphate buffer at pH 8 containing 5 mM dithiothreitol. The homogenate was centrifuged for 10 min at $10,000 \times g$. The resulting supernatant was spun for 90 min at $100,000 \times g$ in a Sorvall OTD-50 ultracentrifuge. The pellet was resuspended in 4 volumes of 50 mM Tricine buffer, pH 8, with 5 mM dithiothreitol and recentrifuged under the same condition as above. The final pellet was manually homogenized with 0.4 volumes of 50 mM Tricine (pH 8, 5 mM dithiothreitol) using a glass tissue grinder.

Enzyme Assays—The conversion of tyrosine into taxiphyllin-related products by the triglochin microsomal preparations was measured by two methods. One of these techniques was based on the colorimetric determination of HCN released by the spontaneous decomposition of *p*-hydroxymandelonitrile, the final compound formed by the microsomal system (see Fig. 1). The other method for measuring the metabolic activity of triglochin microsomes involved incubation with radiolabeled substrates and separation of the products by TLC.

The assays in which free cyanide was determined were performed in Tricine buffer, pH 8. The reaction mixtures contained 0.1 M Tricine, 800 nmol of NADPH, and varying quantities of substrate. The microsomal protein comprised one-tenth of the total volume, which was 500, 750, or 1000 μ l. The assays were performed in small septum-covered vials at 32 °C for 1 h on a water bath shaker and were terminated by addition of 100 μ l of 2 N H_2SO_4 .

The free HCN released was trapped overnight in a center well containing 250 μ l of 1 N NaOH. Colorimetric determinations were performed by means of the modified Lambert assay (13), except that the total volume of the assay mixtures was reduced by a factor of four to 1875 μ l. As little as 1 nmol of cyanide could be detected.

Radioactive assays typically contained 50 μ mol of Tricine (pH 8), 100 μ l of microsomal protein, 1 μ mol of NADPH, and 312 nmol of tyrosine (1.56 μ Ci) in a total volume of 250–350 μ l. After 1 h at 32 °C, the reactions were terminated by addition of 100 μ l of 5 N acetic acid, or 100 μ l of 95% ethanol when the reaction mixtures contained *p*-hydroxyphenylacetaldoxime. Products were separated on Bakerflex Silica Gel 1B flexible sheets with toluene:ethyl acetate (4:1, v/v) as the developing solvent. The TLC plates were streaked with unlabeled standards and visualized under ultraviolet light after spraying with a 0.2% ethanolic solution of 2',7'-dichlorofluorescein. The radioactivity in each compound was determined by cutting appropriate areas up into scintillation vials and counting as described below. The R_F values observed were: *p*-hydroxyphenylacetaldoxime, 0.32; *p*-hydroxybenzaldehyde, 0.50; *p*-hydroxyphenylacetoneitrile, 0.64; and tyrosine, 0.00.

Intermediates were sometimes separated by paper chromatography (Whatman 3MM) using butanol:ammonia, 29% (4:1, v/v). The R_F values observed were: *p*-hydroxyphenylacetoneitrile and *p*-hydroxyphenylacetaldoxime, 0.88; *p*-hydroxybenzaldehyde, 0.45; and tyrosine,

0.08. In this case intermediates were usually located by use of a Packard Model 7201 radiochromatogram scanner.

Data were corrected for loss of free CO_2 and HCN when appropriate. All enzyme assays were normally performed in duplicate and values differed by less than 10%.

Other methods—Protein was determined by the method of Lowry *et al.* (14) with bovine serum albumin as standard. The protein concentration of the microsomal samples was typically 5.0–5.5 mg/ml of microsomal protein. Radioactivity was determined in a Beckman scintillation counter with aqueous (10%) Triton-toluene scintillation fluid (15). Quench corrections were made using the external standard.

RESULTS

The microsomal particles exhibited good activity when harvested 2 to 4 days after transfer to the dark, but no enzyme activity could be obtained if the seedlings were homogenized with the seed coats present. Green tissue showed lower activity than etiolated material used in this paper. Under the conditions reported, product formation was linear with time and directly proportional to the quantity of microsomal particles. The data presented in Table I show that the pH optima of the substrates vary over a pH range of 2 units but are predominantly alkaline.

Estimates of the kinetic parameters were made for each of the substrates, and the results are shown in Table II. All measurements were performed at pH 8, since all substrates showed good activity under these conditions and since the channeling experiments discussed below were performed at this pH. *N*-Hydroxytyrosine and *p*-hydroxyphenylacetaldoxime exhibited substrate inhibition above about 0.1 mM, and *p*-hydroxyphenylacetoneitrile above about 0.2 mM. Michaelis constants were calculated from the linear portions of Eadie-Hofstee plots by linear regression analysis. The corresponding V_{max} values are also shown in Table II, but it should be born

TABLE I

pH optima for the metabolism of pathway intermediates in Tricine and potassium phosphate buffers

The assays were performed over the pH ranges 6–9.5 for Tricine and 6–9 for potassium phosphate, with final buffer concentrations of 0.1 M. Tyrosine metabolism was determined by the radioactive assay and metabolism of the other intermediates by the colorimetric determination of free cyanide as described under "Materials and Methods."

Substrate	Tricine	Phosphate
	Optimal pH values	
Tyrosine	8.5	9.0 ^a
<i>N</i> -Hydroxytyrosine	7.5	7.5
<i>p</i> -Hydroxyphenylacetaldoxime	7.0	7.5
<i>p</i> -Hydroxyphenylacetoneitrile	8.0	8.0

^a This value is not strictly a pH optimum since there was no phosphate buffer over pH 9. In diethanolamine HCl buffer the pH optimum was 9 (over the range 8.5–10) and in carbonate buffer the highest rate was observed at pH 9 (from the range 9–10.8).

TABLE II

Apparent values of K_m and V_{max} for cyanide formation from various intermediates

All assays were carried out at pH 8 as described under "Materials and Methods." Colorimetric cyanide determinations were employed for *N*-hydroxytyrosine, *p*-hydroxyphenylacetaldoxime, and *p*-hydroxyphenylacetoneitrile. Tyrosine metabolism was measured by the radioactive assay.

Substrate	K_m	V_{max}
	mM	nmol HCN/h/g fresh wt
L-Tyrosine	0.240	18.8
<i>N</i> -Hydroxytyrosine	0.016	49.2
<i>p</i> -Hydroxyphenylacetaldoxime	0.014	47.4
<i>p</i> -Hydroxyphenylacetoneitrile	0.024	224.0

in mind that these velocities are lower than those that would be observed at infinite substrate concentration due to the substrate inhibition. In addition, the activity of the microsomes varied somewhat from preparation to preparation. The K_m values of the intermediates are lower than the value for tyrosine by a factor of at least 10. Correspondingly, the V_{max} for tyrosine is much lower than that of the other substrates. When the concentrations of *N*-hydroxytyrosine and *p*-hydroxyphenylacetonitrile were held constant and NADPH varied, the rate of cyanide production increased up to a concentration of 0.5 mM (data not shown) and no substrate inhibition was observed.

In a previous paper (1) it was noted that the nature of the products of the microsomal assay (*p*-hydroxybenzaldehyde and *p*-hydroxyphenylacetonitrile) depended on the presence or absence of dithiothreitol. This relationship is investigated further in Fig. 2. In this experiment, the initial homogenization buffer contained dithiothreitol, since this improved the total activity of the microsomal particles in some cases. Dithiothreitol was added in the assays to the concentrations indicated. It is clear that some dithiothreitol was required for optimal activity, but at high concentrations the conversion of *p*-hydroxyphenylacetonitrile to *p*-hydroxymandelonitrile (and hence to *p*-hydroxybenzaldehyde) was inhibited.

The data in Fig. 3 show the effects of varying concentrations of unlabeled *p*-hydroxyphenylacetonitrile on the metabolism of 14 C-labeled tyrosine by the microsomal particles. The data here and in Figs. 4 and 5 are on a semi-logarithmic plot in order to emphasize the low concentration points in which inhibition effects do not complicate interpretation of the data. The amount of 14 C-labeled *p*-hydroxybenzaldehyde produced

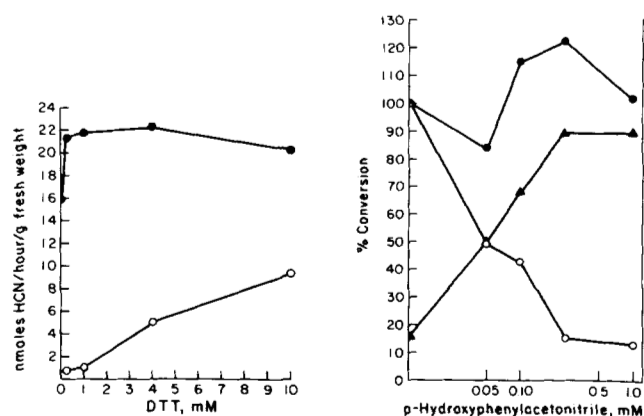


FIG. 2 (left). The effect of dithiothreitol on the metabolism of tyrosine by the microsomal particles. The microsomes were prepared as described under "Materials and Methods," except that they were washed and resuspended in buffer containing no dithiothreitol (DTT). ●, total amount of product (*p*-hydroxyphenylacetonitrile plus *p*-hydroxybenzaldehyde) formed; ○, the amount of *p*-hydroxyphenylacetonitrile produced at each dithiothreitol concentration.

FIG. 3 (right). The metabolism of tyrosine in the presence of varying concentrations of *p*-hydroxyphenylacetonitrile. ●, total products produced from tyrosine (*p*-hydroxyphenylacetonitrile plus *p*-hydroxybenzaldehyde) expressed as a percentage of the amount produced when there is no *p*-hydroxyphenylacetonitrile present (12.0 nmol/h/g, fresh wt). ○, amount of *p*-hydroxybenzaldehyde produced from tyrosine expressed as a percentage of the amount produced when no *p*-hydroxyphenylacetonitrile is present (10.08 nmol/h/g, fresh wt). ▲, the percentage of the total tyrosine products consisting of *p*-hydroxyphenylacetonitrile evaluated at each *p*-hydroxyphenylacetonitrile concentration. Assays were performed using L-[U- 14 C]tyrosine as described under "Materials and Methods" with the indicated concentration of unlabeled *p*-hydroxyphenylacetonitrile present. The total assay volume was 300 μ l.

from tyrosine is drastically reduced (*i.e.* 50%) even at low *p*-hydroxyphenylacetonitrile concentrations (0.05 mM). Correspondingly, the percentage of radioactive product consisting of 14 C-labeled *p*-hydroxyphenylacetonitrile increases sharply and is 50% in the presence of 0.05 mM *p*-hydroxyphenylacetonitrile. (The total amount of products derived from tyrosine shows a small inhibition at the lowest concentration of the nitrile, and a slight stimulation at the higher concentrations.) Such data can be interpreted as either demonstrating the effective trapping of *p*-hydroxyphenylacetonitrile by a small unlabeled pool of this compound or as showing the inhibition of the conversion of the nitrile to *p*-hydroxymandelonitrile. Since our kinetic data, discussed in relation to Table II, indicate that the inhibition effects only become important as the concentrations of the pathway intermediates are raised above 0.1 mM, the accumulation of the radioactive nitrile at 0.05 and 0.1 mM concentrations in Fig. 3 can therefore only be interpreted as a trapping effect.

In contrast, Fig. 4 shows that the trapping of radioactivity in added *p*-hydroxyphenylacetaldoxime becomes significant only when the aldoxime concentration is relatively high (*i.e.* 0.25 mM and 1 mM). At these concentrations, however, the oxidation of tyrosine to products is drastically reduced.

All enzyme assays described hitherto have involved resolution of the products by thin layer or paper chromatography, but *N*-hydroxytyrosine decomposes to *p*-hydroxyphenylacetaldoxime under these conditions (11). Previous studies on the metabolism of *N*-hydroxytyrosine have involved gas chromatography (16). However, detection of small quantities of radioactive intermediates by means of a gas chromatograph coupled to a gas proportional counter is a relatively insensitive technique (3), and in addition, lyophilized triglochin microsomal extracts contained a factor which interfered with the silylation of some intermediates. These considerations plus

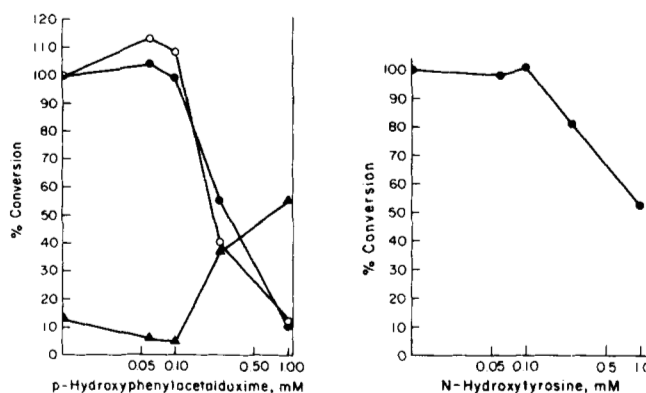


FIG. 4 (left). The metabolism of tyrosine in the presence of varying concentrations of *p*-hydroxyphenylacetaldoxime. ●, total products produced from tyrosine (*p*-hydroxyphenylacetonitrile, *p*-hydroxybenzaldehyde, and *p*-hydroxyphenylacetaldoxime) expressed as a percentage of the products formed when no unlabeled *p*-hydroxyphenylacetaldoxime was added (40.8 nmol/h/g, fresh wt). ▲, the percentage of the total products of tyrosine metabolism consisting of *p*-hydroxyphenylacetaldoxime evaluated at each *p*-hydroxyphenylacetaldoxime concentration. ○, the sum of *p*-hydroxyphenylacetonitrile and *p*-hydroxybenzaldehyde expressed as a percentage of the sum when no trap was added (35.4 nmol/h/g, fresh wt). The assays were performed as described in Fig. 2.

FIG. 5 (right). The metabolism of tyrosine in the presence of varying concentrations of *N*-hydroxytyrosine. At each *N*-hydroxytyrosine concentration the total measurable metabolic products produced from tyrosine (*p*-hydroxybenzaldehyde) is plotted and expressed as a percentage of the amount of products formed when no *N*-hydroxytyrosine was present. A total of 25.2 nmol/h/g, fresh wt of products was formed in the latter case. The assays were performed as described in Fig. 2.

the low activity of triglochin extracts precluded us from measuring *N*-hydroxytyrosine directly. The data in Fig. 5 show the extent of product formation (*p*-hydroxyphenylacetonitrile plus *p*-hydroxybenzaldehyde) from tyrosine with a varying concentration of *N*-hydroxytyrosine as a trap. These data can be interpreted by consideration of the corresponding data in Figs. 3 and 4 for *p*-hydroxyphenylacetonitrile and *p*-hydroxyphenylacetaldoxime, respectively. Insofar as *N*-hydroxytyrosine does not appear to reduce product formation at low concentrations (up to 0.1 mM), it resembles *p*-hydroxyphenylacetaldoxime, and this is consistent with the idea that *N*-hydroxytyrosine is not trapped under these conditions. It is interesting to note that the proportion of the radioactive products accounted for by *p*-hydroxyphenylacetonitrile increases steadily from 60%, when no unlabeled pool is present, to 85% when the *N*-hydroxytyrosine is at 1 mM (data not shown). This indicates some inhibitory effect of *N*-hydroxytyrosine on the last step of the microsomal pathway.

In order to study in more detail the competition between exogenously added intermediate and the same compound generated *in situ*, the experiments shown in Tables III–V were performed. Table III shows the composition of the reaction mixture after incubation for 1 h with ³H-labeled tyrosine and/or ¹⁴C-labeled *p*-hydroxyphenylacetonitrile. The low concentration of *p*-hydroxy[¹⁴C]phenylacetonitrile (0.1 mM) was chosen so as to minimize complications of interpretation due to substrate inhibition effects. In the presence of *p*-hydroxy[¹⁴C]phenylacetonitrile, tritiated *p*-hydroxyphenylacetonitrile accumulated, and there was a concomitant reduction from 5.7 to 1.7 nmol (70%) in production of tritiated *p*-hydroxybenzaldehyde compared to the assays in which no exogenous nitrile was added. It should be noted that the presence of *p*-hydroxy[¹⁴C]phenylacetonitrile caused a 32% increase (15.4 to

20.4 nmol) in the metabolism of tyrosine; a similar effect was observed in Fig. 3. The metabolism of *p*-hydroxy[¹⁴C]phenylacetonitrile was reduced in the presence of tritiated intermediate by 30% (from 7.6 to 5.2 nmol), this effect being most probably due to dilution of the ¹⁴C-labeled compound by ³H-labeled material produced *in situ*. The total amount of *p*-hydroxybenzaldehyde produced is little changed by the presence or absence of tyrosine in the assays containing *p*-hydroxy[¹⁴C]phenylacetonitrile.

In contrast to the data in Table III, *p*-hydroxyphenylacetaldoxime caused a less pronounced trapping of ³H-labeled intermediate and a smaller reduction (9.6 to 8.3 nmol) in tritiated product formation (Table IV). When [³H]tyrosine and *p*-hydroxy[¹⁴C]phenylacetaldoxime were incubated simultaneously, there was a 31% increase (21.5 to 28.1 nmol) in the total amount of product formation over that which is produced with the oxime alone. However, the formation of ¹⁴C-labeled products is relatively unaffected (21.5 to 19.8 nmol) by the presence of tyrosine.

As discussed above, we were not able to measure *N*-hydroxytyrosine directly. Also, direct quantitation of tyrosine was impossible because *N*-hydroxytyrosine remaining will stay at the origin together with tyrosine in the solvent systems employed. When the TLC bands corresponding to *p*-hydroxyphenylacetaldoxime were counted, no difference in the counts measured was observed between samples containing [¹⁴C]tyrosine alone and those containing [¹⁴C]tyrosine plus unlabeled *N*-hydroxytyrosine (data not shown). Since we would expect *p*-hydroxyphenylacetaldoxime to be formed from decomposition of trapped *N*-hydroxytyrosine during TLC, there was no indication that trapping occurred to any significant extent. This is consistent with the fact that the rate of product formation from tyrosine was unaffected (5.7 *versus*

TABLE III

Simultaneous metabolism of tyrosine and *p*-hydroxyphenylacetonitrile by the microsomal system

Assays contained 312 nmol (0.95 mM) of L-[3,5-³H]tyrosine (1.73 μCi) and/or 33 nmol (0.1 mM) of *p*-hydroxy[¹⁴C]phenylacetonitrile (0.18 μCi) in a total volume of 330 μl. They were otherwise performed as described under "Materials and Methods." Reactions were terminated by addition of 100 μl of 95% ethanol. Radioactive products were

separated by paper chromatography using butanol:ammonia, 29% (4:1, v/v) as the developing solvent. Radioactive material was eluted from the paper with ethanol and counted as described under "Materials and Methods."

Substrates	Composition of the reaction mixture				
	[³ H]Tyrosine	<i>p</i> -Hydroxy[³ H]-phenylacetonitrile	<i>p</i> -Hydroxy[³ H]-benzaldehyde	<i>p</i> -Hydroxy[¹⁴ C]-phenylacetonitrile	<i>p</i> -Hydroxy[¹⁴ C]-benzaldehyde
					Total <i>p</i> -hydroxybenzaldehyde
					nmol
[³ H]Tyrosine	296.6	9.7	5.7		5.7
<i>p</i> -Hydroxy[¹⁴ C]phenylacetonitrile				23.7	7.6
[³ H]Tyrosine + <i>p</i> -hydroxy[¹⁴ C]-phenylacetonitrile	291.6	18.7	1.7	24.2	5.2
					6.9

TABLE IV

Simultaneous metabolism of tyrosine and *p*-hydroxyphenylacetaldoxime by the microsomal system

Assays contained 309 nmol (0.94 mM) of L-[3,5-³H]tyrosine (3.46 μCi) and/or 30 nmol (0.09 mM) of *p*-hydroxy[¹⁴C]phenylacetaldoxime (0.098 μCi) in a total volume of 328 μl. Reactions were terminated by addition of 100 μl of 95% ethanol and were otherwise performed as described under "Materials and Methods." Radioactive products were separated by silica gel TLC and scintillation counted as described under "Materials and Methods."

Substrates	Composition of the reaction mixture				
	[³ H]Tyrosine	<i>p</i> -Hydroxy[³ H]-phenylacetaldoxime	<i>p</i> -Hydroxy[³ H]-phenylacetonitrile + <i>p</i> -hydroxy[³ H]benzaldehyde	<i>p</i> -Hydroxy[¹⁴ C]-phenylacetaldoxime	<i>p</i> -Hydroxy[¹⁴ C]-phenylacetonitrile + <i>p</i> -hydroxy[¹⁴ C]-benzaldehyde
					Total <i>p</i> -hydroxyphenylacetonitrile + <i>p</i> -hydroxybenzaldehyde
					nmol
[³ H]Tyrosine	301.9	2.0	9.6		9.6
<i>p</i> -Hydroxy[¹⁴ C]phenylacetaldoxime				9.6	21.5
[³ H]Tyrosine + <i>p</i> -hydroxy[¹⁴ C]-phenylacetaldoxime	301.7	3.5	8.3	10.4	19.8
					28.1

TABLE V

Simultaneous metabolism of tyrosine and *N*-hydroxytyrosine by the microsomal system

Microsomes were prepared in the usual way as described under "Materials and Methods" except that the washing and final suspension buffers contained only 2 mM dithiothreitol. Assays contained 309 nmol of L-[U-¹⁴C]tyrosine (1 μ Ci) and/or 16 nmol (0.059 mM) of *N*-hydroxytyrosine in a total volume of 273 μ l. Reactions were terminated by addition of 100 μ l of 2 N H₂SO₄. Cyanide was determined colorimetrically and *p*-hydroxybenzaldehyde radioactivity was determined by silica gel chromatography and scintillation counting as described under "Materials and Methods."

Substrates	Composition of the reaction mixture			
	<i>p</i> -Hydroxybenzaldehyde derived from tyrosine	<i>N</i> -Hydroxytyrosine remaining	<i>p</i> -Hydroxybenzaldehyde from <i>N</i> -hydroxytyrosine	Total <i>p</i> -hydroxybenzaldehyde
	nmol			
Tyrosine	5.7			5.7
<i>N</i> -Hydroxytyrosine		8.0	8.0	8.0
Tyrosine + <i>N</i> -hydroxytyrosine	5.7	7.7	8.3	14.0

5.7 nmol) by the addition of *N*-hydroxytyrosine (Table V). Under the conditions that this experiment was performed, *p*-hydroxyphenylacetonitrile constituted less than 4% of the measured products; consequently we report data for *N*-hydroxytyrosine based on colorimetric determinations of HCN, since the production of free cyanide is equivalent to *p*-hydroxybenzaldehyde. The conversion of *N*-hydroxytyrosine when both substrates were present was determined by subtracting the rate determined from TLC of ¹⁴C-labeled products from that observed for total cyanide by the colorimetric method. Such data (8.0 versus 8.3 nmol) indicated that the metabolism of *N*-hydroxytyrosine was unaffected by the presence or absence of tyrosine.

DISCUSSION

The pH optima of microsomal intermediates were in the range 7–8 and were similar to the values obtained with sorghum microsomes (5). Tyrosine had noticeably more alkaline pH optima than the other substrates and also than the corresponding values in sorghum (3).

One of the most unexpected features of the triglochin microsomal system was that all the intermediates exhibited substrate inhibition. The substrate inhibition effect appeared to be complex. Both *p*-hydroxyphenylacetaldoxime and *N*-hydroxytyrosine inhibited tyrosine metabolism (Figs. 4 and 5), and all of the intermediates to some extent inhibited the conversion of *p*-hydroxyphenylacetonitrile to the corresponding cyanohydrin (data not shown). However, even at very high substrate concentrations significant metabolism occurred. A similar effect appears in sorghum where *p*-hydroxyphenylacetonitrile strongly inhibits tyrosine metabolism (5), and also in the kaurene synthetase of the fungus *Fusarium moniliforme* (18), where high levels of the intermediate copalylpyrophosphate reduce kaurene production. All of the intermediates are polar compounds, and it is possible that they act in a way similar to chaotropic reagents and reduce enzyme activities by perturbing membrane structure. Alternatively, high concentrations may result in alternate reaction pathways becoming operative with a net decrease in overall conversion.

Tyrosine is by far the poorest possible substrate for cyanide production (Table II), and tyrosine metabolism is likely to be the rate-limiting step in the production of triglochin cyanogenic glycosides *in vivo*. The ratio of V_{\max} to K_m for tyrosine utilization in triglochin is 78, the corresponding ratio in

sorghum (after conversion of V_{\max} to the same units) is 5667 (3). The sorghum and triglochin systems are clearly very different in the efficiency with which tyrosine is utilized, and at least part of this difference is due to an inherent difference between the substrate affinities of the respective enzymes. The significance of this difference remains to be determined. There are several other differences between the kinetics of the sorghum and triglochin systems, the most obvious being that *p*-hydroxyphenylacetonitrile is the best substrate by far for cyanide production in the latter system, but it is much the worst in the former (V_{\max} = 59 nmol/h/g, fresh wt). The reason for this is discussed below.

The ratio of the observed products (*p*-hydroxyphenylacetonitrile and *p*-hydroxybenzaldehyde) varied considerably from preparation to preparation. The concentration of dithiothreitol (Fig. 2) constituted an important factor in determining how much *p*-hydroxybenzaldehyde we observed. Nevertheless, some variability remained in the amounts and ratios of the products obtained and the reason for this is unknown. In sorghum, *p*-hydroxyphenylacetonitrile did not appear as a product under any conditions (2, 3).

One of the most puzzling observations in the corresponding sorghum microsomal system was the apparent requirement of NADPH for *p*-hydroxyphenylacetaldoxime metabolism (2, 4). We have confirmed this observation in the triglochin system. Metabolism of *p*-hydroxy[U-¹⁴C]phenylacetaldoxime depended entirely on the presence of NADPH (data not shown). The conversion of the oxime to the nitrile appears to be a dehydration step, and no simple rationale for the involvement of a reducing agent is possible.

We may define channeling as the preferential utilization of a biochemical intermediate generated *in situ* when the exogenous added intermediate is also present. It follows from this that the channeled compound is not entirely free to exchange by diffusion with the exogenous material (*i.e.* to be trapped by the exogenous material). We have explored the extent to which each intermediate can be trapped in Figs. 3, 4, and 5 by an isotopic dilution technique similar to that used by Matchett in an informative study of channeling in the tryptophan synthase of *Neurospora crassa* (17). It is quite clear that *p*-hydroxyphenylacetonitrile generated from tyrosine equilibrates readily with added material, but that is not the case with *p*-hydroxyphenylacetaldoxime or *N*-hydroxytyrosine.

There are several ways of quantitating channeling in double label experiments such as those performed in Tables III and IV. A procedure involving division of the tritium to carbon 14 ratio of the products by the tritium to carbon 14 ratio of the intermediate in question was developed by Czichi and Kindl (6) and used by Møller and Conn in the channeling studies on sorghum (5). A completely analogous calculation can be made from the data in Table V for *N*-hydroxytyrosine, an experiment in which only carbon 14 labeling was employed. Since we assume that *N*-hydroxytyrosine derived from tyrosine is completely converted to products, (there being no evidence to indicate that any significant accumulation occurred) the above calculation gives an infinite value when applied to this case. In general, the values obtained for the channeling ratio vary greatly with small changes in the amount of trapped intermediate detected. Such amounts are likely to be very small (and subject to a significant error in determination) when the intermediate is highly channeled. Therefore, in this study we have quantitated channeling by dividing the percentage of conversion to products of the intermediate generated from tyrosine by the percentage of conversion of the added intermediate. A value of 1 or more indicates that significant channeling occurs, since the endogenous material is being more efficiently utilized. This calculation method shows a clear

relationship to the definition of channeling that we have employed, and although in general the values obtained show smaller differences between channeled and unchanneled intermediates than the other method, the difficulties associated with that calculation are obviated. From the data in Tables III, IV, and V, the values for *p*-hydroxyphenylacetonitrile, *p*-hydroxyphenylacetaldoxime, and *N*-hydroxytyrosine are: 0.47, 1.1, and 1.9, respectively; the corresponding values in sorghum are 12.9, 0.8, and 9.5 (from data in Ref. 5). We have assumed that the *N*-hydroxytyrosine derived from tyrosine is completely converted to products, since we obtained no indication that any significant accumulation occurred. It should be noted that if an intermediate is indeed freely exchangeable, re-utilization of this material becomes significant due to the small size of added (^{14}C -labeled) pool, and this will tend to raise the apparent value of the channeling parameter. Conversely, for the channeled intermediates, the small pool size of the exogenous intermediates leads to a high percentage of conversion (since they are good substrates relative to tyrosine), and therefore a reduction in the value of the channeling parameter. The problem created by substrate inhibition (allowing us only to use small pool sizes) obscures the distinction between channeled and unchanneled intermediates and makes direct comparison of channeling values with those of sorghum unfruitful.

Nevertheless, the data show that endogenous *N*-hydroxytyrosine and *p*-hydroxyphenylacetaldoxime are both utilized at least as effectively as the material comprising the added pool, despite the fact that initially there is a large amount of added material but no endogenous intermediate. In other words, the intermediate generated *in situ* is used preferentially over exogenous added material.

It is also clear from Tables IV and V that total product formation was higher when both tyrosine and the intermediate were present. There are two possible explanations. The first is that a stimulation of one or both of the metabolites occurred by the presence of the other. In Fig. 4 tyrosine metabolism was stimulated somewhat by exogenous *p*-hydroxyphenylacetaldoxime at low concentrations, but this effect, although varying from preparation to preparation, was always small. The data in Tables IV and V do not indicate that metabolism of either of the intermediates was stimulated by the other, but rather that the rates tended to be additive. This suggests a second explanation in which the exogenous compounds do not saturate their respective enzymes and that the maximum rate observable from the two intermediates is determined by a rate-limiting step other than the inherent V_{max} of the enzyme (such as a diffusion barrier). We have already noted that the best substrate for the triglochin microsomal system is *p*-hydroxyphenylacetonitrile, whereas in sorghum it is *p*-hydroxyphenylacetaldoxime. It is unlikely to be a coincidence that the unchanneled intermediates constitute the best substrates and probably reflects the fact that these compounds have relatively free access to their respective active sites.

Our ability to obtain cell-free activity from *T. maritima* has enabled us to compare the channeling of intermediates in a multistep biosynthetic pathway obtained from two different organisms. We conclude that in the triglochin microsomal preparation *p*-hydroxyphenylacetaldoxime and *N*-hydroxytyrosine are significantly channeled, but that *p*-hydroxyphenylacetonitrile is not. This result contrasts interestingly with the results from sorghum (5), in which the oxime was not channeled but the other two intermediates were. It is possible that the different arrangement of the pathway in triglochin is related to the presence of a second tyrosine-derived cyanogenic glucoside, triglochinin, *in vivo* (19). Preliminary evidence indicates that *p*-hydroxyphenylacetonitrile may be a branch point in the pathways leading to these two different cyanogenic glycosides.²

The simplest rationale for the channeling described here involves a localized domain into which a product is released. The domain also contains the active site of the next enzyme in the pathway. Diffusion into and out of this region is hindered. This explanation is consistent with the data obtained from sorghum (5) and for the phenylalanine ammonia-lyase and cinnamic acid hydroxylase system in cucumber (6).

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² W. Hösel, unpublished results.