The Biosynthesis of Cyanogenic Glucosides in Higher Plants

IDENTIFICATION OF THREE HYDROXYLATION STEPS IN THE BIOSYNTHESIS OF DHURRIN IN SORGHUM BICOLOR (L.) MOENCH AND THE INVOLVEMENT OF 1-ACI-NITRO-2-(p-HYDROXYPHENYL)ETHANE AS AN INTERMEDIATE*

Barbara Ann Halkier and Birger Lindberg Møller‡

From the Department of Plant Biology, Plant Biochemistry Laboratory, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark

N-Hydroxytyrosine, (E)- and (Z)-p-hydroxyphenylacetaldehyde oxime, p-hydroxyphenylacetonitrile, and p-hydroxymandelonitrile are established intermediates in the biosynthesis of the tyrosine-derived cyanogenic glucoside dhurrin (Halkier, B. A., Olsen, C. E., and Møller, B. L. (1989) J. Biol. Chem. 264, 19487-19494). Simultaneous measurements of oxygen consumption and biosynthetic activity using a microsomal enzyme system isolated from etiolated sorghum seedlings demonstrate a requirement for three oxygen molecules in the conversion of tyrosine to p-hydroxymandelonitrile. Two oxygen molecules are consumed in the conversion of tyrosine to (E)-p-hydroxyphenylacetaldehyde oxime, indicating the existence of a previously undetected hydroxylation step in addition to that resulting in the formation of N-hydroxytyrosine. Radioactively labeled 1-nitro-2-(p-hydroxyphenyl)ethane was chemically synthesized and tested as a possible intermediate. Biosynthetic experiments demonstrate that the microsomal enzyme system metabolizes the nitro compound to the subsequent intermediates in dhurrin synthesis ($K_m = 0.05$ mm; $V_{max} = 14$ nmol/mg of protein/h). Low amounts of 1-nitro-2-(p-hydroxyphenyl)ethane are produced in the microsomal reaction mixtures when tyrosine is used as substrate. These data support the involvement of 1-nitro-2-(p-hydroxyphenyl)ethane or more likely its aci-nitro tautomer as an intermediate between N-hydroxytyrosine and p-hydroxyphenylacetaldehyde oxime. The conversion of (E)-p-hydroxyphenylacetaldehyde oxime into p-hydroxyphenylacetamide and p-hydroxyphenylacetonitrile dissociates into p-hydroxybenzaldehyde and cyanide, which are therefore the end products obtained in vitro using the microsomal enzyme system. The biosynthetic pathway involves N-hydroxytyrosine, (E)- and (Z)-p-hydroxyphenylacetaldehyde oxime, p-hydroxyphenylacetonitrile, and (S)-p-hydroxymandelonitrile as intermediates and thus includes two hydroxylation steps (Fig. 1) (2, 4). The participation of each of these intermediates is well established. However, as indicated in Fig. 1, two of the biosynthetic steps have been suggested to represent multistep conversions. The conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime represents a four-electron oxidative de-carboxylation. N-Hydroxytyrosine has been identified as an intermediate in this conversion (4). The retention of the α-hydrogen atom of tyrosine in the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime excludes p-hydroxyphenylpyruvic acid oxime as intermediate between N-hydroxytyrosine and p-hydroxyphenylacetaldehyde oxime (2). Instead, the participation of 3-(p-hydroxyphenyl)-2-nitrosopropanio acid has been suggested (2, 4). This compound, which can be envisioned formed by dehydrogenation of N-hydroxytyrosine, is labile and produces the oxime upon decarboxylation. The lability of the α-nitrosocarboxylic acid could explain why this intermediate has not been isolated. The conversion of p-hydroxyphenylacetaldehyde oxime to p-hydroxyphenylacetamide and p-hydroxyphenylacetonitrile has been reported to require NADPH as a cofactor (3–5), suggesting that this represents a multistep conversion embodying a redox reaction. In the biosynthesis of glucosinolates, an aci-nitro compound has been proposed as the intermediate following the oxime (7, 8). Based on the apparent similarities between the biosynthetic pathways for glucosinolates and cyanogenic glucosides, an aci-nitro compound has subsequently been suggested as intermediate between the oxime and the nitrile in the biosynthetic pathway for cyanogenic glucosides (9, 10). Chemical conversion of nitro compounds into nitriles is well established (11).

In this study, we have used quantitative measurements of oxygen consumption and biosynthetic activity to test the proposals regarding the existence of additional intermediates in the biosynthesis of the cyanogenic glucoside dhurrin. We demonstrate that the conversion of tyrosine to p-hydroxymandelonitrile proceeds with the consumption of three oxygen molecules and show that 1-aci-nitro-2-(p-hydroxyphenyl)ethane is an intermediate in the pathway positioned between N-hydroxytyrosine and (E)-p-hydroxyphenylacet-

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‡To whom correspondence should be addressed.

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The incorporation of 1-aci-nitro-2-(p-hydroxyphenyl)ethane as an additional intermediate in the pathway is in agreement with the oxygen consumption data.

**EXPERIMENTAL PROCEDURES**

**Chemicals.**—L-[U-¹⁴C]Tyrosine (516 mCi/nmol) was purchased from Du Pont-New England Nuclear. Unlabeled N-hydroxytyrosine was synthesized by chemical reduction of p-hydroxyphenylpyruvic acid oxime (12). Unlabeled (E)- and (Z)-p-hydroxyphenylacetalddehyde oxime were produced by oxidative decarboxylation of N-hydroxytyrosine in 1 N HCl (13). [U-¹⁴C](E) and (Z) p-hydroxyphenylacetaldehyde oxime were produced enzymatically from [U-¹⁴C]tyrosine using the -SH microsomal enzyme system as described earlier (2). 1-Nitro-2-(p-hydroxyphenyl)ethane was chemically synthesized by sodium borohydride reduction of 1-nitro-2-(p-hydroxyphenyl)ethane obtained from condensation of benzaldehyde and nitromethane (14). A racemate of (R)- and (S)-[2-³H]1-nitro-2-(p-hydroxyphenyl)ethane was obtained using sodium [3H]borohydride (58.1 Ci/mmol) as reductant. All other chemicals were of analytical grade.

**Plant Material and Microsomal Preparations.**—Seeds of S. bicolor (L) Moench (hybrid Redland 400, grown by seeds International Inc. (Hereford, TX). Seeds were soaked in water for 24 h in the dark at 28°C and germinated in the dark for 2 days at 28°C on metal screens covered with gauze (1). Two types of microsomal preparations were made (1). One type was prepared with 2 mM diithiostreitol in all buffers and is referred to as the +SH microsomal enzyme system. The second type was prepared in the absence of diithiostreitol and is referred to as the -SH microsomal enzyme system. The +SH microsomal enzyme system catalyzes the multistep conversion of tyrosine to (S)-p-hydroxymandelonitrile, i.e., all but the last step in the biosynthesis of dhurrin. The -SH microsomal enzyme system converts tyrosine to p-hydroxyphenylacetalddehyde oxime (4).

**Determination of Biosynthetic Activity.**—Biosynthetic studies were carried out using reaction mixtures containing 50 µl of the +SH microsomal enzyme system (1.5 mg of protein/ml), 2.5 µmol of Tricine or KP, (pH 7.9, 7.5, 7.0, 6.5, 6.0, 5.5, or 5.0), 100–250 nmol of substrate (saturating concentration), and 0.5 µmol of NADPH in a total volume of 250 µl. The reaction mixtures were incubated for 30 min at 30°C. Three different techniques were used to measure biosynthetic activity. One method is based on spectrophotometric determination of HCN formed as described by Halkier and Møller (1). The use of this method is restricted to assay conditions where p-hydroxymandelonitrile formation occurs, i.e., when the substrate (tyrosine or any other intermediate) is administered to the +SH microsomal enzyme system in the presence of NADPH and O₂ (4). The second and third methods for measuring biosynthetic activity involve the use of radioactively labeled substrates and separation of intermediates and end products by TLC (4) and HPLC (2). These methods are more informative since they permit the monitoring of individual steps in the pathway and the determination of intermediates accumulating in the reaction mixtures. In addition, the HPLC method allows the separation of the E- and Z-isomers of p-hydroxyphenylacetaldehyde oxime (2). Conversion of (S)-[2-³H]1-nitro-2-(p-hydroxyphenyl)ethane to (S)-p-hydroxyphenylacetonitrile results in a complete loss of the radioactive labeling, whereas conversion of (R)-[2-³H]1-nitro-2-(p-hydroxyphenyl)ethane to (S)-p-hydroxyphenylacetonitrile results in retention of the labeling. Since a racemate of (R)- and (S)-[2-³H]1-nitro-2-(p-hydroxyphenyl)ethane was administered to the microsomal enzyme system, corrections were made for the loss of 50% of the radioactivity in the production of (S)-p-hydroxyphenylacetonitrile. Using ³C-labeled substrates, corrections were made for the loss of one or two carbon atoms as products were formed.

**Quantitative Measurements of Oxygen Consumption and Biosynthetic Activity.**—Microsomal reaction mixtures were prepared in the chamber of an oxygen electrode (Rank Brothers, Bottisham, United Kingdom) to permit measurements of oxygen consumption and biosynthetic activity. Typically, the reaction mixture contained the +SH or -SH microsomal enzyme system (500 µg of protein), 100 nmol of substrate (tyrosine (5.9 nCi/nmol) or p-hydroxyphenylacetaldehyde oxime (4.4 nCi/nmol)), and 22 µmol of Tricine (pH 7.9) in a total volume of 565 µl. After 2 min of preincubation, the reaction was initiated by injection of 40 µl of NADPH (130 nmol) into the chamber. Oxygen electrode experiments where either substrate or NADPH was omitted from the reaction mixture served as controls. The consumption of oxygen was continuously measured during the incubation period (13 min) at 26°C. At the end of the incubation, the intermediates and end products accumulated in the reaction mixture were quantified by TLC analysis.

**Metabolism of [2-³H]1-nitro-2-(p-hydroxyphenyl)ethane by +SH and -SH Microsomal Enzyme Systems as Function of pH.**—The pH optimum for the metabolism of [2-³H]1-nitro-2-(p-hydroxyphenyl)ethane was determined using reaction mixtures containing the +SH or -SH microsomal enzyme system (150 and 300 µg of protein, respectively), 1.1 nmol of [2-³H]1-nitro-2-(p-hydroxyphenyl)ethane (92.5 µCi/nmol), 1.4 µmol of NADPH, and 10 µmol of KP, (pH 7.9, 7.5, 7.0, 6.5, or 6.0) in a total volume of 230 µl. After incubation for 30 min at 30°C, the accumulation of intermediates in the reaction mixtures was analyzed by TLC.

**Results**

**Determination of Number of Hydroxylation Steps Involved in Biosynthesis of the Cyanogenic Glucoside Dhurrin in Sorghum.**—Molecular oxygen is considered as a substrate in the conversion of tyrosine to N-hydroxytyrosine (4) and in the conversion of p-hydroxyphenylacetonitrile to p-hydroxyphenylacetalddehyde oxime (Fig. 1) (14). The validity of this pathway was tested by preparing reaction mixtures containing 25 nmol of [U-¹⁴C]p-hydroxyphenylacetalddehyde oxime (5 nCi/nmol) or 250 nmol of p-hydroxyphenylacetonitrile, 25–100 nmol of 1-nitro-2-(p-hydroxyphenyl)ethane as trap, 0.3 µmol of NADPH, and 14 µmol of KP, (pH 7.9) in a total volume of 230 µl. After incubation for 30 min at 30°C, the accumulation of intermediates in the reaction mixtures was analyzed by TLC.

The consumption of intermediates in the reaction mixture was analyzed by TLC or HPLC.

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Biosynthesis of the Cyanogenic Glucoside Dhurrin in Sorghum

**Fig. 1.** Biosynthetic pathway for tyrosine-derived cyanogenic glucoside dhurrin from *S. bicolor*. The double arrows indicate potential multi-step conversions.

**Fig. 2.** Oxygen consumption during tyrosine metabolism. Oxygen consumption was measured in the oxygen electrode in the presence of the +SH microsomal enzyme system, tyrosine, and NADPH (TYR trace). Tyrosine was absent in the control.

Demonstrate that the conversion of oxime to p-hydroxybenzaldehyde involves a single hydroxylation reaction independent of the use of the (E)- or (Z)-oxime as substrate. These data are in accord with Pathway B, but not with Pathway C, and indicate that two molecules of oxygen are consumed in the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime. This was investigated experimentally by administration of tyrosine to the +SH microsomal enzyme system, which converts tyrosine to p-hydroxyphenylacetaldehyde oxime.

The oxygen consumption compared to the oxime production demonstrates that two oxygen molecules are required in the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime (Table I, Pathway B).

**Metabolism of 1-Nitro-2-(p-hydroxyphenyl)ethane by Microsomal Enzyme System—**N-Hydroxytyrosine is an established intermediate in the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime (4). The consumption of two oxygen molecules in this conversion reveals the existence of an additional intermediate. 1-Nitro-2-(p-hydroxyphenyl)ethane was tested as a putative candidate. In the presence of NADPH, administration of [2-3H]1-nitro-2-(p-hydroxyphenyl)ethane to the +SH microsomal enzyme system results in the production of [1-3H]p-hydroxybenzaldehyde, the end product of the +SH microsomal enzyme system (Fig. 3). Usually, only insignificant amounts of the intermediates positioned between the administered substrate and p-hydroxybenzaldehyde accumulate in the +SH microsomal reaction mixtures (2, 4, 15). However, using 1-nitro-2-(p-hydroxyphenyl)ethane as substrate, a considerable amount of p-hydroxyphenylacetaldehyde oxime accumulates in the reaction mixture (Fig. 3).

When [2-3H]1-nitro-2-(p-hydroxyphenyl)ethane was incubated in reaction mixtures devoid of NADPH or microsomes, no production of p-hydroxyphenylacetaldehyde oxime, p-hydroxyphenylacetoneitrile, or p-hydroxybenzaldehyde can be detected. Although the metabolic rates are low, the data demonstrate that the microsomal enzyme system is capable of catalyzing an NAPDH-dependent conversion of 1-nitro-2-(p-hydroxyphenyl)ethane to p-hydroxyphenylacetaldehyde oxime and p-hydroxybenzaldehyde. The pH optimum for the production of p-hydroxybenzaldehyde is 7.5. When the pH of the reaction mixture is lowered from 7.5 to 7.0, the aldehyde production is reduced, whereas the accumulation of oxime is increased, indicating that the enzyme converting the oxime to p-hydroxybenzaldehyde is more sensitive to lowering of pH than the enzyme catalyzing the reduction of 1-nitro-2-(p-hydroxyphenyl)ethane to p-hydroxyphenylacetaldehyde oxime. The apparent $K_m$ for 1-nitro-2-(p-hydroxyphenyl)ethane is 0.05 mM, and the $V_{max}$ for the combined rate of oxime and aldehyde production is 14 nmol/mg of protein/h. The values for $K_m$ and $V_{max}$ were calculated from Lineweaver-Burk plots since even at the very high substrate concentrations used, a constant maximum velocity was not reached.

When [2-3H]1-nitro-2-(p-hydroxyphenyl)ethane is administered to the -SH microsomal enzyme system, [2-3H]p-hydroxyphenylacetaldehyde oxime accumulates in the reaction mixture (Fig. 3). The nitro compound is not produced when [14C]-labeled oxime is administered to the -SH microsomal enzyme system. In agreement with the data on the oxygen consumption (Table I, Pathway B), this indicates that...
TABLE I

Quantitative measurements of oxygen consumption and biosynthetic activity to discriminate among Pathways A, B, and C

After incubation, the percentage of radioactivity accumulating in the intermediates was analyzed by TLC, and the amounts of products formed from tyrosine or oxime were calculated. The consumption of oxygen was calculated according to Pathways A, B, and C and compared to the oxygen consumption measured. For experimental details, see "Experimental Procedures."

| Reaction mixture composition after incubation | Measured O₂ consumption | Calculated O₂ consumption |
|---------------------------------------------|--------------------------|----------------------------|
|                                               | Uncorrected | Corrected | Pathway A | Pathway B | Pathway C |
| +SH enzyme, tyrosine, NADPH                  | 77          | 65        | 65        | 67        | 65        |
| +SH enzyme, tyrosine, NADPH                  | 100         | 61        | 44        | 67        | 67        |
| +SH enzyme, (E)-OX, NADPH                    | 66          | 32        | 34        | 34        | 67        |
| +SH enzyme, NADPH                            | 100         | 32        | 34        | 34        | 67        |
| +SH enzyme, (Z)-OX, NADPH                    | 67          | 31        | 33        | 33        | 66        |
| +SH enzyme, NADPH                            | 100         | 31        | 33        | 33        | 66        |
| -SH enzyme, tyrosine, NADPH                  | 83          | 43        | 18        | 36        | 20        |
| -SH enzyme, tyrosine, NADPH                  | 98          | 8         | 8         | 8         | 8         |

*Ty, [U-¹C]tyrosine; OX, p-hydroxyphenylacetaldehyde oxime; CN, p-hydroxyphenylacetonitrile; CHO, p-hydroxybenzaldehyde.

FIG. 3. Metabolism of 1-nitro-2-(p-hydroxyphenyl)ethane by +SH and −SH microsomal enzyme systems as function of pH. [²H₂]-Nitro-2-(p-hydroxyphenyl)ethane was administered to the +SH and −SH microsomal enzyme systems in phosphate buffers at different pH values. After incubation, the percentage of radioactivity accumulating in p-hydroxybenzaldehyde oxime (OX) and p-hydroxyphenylacetaldehyde (CHO) was analyzed by TLC. 100% represents 1.1 nmol. For experimental details, see "Experimental Procedures."

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The nitro compound is positioned before the oxime in the pathway.

Production of 1-Nitro-2-(p-hydroxyphenyl)ethane by Microsomal Enzyme System—When [U-¹C]tyrosine is administered to the +SH or −SH microsomal enzyme system, p-hydroxybenzaldehyde is the main product accumulating in the +SH microsomal reaction mixtures, whereas p-hydroxyphenylacetaldehyde oxime accumulates in the −SH microsomal reaction mixtures (4). Accumulation of ¹C-labeled 1-nitro-2-(p-hydroxyphenyl)ethane in both types of reaction mixtures is demonstrated by a specific blackening of the 1-nitro-2-(p-hydroxyphenyl)ethane band on the autoradiographs of the TLC plates after prolonged exposure. The blackening at this position is not observed in control experiments devoid of NADPH or microsomal enzyme system. The amount of ¹C-labeled nitro compound accumulated is very low (Fig. 4). The +SH and −SH microsomal enzyme systems differ with respect to their ability to accumulate the nitro compound. At pH 7.9, the percentage of radioactivity present as nitro compound in the −SH microsomal reaction mixture is typically 0.9%, whereas the level observed with the +SH microsomal enzyme system only reaches 0.1% (Fig. 4). In an

FIG. 4. Metabolism of tyrosine by +SH and −SH microsomal enzyme systems as function of pH. [¹C]Tyrosine was administered to the +SH and −SH microsomal enzyme systems in phosphate buffers at different pH values. After incubation, the percentage of radioactivity accumulating in p-hydroxybenzaldehyde (CHO), p-hydroxyphenylacetonitrile (CN), (E)- and (Z)-p-hydroxyphenylacetaldehyde oxime (OX), and 1-nitro-2-(p-hydroxyphenyl)ethane (NO₂) was analyzed by TLC. For experimental details, see "Experimental Procedures."
attempt to increase the accumulation of the nitro compound in the reaction mixture, the production of 1-nitro-2-(p-hydroxyphenyl)ethane from tyrosine was analyzed at different pH values (Fig. 4). The maximum amount of 1-nitro-2-(p-hydroxyphenyl)ethane was found to accumulate at pH 7.9. However, at low pH, the nitro compound constitutes a larger proportion relative to the other intermediates produced due to the strong inactivation of the aldehyde-, nitrile-, and oxime-metabolizing enzymes at low pH (4).

Effect of Exogenously Added 1-Nitro-2-(p-hydroxyphenyl)-ethane on Metabolic Activity of Microsomal Enzyme System—

Trapping experiments, in which [U-14C]tyrosine was administered to the +SH microsomal enzyme system in the presence of exogenously added unlabeled 1-nitro-2-(p-hydroxyphenyl)ethane, were designed in an attempt to increase the amount of radioactivity accumulating in the nitro compound. The presence of a trap of the nitro compound, however, does not result in an increase in the amount of radioactivity recovered in the nitro compound (Fig. 5). Using the +SH microsomal system, the amount of radioactivity in the nitro compound accounts for 0.04% of the total radioactivity when no trap is added. Upon the addition of 100 nmol of unlabeled nitro compound, no radioactivity is recovered in the nitro compound. The corresponding percentages using the -SH microsomal enzyme system are 0.6 and 0.2%, respectively (Fig. 5). The addition of a trap of the unlabeled nitro compound results in accumulation of the oxime in the reaction mixtures prepared with the +SH microsomal enzyme system (Fig. 5). Accumulation of the oxime was also observed when the 3H labeled nitro compound was administered to the +SH microsomal enzyme system (Fig. 3). Administration of increasing amounts of unlabeled nitro compound inhibits the metabolism of tyrosine (Fig. 5) as well as of oxime and nitrile (Fig. 6). This general inhibitory effect of the nitro compound on the metabolic activities of the microsomal enzyme system probably explains why the trapping experiments with the nitro compound do not result in the expected accumulation of radioactivity in the nitro compound.

The biosynthetic pathway for cyanogenic glucosides is generally accepted to involve two hydroxylation reactions (2–5). This study on the in vitro biosynthesis of the cyanogenic glucoside dhurrin in sorghum demonstrates the involvement of a third hydroxylation reaction. This conclusion is based on simultaneous quantitative measurements of oxygen consumption and biosynthetic activity which demonstrate that three oxygen molecules are required for the conversion of one molecule of tyrosine into p-hydroxybenzaldehyde. Two of these oxygen molecules are required for the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime and one molecule for the conversion of oxime to p-hydroxybenzaldehyde (Table I, Pathway B). Attempts to quantify the corresponding amount of NADPH being oxidized were unsuccessful due to the formation of varying amounts of an addition product between the enzymatically produced NADPH and the cyanide formed in the reaction mixture during incubation. The addition product has an absorption maximum at 325 nm and interferes with spectrophotometric NADPH determination at 340 nm.

The oxygen measurements demonstrate that the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime involves a hitherto undetected hydroxylation reaction in addition to that resulting in the production of N-hydroxytyrosine. The possible chemical structure of such putative, hitherto undetected intermediates is limited by the experimentally observed quantitative retention of the α-hydrogen of tyrosine in p-hydroxyphenylacetaldehyde oxime (2). In earlier studies (2, 4), 3-(p-hydroxyphenyl)-2-nitrosopropionic acid was suggested as an intermediate. However, this compound is derived from N-hydroxytyrosine by a dehydrogenation reaction, and its presence in the pathway was not explain the consumption of the second oxygen molecule. Another previously suggested intermediate in the pathway is 1-nitro-2-(p-hydroxyphenyl)ethane (9, 10). This compound was envisioned as being formed by oxidation of the oxime (10). Whereas formation of 1-nitro-2-(p-hydroxyphenyl)ethane would be associated with

**Fig. 5.** Metabolism of [U-14C]tyrosine by +SH and −SH microsomal enzyme systems in presence of trap of unlabeled 1-nitro-2-(p-hydroxyphenyl)ethane. The +SH and −SH microsomal enzyme systems were incubated with [U-3H]tyrosine (TYR) in the presence of increasing amounts of unlabeled 1-nitro-2-(p-hydroxyphenyl)ethane. After incubation, the percentage of radioactivity accumulating in the intermediates was analyzed by TLC. 100% represents 50 nmol of tyrosine. For abbreviations, see legend to Fig. 4.

**Fig. 6.** Effect of exogenously added 1-nitro-2-(p-hydroxyphenyl)ethane on production of p-hydroxybenzaldehyde using p-hydroxyphenylacetaldehyde oxime and p-hydroxyphenylacetonitrile as substrates. After incubation with the +SH microsomal enzyme system, p-hydroxybenzaldehyde production from the oxime (OX substrate) and the nitrile (CN substrate) was analyzed by TLC and HPLC, respectively. 100% represents the aldehyde production in the absence of the nitro compound.
the consumption of an additional molecule of oxygen, the previously suggested position of the nitro compound as intermediate between the oxime and the aldehyde is not in accordance with the measured oxygen requirements of the partial reactions. The single oxygen molecule consumed in the conversion of p-hydroxyphenylacetaldehyde oxime to p-hydroxybenzaldehyde serves to hydroxylate p-hydroxyphenylacetonitrile to p-hydroxymandelonitrile. This excludes the involvement of a hydroxylation step between the oxime and the nitrile and strongly supports that the conversion of the oxime to the nitrile proceeds as a simple dehydration. These data indicate that the nitro compound is formed by an alternative mechanism involving N-oxidation of N-hydroxytyrosine to produce 2-nitro-3-p-hydroxyphenylpropionic acid, which by decarboxylation gives rise to the formation of the 1-aci-nitro-2-(p-hydroxyphenyl)ethane (Fig. 7). The aci-form of the nitro compound is in tautomeric equilibrium with the parent nitro compound. Since the aci-nitro compound is the direct product of decarboxylation of the α-nitrocarnitric acid, the aci-tautomer is most likely the biosynthetically active tautomer. The positioning of an aci-nitro compound between N-hydroxytyrosine and p-hydroxyphenylacetaldehyde oxime in the pathway is in agreement with the oxygen stoichiometry data. The presence of the aci-nitro compound as an intermediate in the pathway is justified by the biosynthetic data which demonstrate that the microsomal enzyme system catalyzes the conversion of tyrosine to 1-nitro-2-(p-hydroxyphenyl)ethane as well as the conversion of 1-nitro-2-(p-hydroxyphenyl)ethane to p-hydroxybenzaldehyde (Figs. 3 and 4). When tyrosine is administered to the -SH microsomal enzyme system, p-hydroxyphenylacetaldehyde oxime and 1-nitro-2-(p-hydroxyphenyl)ethane accumulate. Administration of the nitro compound to the -SH microsomal enzyme system results in production of the oxime, whereas no nitro compound is produced when the oxime is used as substrate. Although the incorporation percentages obtained are low, the biosynthetic data indicate that the nitro compound is an intermediate between N-hydroxytyrosine and p-hydroxyphenylacetaldehyde oxime. In a previous study, Møller and Conn (4) stated that the nitro compound is an unlikely intermediate in the biosynthesis of cyanogenic glucosides since administration of 1-nitro-2-(p-hydroxyphenyl)ethane to the sorghum microsomal enzyme system did not result in any detectable production of hydrogen cyanide. This conclusion was based on a spectrophotometric assay, which is less sensitive than the assay used in this study based on radioactively labeled substrates.

The pH optimum for the conversion of 1-nitro-2-(p-hydroxyphenyl)ethane to p-hydroxybenzaldehyde is 7.5 (Fig. 3). Even at the pH optimum, the conversion rate is low as evidenced by the Vmax value of 14 nmol/mg of protein/h for the nitro compound compared to values of 145 and 400 nmol/mg of protein/h for tyrosine and p-hydroxyphenylacetonitrile, respectively (4). One possibility is that the nitro compound does not have free access to the active site of the appropriate enzyme. Another explanation is that the nitro compound is not administered to the enzyme system in the right tautomeric form. Nitro compounds are in equilibrium with tautomeric aci-nitro compounds and their common nitronate anions (17) (Equation 1).

\[
\begin{align*}
\text{aci-Nitro} & \quad \text{Nitronate} \\
\text{Nitro} & \quad \text{(Nitric acid)}
\end{align*}
\]

At pH values around 7, the equilibrium between the tautomers favors the stable parent nitro compound. aci-Nitro compounds are weak acids formed by protonization of the nitronate anion (17). Attempts to increase the rate of the enzymatic reduction of the nitro compound to the oxime by lowering the pH were unsuccessful probably because the pH values necessary to generate significantly elevated amounts of the aci nitro tautomer are too low to maintain enzymatic activity (Fig. 3). The microenvironment of the microsomal enzyme system catalyzing the coordinated formation and utilization of the nitro compound may serve to stabilize the aci-tautomer, thus maintaining the nitro compound on the aci-tautomer form suitable for metabolism. Exogenously added nitro compound exerts an inhibitory effect on the metabolic activity of the microsomal enzyme system as measured with tyrosine as well as with p-hydroxyphenylacetaldehyde oxime and p-hydroxyphenylacetonitrile as substrates (Figs. 5 and 6). Based on the structural similarities between the nitro compound and the oxime, the inhibitory effect on the metabolism of the oxime may be caused by a specific interaction between the nitro compound and the active site on the oxime-metabolizing enzyme. The inhibitory effect of the nitro compound on the metabolism of tyrosine and p-hydroxyphenylacetonitrile indicates that the nitro compound has an unspecific effect on the microsomal enzyme system. The inhibition of the metabolism of the oxime in the presence of the nitro compound may explain why p-hydroxyphenylacetaldehyde oxime accumulates together with p-hydroxybenzaldehyde in the +SH microsomal reaction mixtures where 1-nitro-2-(p-hydroxyphenyl)ethane is administered as substrate (Fig. 3) and why incubation of radioactively labeled tyrosine with the +SH or -SH microsomal enzyme system in the presence of a trap of unlabeled nitro compound results in the accumulation of radioactivity in the oxime rather than in the nitro compound (Fig. 5). The inability to trap 14C-labeled 1-nitro-2-(p-hydroxyphenyl)ethane could indicate that the enzymatically produced nitro compound does not exchange freely with the exogenously added nitro compound. This observation may be explained by permeability barriers imposed by the microsomal vesicles. It could also...
indicate that the nitro compound is metabolically channeled. In previous studies (2, 15), it was shown that the sorghum microsomal enzyme system exhibits catalytic facilitation with respect to N-hydroxytyrosine, (E)-p-hydroxyphenylacetaldehyde oxime, and p-hydroxyphenylacetone, i.e. the enzyme system metabolizes more efficiently the N-hydroxytyrosine, (E)-p-hydroxyphenylacetaldehyde oxime, and p-hydroxyphenylacetone produced in situ compared with the same intermediates when exogenously added. The position of the nitro compound between N-hydroxytyrosine and (E)-p-hydroxyphenylacetaldehyde oxime, which are both channeled intermediates in the pathway, combined with the difficulties in trapping the nitro compound suggest that also 1-nitro-2-(p-hydroxyphenyl)ethane may be a metabolically channeled intermediate.

The +SH and -SH microsomal enzyme systems differ greatly in their ability to accumulate 1-nitro-2-(p-hydroxyphenyl)ethane (Fig. 4). Generally, the level of accumulated nitro compound is 10 times higher in reaction mixtures prepared with the -SH microsomal enzyme system than in those prepared with the +SH microsomal system. The -SH microsomes are manipulated to lose the ability to convert p-hydroxyphenylacetaldehyde oxime by overnight dialysis against a buffer not containing dithiothreitol. The higher percentage of 1-nitro-2-(p-hydroxyphenyl)ethane accumulating in the -SH reaction mixtures may reflect a partial inactivation of the nitro compound-metabolizing enzyme activity during the preparation of the -SH microsomal enzyme system.

Glucosinolates and cyanogenic glucosides are synthesized from amino acids. Oximes have been reported as common intermediates (9). From these similarities, the initial steps in the biosynthesis of these two groups of secondary plant products have been speculated to proceed by a common pathway (9). Matsuo et al. (8) have studied the biosynthesis of benzylglucosinolate in *Tropaeolum majus* and found that 1-nitro-2-(p-hydroxyphenyl)ethane does serve as a precursor (8). Based on trapping experiments where radioactively labeled p-hydroxyphenylacetaldehyde oxime was fed to shoots of *T. majus* in the presence of unlabeled 1-nitro-2-(p-hydroxyphenyl)ethane, the oxime was shown to be a precursor for the nitro compound (8). Prompted by the results of Matsuo et al. on the involvement of an aci-nitro compound in glucosinolate biosynthesis, an aci-nitro compound has been suggested as the intermediate following the oxime in the biosynthesis of cyanogenic glucosides (9). The study presented here indicates that the aci-nitro compound is an intermediate in the biosynthesis of cyanogenic glucosides. However, in contrast to the studies of Matsuo et al., this study indicates that the nitro compound is positioned before and not after the oxime in the pathway. We are therefore tempted to suggest that the conversion of oxime to the aci-nitro compound observed by Matsuo et al. with seedlings of *T. majus* reflects a secondary metabolic transformation not directly related to the biosynthetic pathway. Similar results as those reported by Matsuo et al. regarding oximes as precursors for the nitro compound have been reported by Høsel et al. (18) with osmotically stressed cell suspension cultures of *Brassica poppy* (Eschscholzia californica Cham.). This plant contains the two tyrosine-derived cyanogenic glucosides triglochinin and dhurrin. The cyanogenic glucosides were not produced in the cell suspension cultures, which, however, were shown to accumulate the glucoside of 1-nitro-2-(p-hydroxyphenyl)ethane. Conversely, microsomes isolated from 6-day-old seedlings of California poppy did not accumulate detectable amounts of 1-nitro-2-(p-hydroxyphenyl)ethane. Whether accumulation of 1-nitro-2-(p-hydroxyphenyl)ethane occurs in the intact plant under osmotic stress or only in osmotically stressed cell suspension cultures was not reported. The production of the glucoside of the nitro compound in the osmotically stressed cell suspension cultures may represent a secondary unspecific oxidation of the oxime induced as a result of the inability of the cultures to convert the oxime to the cyanogenic glucosides which are the natural constituents of the plant.

The occurrence of nitro compounds in biological material is rare. 1-Nitro-2-(p-hydroxyphenyl)ethane has been found in the plant *Thalictrum aquilegifolium*, which also contains the tyrosine-derived cyanogenic glucosides dhurrin and taxiphyllin (19), 3-Nitropropionic acid and its alcohol are toxicants mainly found in legume families (e.g. *Astragalus*) (20). Many examples of loss of livestock from grazing on *Astragalus* with poisonous nitro compounds have been reported (21). Several nitro compounds isolated from bacteria have an antibiotic effect, the most important being chloramphenicol. It appears that nitro compounds are potential inhibitors of many reactions.

In conclusion, we have demonstrated the requirement of three oxygen molecules in the biosynthesis of cyanogenic glucosides. The two oxygen molecules consumed in the conversion of tyrosine to p-hydroxyphenylacetaldehyde oxime demonstrate the involvement of a hitherto unknown hydroxylation step. The one oxygen molecule consumed between p-hydroxyphenylacetaldehyde oxime and p-hydroxybenzaldehyde indicates that the conversion of p-hydroxyphenylacetaldehyde oxime to p-hydroxyphenylacetone proceeds by a simple dehydration. The occurrence of the additional hydroxylation step compared with the biosynthetic data presented shows the involvement of 1-aci-nitro-2-(p-hydroxyphenyl)ethane as an additional intermediate between N-hydroxytyrosine and p-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin (Fig. 7).

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B A Halkier and B L Møller

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