The Biosynthesis of Cyanogenic Glucosides in Higher Plants

N-HYDROXYTYROSINE AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF DHURRIN BY SORGHUM RICOLOR (LINN.) MONNICH*

Birger Lindberg Møller‡ and Eric E. Conn
From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

The following compounds were tested as early intermediates in the conversion of tyrosine to p-hydroxymandelonitrile by a microsomal preparation from dark grown sorghum seedlings: p-hydroxyphenyllactemamide, 1-nitro-2-p-hydroxyphenylethane, p-hydroxyphenylpyruvic acid oxime, tyramine, N-hydroxytyramine, and N-hydroxytyrosine. Of these, only N-hydroxytyrosine was metabolized to p-hydroxymandelonitrile. N-Hydroxytyrosine was produced from L-[U-14C]tyrosine in tracer experiments when unlabeled N-hydroxytyrosine was added as a trap.

These data indicate N-hydroxytyrosine as the first intermediate in the biosynthesis of dhurrin, the cyanogenic glucoside of sorghum, and represent the first demonstration of the formation of an α-N-hydroxyamino acid in a biological system. The enzyme system involved in this reaction was partially characterized with respect to substrate specificity and the effect of various inhibitors. The enzyme was shown to have properties different than those reported for the mammalian enzyme system(s) involved in the N-hydroxylation of amine drugs. The possible involvement of N-hydroxymonoamino acids in the biosynthesis of other secondary plant products is discussed.

Five protein amino acids, valine, leucine, isoleucine, phenylalanine, and tyrosine, are the primary precursors of the aglycones of some cyanogenic glucosides (1). The C₆-CH₂-N moiety of these amino acids is incorporated intact into the cyanogenic glucosides (2-4). This implies that all intermediates contain the nitrogen atom although, in intact plants, intermediates involved in this conversion cannot normally be detected. Studies on flax seedlings with the valine analogue O-methylythreonine have indicated the involvement of an aldoxime as an intermediate (5). On the basis of this information a general pathway was proposed (6) and is outlined in Fig. 1 for those plants which utilize tyrosine as the parent amino acid.

Sorghum seedlings contain the cyanogenic glucoside dhurrin derived from tyrosine. Strong support for the operation of the proposed pathway in this species was obtained when a membrane-bound (microsomal) system from etiolated sorghum seedlings was found to carry out all but the last step in the conversion of the amino acid tyrosine to dhurrin (7). The microsomal preparation does not contain the glucosyltransferase which converts p-hydroxymandelonitrile to dhurrin (8). Because the former compound rapidly dissociates into p-hydroxybenzaldehyde and hydrocyanic acid, these two compounds are the observed end products in the microsomal system (Fig. 1). Low amounts of p-hydroxyphenylethaldoxime can be detected in the incubation mixtures (7). When p-hydroxyphenylethaldoxime was administered to the microsomal system in the presence of NADPH, it was metabolized to p-hydroxybenzaldehyde and hydrocyanic acid (7). Thus, the results obtained with intact plants and with a cell-free microsomal system strongly indicate an aldoxime as an intermediate in the biosynthetic pathway.

Little is known about the origin of aldoximes from their parent amino acids (9). The conversion involves a 4 electron oxidative decarboxylation, and, with tyrosine as the parent amino acid, one or more of the following compounds could be postulated as intermediates (Fig. 2): 1-nitro-2-p-hydroxyphenylethylamine, p-hydroxyphenyllactemamide, tyramine, N-hydroxytyramine, N-hydroxytyrosine, 3-(p-hydroxyphenyl) 2-nitrosopropionic acid, p-hydroxyphenylpyruvic acid imine, or p-hydroxyphenylpyruvic acid oxime. In this paper data are presented to discriminate between these various possibilities and to establish and characterize more accurately the reaction which constitutes the first step in the biosynthesis of the cyanogenic glucoside dhurrin.

Experimental Procedures

Chemicals—L-[U-14C]Tyrosine (specific activity 440 mCi/mmol) was purchased from New England Nuclear, Boston, MA. 1-Nitro-2-p-hydroxyphenylethene was synthesized from 1-nitro-2-p-hydroxyphenylethene (10). p-Hydroxyphenylpyruvic acid oxime was synthesized by the method of Ahmad and Spenser (11), except that the reaction time used was only 20 min. p-Hydroxyphenylethaldoxime was synthesized by the method of Robbins (12), except that the synthesis was carried out under nitrogen to improve the yield and purity of product. N-Hydroxytyramine (mp. 226-228°C, decomposed) and N-hydroxyphenylalanine (mp. 156°C, decomposed) were synthesized as already reported (13).

N-Hydroxytyramine was obtained by reduction of p-hydroxyphenylethaldoxime (35 mg) with sodium cyanoborohydride (11 mg) (14). Tetrahydrofuran was used as solvent and the pH of the reaction mixture was adjusted to 3 by use of a gentle stream of hydrogen chloride gas. After 10 min, N-hydroxytyramine hydrochloride was precipitated out of the reaction mixture by addition of ethylacetate. When analyzed by thin layer chromatography (TLC)1 (Bakerflex Silica Gel 1B-F flexible thin layer sheets) with benzene/ethylacetate (9:1, v/v) as solvent, an ultraviolet absorbent spot at Rf = 0.09 was observed. This spot produced a red color momentarily when sprayed

1 The abbreviations used are: TLC, thin layer chromatography; GLC, gas-liquid chromatography; GLC/GPC, gas chromatograph coupled to a gas proportional counter.
Cyanogenic Glucoside Biosynthesis

8576

FIG. 1. Proposed pathway for the biosynthesis of the cyanogenic glucoside dhurrin from its precursor amino acid, l-tyrosine.

FIG. 2. Various possibilities for the formation of an aldoxime from its parent amino acid. The heavy arrows represent the sequence of intermediates as indicated by this study.

with triphenyltetrazolium chloride and produced a purple color when sprayed with ninhydrin. Another ultraviolet absorbing spot at the origin also gave a purple color with ninhydrin. This spot was tentatively identified as tyramine, presumably produced by further reduction of N-hydroxytyramine. The aldoxime was no longer present in the supernatant after recentrifugation for 90 min at 74,000 × g. The reaction mixture contained 10 nmol of HCN in a nitrogen atmosphere while β-mercaptoethanol particles were dialyzed in normal atmosphere. All operations were carried out at 0-4°C.

Assay for Protein—Protein interaction was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard.

Determination of Metabolic Activity—Three different techniques were used to measure the metabolic activity of the sorghum particles. One of these techniques was based on the colorimetric determination of the HCN produced upon addition of various compounds to the particles. Since the HCN is formed by the decomposition of p-hydroxymandelonitrile (Fig. 1), this method measures the formation of the least to the intermediate in the biosynthetic pathway. The use of this method, therefore, is restricted to experimental conditions under which p-hydroxymandelonitrile formation occurs, i.e. +/β-mercaptoethanol particles. NADPH-generating system, and oxygen (7).

As low as 10 nmol of HCN can be measured by this method.

The second and third techniques for measuring the metabolic activity of sorghum particles involved the use of 14C-labeled substrates and the separation of intermediates and end products formed by TLC or gas-liquid chromatography (GLC), respectively. These techniques were more informative in that they permitted the monitoring of individual steps of the pathway and the detection of intermediates when they accumulated in the reaction mixtures. However, not all intermediates are separated by either of these two techniques and individual determination of each intermediate is only accomplished by the combined use of both techniques.

Assay for Cyanide Formation—Cyanide production from various compounds examined as substrates in the microsomal system was determined either in phosphate buffer at pH 7.2 or Tricine buffer at pH 8.0. The reaction mixture contained 100 nmol of buffer, 0.3 μmol of NADP+, 6.0 μmol of glucose-6-phosphate, 3.0 μl of glucose-6-phosphate dehydrogenase, 0.02 to 2.5 μmol of substrate, and 0.6 mg of BMD microsomal protein in a total volume of 25 μl and dialyzed against the same buffer.

The sensitivity of the two assays is the same but the Lambert assay is advantageous in that the reagents involved are stable for a longer period of time.

Analysis of Intermediates by Thin Layer Chromatography—TLC separation of known and tested intermediates was performed on Bäckert Silica Gel 60 F254 sheets with benzene:methanol:acetic acid:water (5:3: 1: v/v) as solvent (7). The Rf values of reference compounds were p-hydroxybenzoic acid, 0.56; N-hydroxybenzaldehyde, 0.50; p-hydroxyphenylacetone. 0.17; p-hydroxymandelonitrile, 0.42; and 1-nitro-2-p-hydroxyphenylethane, 0.54. Tyrosine, N-hydroxytyrosine, p-hydroxyphenylpyruvic acid oxime, and tyramine remained at the origin. For enzymatic experiments, the TLC plates were prestreaked with unlabeled standards to allow visualization under ultraviolet light after chromatography. Aliquots from biosynthetic reaction mixtures were streaked directly on the plates. This results in immediate inactivation of the enzyme system as judged by the linearity of reaction with time. After development of the TLC plates, radioactivity in separated compounds was determined by cutting appropriate areas up into counting vials. All radioactivity measurements were done by scintillation counting (19). From the

2 Through the remainder of this paper, these two preparations will be designated as "+/β-mercaptoethanol particles" and "−/β-mercaptoethanol particles."
specific radioactivity of the tyrosine employed, the number of nanomoles corresponding to the measured radioactivity was calculated. Occasionally a 0.2% loss of 1 or 2 carbon atoms as products were formed from tyrosine.

This method effectively separates \( p \)-hydroxyphenylacetaldoxime, \( p \)-hydroxyphenylacetonitrile, and \( p \)-hydroxybenzaldehyde, the labeled products normally encountered in a sorghum particle mixture when \([\text{U}^\text{14C}]\)tyrosine is used as substrate. However, this technique was not always successful in the present study since tyrosine, \( N \)-hydroxytyrosine, and \( p \)-hydroxyphenylpyruvic acid oxime remain at the origin and \( p \)-hydroxybenzoic acid, which can be formed from \( p \)-hydroxybenzaldehyde, is also poorly separated from the origin. Furthermore, some \( N \)-hydroxytyrosine is oxidatively decarboxylated to \( p \)-hydroxyphenylacetoxaldehyde during the TLC procedure (20).

A third analytical technique (15) based on gas chromatographic separation of the trimethyl silyl derivatives of known and possible intermediates was developed because of the instability of \( N \)-hydroxytyrosine in the TLC method just described. This method allowed the examination of all compounds. However, \( p \)-hydroxyphenylacetonitrile is not separated from \( p \)-hydroxybenzoic acid by the GLC procedure.

The analysis of enzymatic reaction mixtures by use of a gas chromatograph coupled to a gas proportional counter (GLC/GPC) was carried out as described elsewhere (15). Aliquots from the reaction mixture were taken at various time intervals and pipetted into small ampules which were immediately immersed into liquid nitrogen to obtain a frozen sample. The samples were stored in dry ice until the end of the experiment. Their contents were then lyophilized to dryness and silylated by heating for 15 min at 90°C with an excess of \( \text{N,O-bis-(trimethylsilyl)} \)trifluoroacetamide in acetonitrile. An unlabeled standard mixture of trimethylsilyl derivatives (MeSi derivatives) was added. Aliquots of 10 \( \mu l \) were injected into the GLC/GPC and each labeled peak obtained was integrated electronically and identified by retention time and its superimposition with the mass peak of the added authentic unlabeled standard.

**Trapping Experiments**—Trapping experiments were performed in reaction mixtures containing 0.3 \( \mu \)m of NADP\(^+\). 3.0 \( \mu \)m of glucose-6-phosphate, 4 \( \mu \)m of glucose-6-phosphate dehydrogenase, 0.12 \( \mu \)m of \( \text{L-}[\text{U}^\text{14C}]\)tyrosine (2.0 \( \mu \)Ci), 1.2 \( \mu \)m of trapping compound, 1.0 \( \mu \)g of Cheeseman protein, and 190 \( \mu \)g of 

In trapping experiments where GLC/GPC analysis had indicated the accumulation of labeled \( N \)-hydroxytyrosine the silylated samples were combined and evaporated to dryness in a stream of dry nitrogen. The residue was dissolved in 250 \( \mu \)l of silyating agent and heated to 90°C for 15 min to ensure complete silylation of the sample. Aliquots (25 \( \mu \)l) were then injected into the GLC which was run isothermally at 190°C with a decreased flow rate of carrier gas (25 ml/min). With the aid of a splitter (1:100) the effluent corresponding to silylated \( N \)-hydroxytyrosine was collected in Pasteur pipettes. The tips of the pipettes which contained the labeled material were crushed in a small vial containing 46 mg of unlabeled \( N \)-hydroxytyrosine carrier dissolved in 6 \( \mu \)l of \( 0.1 \text{ N HCl} \) chilled to 4°C. The vial was shaken for 24 h at room temperature to remove the silyl groups and then frozen in dry ice and lyophilized. The sample was recrystallized from water by heating on a steam bath while flushing the vial with nitrogen. After filtration through a glass filter, the clear supernatant was cooled in ice and the precipitate which formed was isolated by centrifugation. After lyophilization overnight, the specific activity was determined by liquid scintillation counting of a weighed aliquot (approximately 1.5 mg). The crystallization procedure was repeated for five additional times.

**Inhibitor Studies on the Conversion of Tyrosine to \( p \)-Hydroxyphenylacetaldoxime**—Inhibitor experiments were carried out with \( \beta \)-mercaptoethanol micromolar. In this series of experiments, NADPH was used directly and not generated in the reaction mixture from NADP\(^+\) and glucose-6-phosphate dehydrogenase. This approach avoids changes in the NADPH concentration due to inhibition of the dehydrogenase by the various inhibitors. Each reaction mixture contained 95 \( \mu \)mol of Tricine buffer (pH 8.0), 0.3 \( \mu \)mol of NADPH, 0.06 \( \mu \)mol of \( \text{L-}[\text{U}^\text{14C}]\)tyrosine (1 \( \mu \)Ci), 0.5 mg of microsomal pellet, and, unless otherwise stated, 0.363 \( \mu \)mol of inhibitor in a total volume of 383 \( \mu \)l. In some cases a small volume of ethanol was added to the inhibitor to facilitate solubilization. Studies with carbon monoxide were performed as earlier described (19). The incubations were carried out in 1-ml ampules and stopped at 15 min by immersing the ampule in liquid nitrogen. After lyophilization to dryness and silylation, the composition of the reaction mixtures was determined by the GLC/GPC procedure.

**RESULTS**

**Effect of Thiols Reagents on Microsomal Preparations**—Microsomal preparations from etiolated sorghum seedlings of Sordan 70 and Redland \( \times \) Greenleaf varieties convert tyrosine to \( p \)-hydroxybenzaldehyde and cyanide (Table I) when prepared as described previously (7) in the presence of 10 \( \mu \)m \( \beta \)-mercaptoethanol. It was found in this study that 1 \( \mu \)m dithiothreitol can substitute for 10 \( \mu \)m \( \beta \)-mercaptoethanol and when dithiothreitol concentrations in the range of 5 to 10 \( \mu \)m were used, a 30% increase in the specific activity (\( V_{max} \)) was obtained.

In a previous paper (7) it was briefly noted that isolation of sorghum microsomes in the absence of \( \beta \)-mercaptoethanol (\( \beta \)-mercaptoethanol) particles resulted in preparations which still metabolized tyrosine but at a lower rate, and that \( p \)-hydroxyphenylacetaldoxime was the main product. With the sorghum variety (Sordan 70) used in that study and also in the early stages of this study, inactive preparations were frequently obtained (20). The active preparations were yellow while the inactive preparations had a whitish brownish tint. More active preparations were obtained when the initial centrifugation steps at 800 \( \times \) \( g \) and 10,000 \( \times \) \( g \) were shortened from 15 to 5 min each. However, even the most active \( \beta \)-mercaptoethanol preparations from Sordan 70 seedlings metabolized tyrosine only 20% as rapidly as the \( \beta \)-mercaptoethanol preparations from that variety (Table I).

When seedlings of the sorghum variety Redland \( \times \) Greenleaf were used, the absence of \( \beta \)-mercaptoethanol again resulted in a preparation which converted tyrosine only to \( p \)-hydroxyphenylacetaldoxime. However, the specific activity of such microsomal preparations was not affected by omitting \( \beta \)-mercaptoethanol (Table I). Unless otherwise stated, the hybrid Redland \( \times \) Greenleaf was used in experiments with \( \beta \)-mercaptoethanol microsomes. The yield of microsomal protein was 0.85 mg/g of fresh weight of the seedlings and was independent of whether \( \beta \)-mercaptoethanol was employed during the preparation of microsomes.

**Cyanide Production from Various Known and Possible Intermediates**—The ability of the \( \beta \)-mercaptoethanol microsomal system to metabolize known and possible intermediates in dhurrin biosynthesis was examined in two different buffer systems, phosphate at pH 7.2 and Tricine at pH 8.0. Two different systems were examined because it had been shown that tyrosine utilization was dependent on the nature of the buffer used. (The two pH values chosen were the pH

**Table 1**

<table>
<thead>
<tr>
<th>Sorgum variety</th>
<th>( \beta )-mercaptoethanol</th>
<th>Product ( \mu mol/\text{h/mg protein} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( p )-Hydroxyphenyletaldoxime</td>
<td>( p )-Hydroxyphenylacetonitrile</td>
</tr>
<tr>
<td>Sordan 70</td>
<td>+</td>
<td>383</td>
</tr>
<tr>
<td>Redland ( \times )</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Greenleaf</td>
<td>-</td>
<td>205</td>
</tr>
</tbody>
</table>
optima for tyrosine metabolism in the respective buffer systems. For each compound a substrate concentration range from 0.025 to 3 mM was examined and the reaction was monitored by following the production of HCN. All experiments were carried out under aerobic conditions in the presence of a NADPH-regenerating system. No cyanide production could be detected from tyramine, l-nitro-2-p-hydroxyphenylethane, N-hydroxytyrosine and p-hydroxyphenylacetonitrile. Since the last named compound in solution slowly decomposes to p-hydroxyphenylacetonitrile (11), it was dissolved in cold buffer immediately before testing in order to avoid low levels of cyanide production from p-hydroxyphenylacetonitrile produced nonenzymatically.

The apparent values for $K_m$ and $V_{max}$ for the compounds which were metabolized are presented in Table II. The values for tyrosine and p-hydroxyphenylacetaldoxime were reproducible in different experiments, while the values obtained for N-hydroxytyrosine and p-hydroxyphenylacetamide were more variable. One important factor causing this variation was the concentration of $\beta$-mercaptoethanol in the assay mixture. Thus, when $\beta$-mercaptoethanol was added to the assay mixture, cyanide production from N-hydroxytyrosine decreased dramatically and sometimes no maximum was obtained within the substrate range tested. To avoid this inhibition of N-hydroxytyrosine metabolism, $\beta$-mercaptoethanol was not included in the standard assay mixture. Since the microsomes had to be prepared in the presence of $\beta$-mercaptoethanol to remain active in cyanide production, the amount of $\beta$-mercaptoethanol still present in the assay mixture after dialysis could vary from experiment to experiment.

Cyanide formation from the four compounds listed in Table II was linear with time and proportional to the amount of enzyme employed. Initial experiments with N-hydroxytyrosine did not show this proportionality. This was because various amounts of $\beta$-mercaptoethanol buffer were added to the assay mixtures to compensate for the different volumes of enzyme used. When the volume was adjusted with buffer not containing $\beta$-mercaptoethanol, a linear relationship between the amount of enzyme and cyanide production was obtained, indicating that most of the $\beta$-mercaptoethanol contained in the buffer used for enzyme preparation apparently was oxidized during enzyme preparation and dialysis.

The effect of pH on cyanide production from the different compounds tested was determined in phosphate and Tricine buffer (Fig. 3). For every compound, the pH optima were more alkaline in Tricine than in phosphate buffer. p-Hydroxyphenylacetaldoxime was utilized more rapidly in Tricine compared to phosphate buffer, while the opposite result was obtained with N-hydroxytyrosine.

Studies on N-Hydroxytyrosine as a Substrate—The ability of sorghum microsomes to catalyze the formation of HCN from N hydroxytyrosine suggests that the N-hydroxyamino acid is an intermediate in the conversion of tyrosine to p-hydroxyphenylacetaldoxime. However, N-hydroxytyrosine had earlier been reported to disproportionate to tyrosine and p-hydroxyphenylacetaldoxime (13). Furthermore, under certain conditions, N-hydroxytyrosine is slowly converted to p-hydroxyphenylacetaldoxime by oxidizing agents such as molecular oxygen and dyes (20). Because the decomposition products formed are known precursors of dithrinn (6), the cyanide formation observed upon addition of N-hydroxytyrosine to the microsomal system could reflect the metabolism of the decomposition products instead of N-hydroxytyrosine. This possibility was tested by two independent methods.

It was first shown that both the rate and the amount of cyanide formed upon NADPH addition was the same after preincubation of the microsomal system for 90 min with a range of concentrations of N-hydroxytyrosine (0.026 to 3.3 mM), as was observed without preincubation. This would not be expected if the decomposition products tyrosine and p-hydroxyphenylacetaldoxime were formed and were acting as substrates instead of N-hydroxytyrosine. It was then shown that the rate of cyanide formation with N-hydroxytyrosine as substrate remained unchanged when tyrosine decarboxylase (0.50 i.u.) was added to the assay mixture. However, when tyrosine was used as substrate, the addition of tyrosine decarboxylase resulted in a large decrease in cyanide production, i.e. at a tyrosine concentration of 0.2 mM (saturation conditions) cyanide production decreased from 145 to 22 nmol/h/mg of protein. Thus, neither the nonenzymatic disproportionation reaction converting N-hydroxytyrosine to tyrosine and p-hydroxyphenylacetaldoxime nor the oxidative decarboxylation of N-hydroxytyrosine into p-hydroxyphenylacetaldoxime can account for the observed metabolism of N-hydroxytyrosine. The experiment with tyrosine decarboxylase also excluded the possibility that metabolism takes place only after an initial microsomal reduction of N-hydroxytyrosine to tyrosine.

**Metabolism of l-[U-$^14$C]Tyrosine by Microsomal Prepa-
Microsomes prepared with β-mercaptoethanol efficiently metabolized L-[U-¹⁴C]tyrosine upon addition of a NADPH-regenerating system. The p-hydroxy-[U-¹⁴C]mandelonitrile formed is labile and dissociates into p-hydroxy-[U-¹⁴C]benzaldehyde and [¹⁴CHCN. p-Hydroxy-[U-¹⁴C]phenylacetaldoxime and p-hydroxy-[U-¹⁴C]phenylacetocitrite were present only in low levels during the course of the reaction, the latter compound always in the lower quantity (Fig. 4I). The p-hydroxy-[U-¹⁴C]benzaldehyde formed was further metabolized, and the reaction mixtures, therefore, did not contain strictly equimolar amounts of p-hydroxybenzaldehyde and cyanide. The product formed from p-hydroxybenzaldehyde, based on its Rf value on TLC and retention time on GLC when compared with an authentic standard, was tentatively identified as p-hydroxybenzoic acid. No other labeled products could be detected either by TLC or GLC/GPC analysis of the reaction mixture.

p-Hydroxy[U-¹⁴C]phenylacetaldoxime was the main product of metabolism of L-[U-¹⁴C]tyrosine when the microsomes were prepared in the absence of β-mercaptoethanol (Fig. 4II).

The earlier study (7) had shown that L-tyrosine was the only amino acid utilized by the sorghum microsomes. This was confirmed by the present work (Fig. 4). The microsomes prepared with/β-mercaptoethanol efficiently metabolized L-[U-¹⁴C]tyrosine upon addition of a NADPH-regenerating system. The p-hydroxy-[U-¹⁴C]mandelonitrile formed is labile and dissociates into p-hydroxy-[U-¹⁴C]benzaldehyde and [¹⁴CHCN. p-Hydroxy-[U-¹⁴C]phenylacetaldoxime and p-hydroxy-[U-¹⁴C]phenylacetocitrite were present only in low levels during the course of the reaction, the latter compound always in the lower quantity (Fig. 4I). The p-hydroxy-[U-¹⁴C]benzaldehyde formed was further metabolized, and the reaction mixtures, therefore, did not contain strictly equimolar amounts of p-hydroxybenzaldehyde and cyanide. The product formed from p-hydroxybenzaldehyde, based on its Rf value on TLC and retention time on GLC when compared with an authentic standard, was tentatively identified as p-hydroxybenzoic acid. No other labeled products could be detected either by TLC or GLC/GPC analysis of the reaction mixture.

p-Hydroxy[U-¹⁴C]phenylacetaldoxime was the main product of metabolism of L-[U-¹⁴C]tyrosine when the microsomes were prepared in the absence of β-mercaptoethanol (Fig. 4II).

With longer incubation times ¹⁴C-labeled p-hydroxybenzaldehyde and p-hydroxybenzoic acid were observed but only low amounts of ¹⁴C-labeled p-hydroxyphenylacetonitrile were present. Again no other labeled compounds were observed. Metabolism of L-[U-¹⁴C]Tyrosine by Microsomal Preparations in the Presence of Unlabeled Compounds which Might Be Intermediates—The addition of various unlabeled compounds which might serve as intermediates generally inhibited the metabolism of L-[U-¹⁴C]tyrosine by sorghum microsomes. However, no radioactivity could be trapped in tyramine, N-hydroxytyramine, p-hydroxyphenylacetamide, p-hydroxyphenylpyruvic acid oxime, or 1-nitro-2-p-hydroxyphenylethane when they were incubated with sorghum microsomes and L-[U-¹⁴C]tyrosine. Only when unlabeled N-hydroxytyrosine was added to reaction mixtures containing L-[U-¹⁴C]tyrosine and microsomes prepared in Tricine buffer did radioactivity accumulate in the N-hydroxytyrosine. Mass and radioactivity tracings obtained using the GLC/GPC procedure (15) are shown in Fig. 5. The amount of radioactivity trapped ranged between 3 and 10% of the activity administered as labeled tyrosine. Due to the low amounts of radioactivity obtained, the quantitation of N-hydroxy-[U-¹⁴C]tyrosine could not be done with any great accuracy although the radioactivity generally increased with incubation time. The identification of the radioactive peak as N-hydroxy-[U-¹⁴C]tyrosine was by recrystallization to constant specific activity. The effluent corresponding to the radioactive peak was collected from GLC, unlabeled N-hydroxytyrosine was added as carrier, the material was desilylated in 10 n HCl, and recrystallized from water (nitrogen atmosphere). The specific radioactivity of successive lots of crystals was 1170, 940, 910, 890, 900, and 910 dpm/mg. Part of the crystalline material obtained in the last recrystallization step was resilylated and by GLC analysis (15) produced only one peak corresponding to N-hydroxytyrosine. Thus, the product obtained after repeated recrystallizations was N-hydroxytyrosine and not a decomposition product. When the microsomes were prepared in phosphate buffer, the amount of radioactivity which accumulated in N-hydroxytyrosine was much lower and in some experiments not even detectable. This observation can perhaps be explained from the pH curves for N-hydroxytyrosine utilization (Fig. 3), which show that N-hydroxytyrosine is metabolized approximately twice as rapidly in phosphate than in Tricine buffer at the pH values used.

The earlier study (7) had shown that L-tyrosine was the only amino acid utilized by the sorghum microsomes. This
was confirmed in the present study since neither d-tyrosine, L-phenylalanine, nor d- and l-histidine were metabolized. Moreover, when L-[U-\textsuperscript{14}C]phenylalanine was incubated in the standard reaction mixture in the presence of a cold N-hydroxyphenylalanine trap, no labeled N-hydroxyphenylalanine was detected with the GLC/GPC procedure (15).

**Metabolism of L-[U-\textsuperscript{14}C]Tyrosine by Microsomal Preparations in the Presence of Various Inhibitors**—The effect of numerous metabolic inhibitors on the conversion of L-tyrosine to \(\beta\)-hydroxyphenylacetaldoxime by \(\beta\)-mercaptoethanol microsomes was examined in order to learn more about the two step reaction sequence (Fig. 2). The initial N-hydroxylation reaction is presumably responsible for the demonstrated requirement of NADPH in the reaction mixture. Therefore, oxidizing agents which could reduce the concentration of NADPH by oxidizing it to NADP\(^+\) were expected to be inhibitory. The conversion of N-hydroxytyrosine to p-hydroxyphenylacetaldoxime is a 2-electron oxidative decarboxylation that might require NADP\(^+\), NAD\(^+\), FMN, or FAD. Therefore, reducing agents capable of reducing NADPH to NADPH might inhibit the oxidative decarboxylation reaction and cause N-hydroxytyrosine to accumulate. Finally the NADPH produced during the initial oxygenation of tyrosine to N-hydroxytyrosine might in turn be tightly coupled to the subsequent oxidative decarboxylation of N-hydroxytyrosine in the microsomal system and little or no effect of inhibitors might be expected.

Table III shows that reducing agents such as sodium dithionite, methylviologen, sodium arsenite, and sodium ascorbate do inhibit the production of p-hydroxy-[U-\textsuperscript{14}C]phenylacetaldoxime from L-[U-\textsuperscript{14}C]tyrosine to variable extents; however, N-hydroxytyrosine did not accumulate in the partially inhibited reaction mixtures when these were monitored by the GLC/GPC procedure. Numerous oxidizing agents, including several quinones, were also strongly inhibitory as were FAD and FMN. This strong inhibition presumably was exerted through the oxidation of NADPH as little or no metabolism of L-[U-\textsuperscript{14}C]tyrosine, as monitored by GLC/GPC, could be detected. The inhibition by quinones could also be due to their reaction with an essential sulphydryl group on an enzyme.

Neither carbon monoxide nor any of four other inhibitors of cytochrome P\(\text{_{450}}\) (SKF 525A, 2-(3,4-dichloro-o-phenylphenoxy)ethylamine, metyrapone, and cobalt chloride) had an effect on the conversion of tyrosine to p-hydroxyphenylacetaldoxime. However, metal chelating compounds known to complex iron (i.e. \(\alpha\)-dipyridyl, 8-hydroxyquinoline and \(\alpha\)-phenanthroline) were moderately to strongly inhibitory. The sulfhydryl oxidants examined (Table III) showed different degrees of inhibition as might be expected due to their different mode of action. The flavoenzyme inhibitors tested (quinacrine, rotenone, thenoyltrifluoroacetone, antymycin A, sodium amytal, phenylmethane, pyrrolnitrine, and riboflavin) were without effect except for riboflavin which was strongly inhibitory. Riboflavin may act as an electron sink like other oxidizing agents. Inhibitors of pyridoxal phosphate enzymes (sodium azide, deoxyribofuranose, and hydroxylamine) had little or no effect. Agents such as phospholipase A and phospholipase C which can destroy membrane organization were strong inhibitors as were the detergents thiourea and sodium dodecyl sulfate.

p-Hydroxybenzaldehyde and cyanide, the nonenzymatic degradation products of p-hydroxymandelonitrile, were not inhibitory even at high concentrations. Thus, the first part of the biosynthetic pathway leading to dhurrin is insensitive to cyanide which can eventually be formed in vivo by catabolism of the cyanogenic glucoside.

These inhibitor studies permit only general conclusions to be drawn regarding the nature of the enzymes involved since in most cases only one concentration of inhibitor was tested. It must also be noted that the observed inhibition of metabolic rate could be the result of the inhibitor disturbing in some manner the organization of the membrane bound mutlinzyme system rather than by direct action on the individual enzymes.

**DISCUSSION**

The production and utilization of N-hydroxytyrosine by the sorghum microsomal system strongly implies N-hydroxytyrosine as the first intermediate in the biosynthesis of the cyanogenic glucoside dhurrin. This interpretation is further supported by experiments with \(\textsuperscript{18}O\), which show the oxygen atom introduced into N-hydroxytyrosine (as well as the oxygen atom of the oxime group in p-hydroxyphenylacetaldoxime) to be derived from molecular oxygen (21). Other experiments with L-\(\alpha\)-d-\(\alpha\)-tyrosine show the \(\alpha\)-hydrogen atom of tyrosine to be retained in N-hydroxytyrosine and p-hydroxyphenylacetaldoxime (Fig. 6) (21).

The conversion of N-hydroxytyrosine to p-hydroxyphenylacetaldoxime could proceed by decarboxylation forming N-hydroxytyramine or by dehydrogenation forming either 3-p-hydroxyphenyl-2-nitrosopropionic acid or p-hydroxyphenylpyruvic acid oxime (ketoxime) (Fig. 2). The deuterium experiments cited above (21) eliminate the ketoxime as an intermediate. However, it has not been possible to decide conclusively on the other two intermediates.

If N-hydroxytyramine is an intermediate, the enzyme which

**TABLE III**

<table>
<thead>
<tr>
<th>Class</th>
<th>Compound</th>
<th>Nanomoles p-hydroxyphenylacetaldoxime/h/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No inhibitor</td>
<td>155</td>
</tr>
<tr>
<td>Reducing/oxygenizing agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium dithionite</td>
<td>132</td>
<td></td>
</tr>
<tr>
<td>Methylviologen</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Sodium arsenite</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Sodium ascorbate</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>FMN</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>FAD</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Triphenyltetrazolium chloride</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Methylene blue</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c, 0.01 mm</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Horseradish peroxidase (30 i.u.)</td>
<td>0 + peroxide</td>
<td></td>
</tr>
<tr>
<td>Quinones</td>
<td>p-Benzoinoquinone</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Menadione</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1.4 Naphthoquinone</td>
<td>0</td>
</tr>
<tr>
<td>Sulphydryl agents</td>
<td>p-Chloromercuroxycarbazate</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>N-ethylmaleimide</td>
<td>74</td>
</tr>
<tr>
<td>Others</td>
<td>Phospholipase A, 10 i.u.</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Phospholipase C, 10 i.u.</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Thiourea</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Sodium diethyldithiocarbamate</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>p-Hydroxybenzaldehyde</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>Sodium cyanide</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>Nitrogen atmosphere</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Darkness</td>
<td>155</td>
</tr>
</tbody>
</table>
catalyzes its further oxidation to p-hydroxyphenylacetaldoxime must act stereospecifically on the prochiral carbon atom C₂ to retain the hydrogen atom originating from tyrosine (Fig. 6). This requirement is reasonable since the enzyme catalyzing the subsequent hydroxylation of p-hydroxyphenylacetaldoxime does discriminate between the enantiotopic hydrogens of its achiral substrate (Fig. 6) (22). However, the microsomal enzyme system did not utilize N-hydroxytyramine as a substrate nor was the N-hydroxyamine labeled in trapping experiments. (The other possible intermediate, 3-p-hydroxyphenyl-2-nitroso propionic acid, could be formed by enzymatic oxidation of N-hydroxytyrosine. Such secondary α-nitrosoacids can be synthesized chemically by gentle oxidation of α-N-hydroxy amino acids. Since they are extremely labile, decomposing into an aldoxime and carbon dioxide (Fig. 7) (23), it has not been possible to examine 3-p-hydroxyphenyl-2-nitroso propionic acid as a substrate in the microsomal system. Secondary α-nitrosoacids are in tautomeric equilibrium with their ketoximes (Fig. 7) (23) which in turn decompose to the homologous nitrile. Since equilibrium strongly favors the keto xime, the only decomposition product observed when p-hydroxyphenylpyruvic acid oxime was dissolved in buffer, incubated, and analyzed by use of the GLC/GPC procedure was p-hydroxyphenylacetaldoxime. If any tautomeric α-nitrosoacid had been formed, p-hydroxyphenylacetaldoxime should also have been obtained as a degradation product. As noted above, the stable isotope experiments exclude the aldoxime as an intermediate in the pathway (21). Therefore, if the α-nitrosoacid is an intermediate between N-hydroxytyrosine and p-hydroxyphenylacetaldoxime, the subsequent conversion to the aldoxime must occur rapidly excluding tautomization to the keto xime. Based on the negative biosynthetic results obtained with N-hydroxytyramine, 3-p-hydroxyphenyl-2-nitro so propionic acid seems the more likely intermediate between Nhydroxytyrosine and p-hydroxyphenylacetaldoxime if oxidation and decarboxylation occur in separate steps.

p-Hydroxyphenylacetaldoxime might also be formed by the oxidative decarboxylation of N-hydroxytyrosine in a concerted manner at the catalytic site, in which case no intermediate would be detected.

Although other compounds shown in Fig. 2 (p-hydroxyphenylacetamide, p-hydroxyphenylpyruvic acid imine, 1-nitro-2-p-hydroxyphenylethane, and tyramine) can be proposed instead of N-hydroxytyrosine as intermediates between tyrosine and p-hydroxyphenylacetaldoxime, the available evidence appears to exclude them. Thus, the amide, nitro compound, and amine were neither utilized by the sorghum microsomes nor were they labeled in trapping experiments. (The imine being unstable was not available for experimental test-

![Fig. 6. Stereochemical aspects of dhurrin biosynthesis with N-hydroxytyramine as a hypothetical intermediate.](http://www.jbc.org/)

![Fig. 7. Tautomeration and decomposition of secondary α-nitroso acids and ketoximes.](http://www.jbc.org/)
ther D-tyrosine, L-phenylalanine, nor L- and D-histidine gave rise to cyanide production. The pathway for cyanide formation by the *Chlorella* extracts appears to proceed via the imino acid (Fig. 2) (31). This compound is excluded as an intermediate in the sorghum system by the mass spectrometry data obtained with L-α-dL-tyrosine (21). The pathways for cyanide formation by the *Chlorella* and sorghum systems must, therefore, differ.

To our knowledge this is the first report where an α-N-hydroxyamino acid has been isolated from biological material. Evidence for the involvement of N-hydroxyamino acids in the biosynthesis of the hydroxamic acids hadacin (32) and myrtenol (33) has been presented but, in spite of concerted efforts (34, 35), the corresponding N-hydroxyamino acids were never isolated from the fungi involved. In another study, N-hydroxyphenylalanine was reported as a precursor for the glucosinolate glucotropaeolin (36). In that paper the authors considered the possibility of N-hydroxyphenylalanine being converted nonenzymatically to products which subsequently could be metabolized to glucotropaeolin. However, the calculations were based on a reported disproportionation of N-hydroxyphenylalanine to phenylalanine and phenylpyruvic acid oxime (37). Later investigations (20) have shown that the major decomposition reaction of N-hydroxyamino acids is the oxidative decarboxylation to the corresponding aldoxime, in this case, phenylacetaldoxime. If N-hydroxyamino acids do in fact disproportionate under physiological conditions, the products obtained will be the corresponding amino acid and aldoxime (13), i.e. in this case phenylalanine and phenylacetaldoxime. Phenylacetaldoxime is already established as an intermediate in the biosynthesis of glucotropaeolin and is a much better substrate than the ketoaldehyde. The calculations made in the above paper (36) were, thus, based on incorrect assumptions. Furthermore, the above studies with intact plants required long times for feeding the N-hydroxyphenylalanine (24 h) compared to the short time experiments (2 min to 2 h) in the present study. Even though N-hydroxyamino acids may have different rates of decomposition, the prolonged experimental time and the possibility for the N-hydroxyamino acid to react with various oxidizing agents, especially during the heating process also involved, would suggest that a significant portion of the N-hydroxyphenylalanine originally administered was incorporated only after a preliminary nonenzymatic conversion to phenylacetaldoxime. Because of these decomposition reactions, the involvement of N-hydroxyamino acids as intermediates in the biosynthesis of cyanogenic glucosides should preferably be demonstrated by their production in biological systems rather than by their utilization. The GLC/GPC procedure already described (15) should prove advantageous in monitoring such reactions.

In contrast to the N-hydroxylation of amino acids, the N-hydroxylation of various medicinal amine drugs is well established (38). N-Hydroxyamines have been detected in the blood, bile, and urine of numerous species following the administration of amine drugs. *In vitro* experiments have shown that this N-hydroxylation step is mediated by a complex enzyme system obtained as a microsomal fraction from disrupted cells (38). Considering the vast number and types of drugs which are N-hydroxylated, the enzyme system(s) involved must be hypothesized to show a broad range of substrate specificity. This is in strong contrast to the sorghum microsomal system described here and suggests that the plant enzyme system differs significantly from the mammalian drug-metabolizing system(s). Little is known about the quantitative importance of N-hydroxylation as a metabolic route for amine drugs. However, this route leads to pharmacologically and pathologically important metabolic intermediates. N-Hydroxylation is the initial activation step required for some amine drugs to express their known carcinogenicity (39, 40). To test N-hydroxyamino acids for carcinogenic properties, N-hydroxytyrosine was examined by the Ames procedure (41, 42) as a frameshift mutagen and as a mutagen causing base pair substitution. N-hydroxytyrosine was found not to be toxic to the Salmonella strains used and did not show any mutagenic effect.

The studies on dhurrin biosynthesis described here and elsewhere (6, 43) demonstrate that the biosynthesis of cyanogenic glucosides involves a series of compounds not commonly found in biological systems. The difficulties encountered in their identification have in part been due to the low levels at which these intermediates are present. No intermediates have been isolated from intact untreated plants and even in the cell-free system used here, accumulation of intermediates occurs only after special treatment of the microsomal preparation. Such results suggest that the biosynthetic pathway is catalyzed by a highly organized multienzyme complex or by a multifunctional enzyme (44, 45). This suggestion has recently been substantiated by feeding experiments with double labeled precursors. These results in turn can explain our failure to inhibit biosynthesis at the N-hydroxytyrosine level or to determine the co-factor requirements for the conversion of N-hydroxytyrosine to p-hydroxyphenylacetaldoxime.

Acknowledgments—We are indebted to Dr. Dennis Hsieh, Environmental Toxicology, University of California, Davis, and Dr. Linda B. Jacobsen, Purdue University Cancer Center, West Lafayette, Indiana, for testing the mutagenic properties of N-hydroxytyrosine. Prof. Peder Olesen Larsen, Chemistry Institute, Royal Veterinary and Agricultural University, Copenhagen, is thanked for a critical review of the manuscript.

REFERENCES


3B. L. Moller and E. E. Conn, unpublished results.
Cyanogenic Glucoside Biosynthesis

The biosynthesis of cyanogenic glucosides in higher plants. N-Hydroxytyrosine as an intermediate in the biosynthesis of dhurrin by Sorghum bicolor (Linn) Moench.

B L Møller and E E Conn


Access the most updated version of this article at http://www.jbc.org/content/254/17/8575

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/17/8575.full.html#ref-list-1