Study of Nicotine Demethylation in *Nicotiana otophora*

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Microsomes from *Nicotiana otophora* catalyze the demethylation of nicotine to nornicotine in the presence of NADPH and oxygen. Activity was maximal at pH 7.0–7.5 and 30 °C. The enzyme appeared to be most stable in the presence of both nicotine and NADPH. Phosphate, magnesium, nornicotine, and concentrations of microsomal protein higher than 2 mg/mL were inhibitory. Typical rates of nornicotine formation were 10–50 pmol min^{-1} (mg of protein)$^{-1}$, while V_{max} and the apparent K_m (nicotine) were estimated to be 105 pmol/min and 51 μM, respectively. Early studies suggest that Triton X-100 may be able to solubilize active nicotine demethylase. Nicotine demethylation satisfies some of the primary criteria for cytochrome P-450 involvement, including inhibition by anticytochrome P-450 reductase, but inhibition by carbon monoxide was not demonstrated.

INTRODUCTION

Nornicotine is a major alkaloid in *Nicotiana* and is the principal alkaloid in 30–40% of the species (Bush and Crowe, 1983). Virtually all nornicotine is formed from *N*-demethylation of nicotine (Dawson, 1951; Bush, 1981; Leete, 1984), and depending on the species, much of the nornicotine accumulation in *Nicotiana* occurs as the leaf matures (*e.g.*, *N. otophora*) (Dawson, 1945) or during curing (*e.g.*, *N. tabacum*) (Wada, 1956).

The demethylation of nicotine has been described to be controlled by one of two dominant genes (Griffith *et al.*, 1955). This demethylation is not specific for the naturally occurring stereoisomer of nicotine, and partial racemization takes place during the process (Kisaki and Tamaki, 1961). The demethylation of nicotine as the primary source of nornicotine was first reported over 45 years ago by Dawson (1945). Nevertheless, the enzyme catalyzing this process has not been characterized or isolated. Schröter (1966) did detect *in vitro* nicotine demethylation in extracts from *N. alata*, but he did not present any *in vitro* properties of the enzyme catalyzing this process.

The objective of this study was to characterize the *in vitro* properties of nicotine demethylation from *N. otophora*.

MATERIALS AND METHODS

Plant Material. *N. otophora* readily converts nicotine to nornicotine (Saitoh *et al.*, 1985) and was used as the enzyme source. Lamina or pith was obtained from 6-month-old plants grown in a greenhouse with supplemental light from high-pressure sodium lamps.

Preparation of Microsomes. Microsomes were isolated at 4 °C by a modification of methods used for the extraction of microsomes from higher plants (Reichhart *et al.*, 1980; Dohn and Krieger, 1984; Mougin *et al.*, 1990). Pith or lamina tissue was disrupted separately in a Waring blender in the presence of 8 volumes of *N*-(2-hydroxyethyl)piperazine-*N*'-[2-ethanesulfonic acid] (HEPES) buffer (50 mM, pH 7.5) supplemented with 250 mM sucrose, polyvinylpolypyrrolidone (PVPP) (0.2 g/g of tissue), and 15 mM mercaptoethanol. The crude extract was filtered through two layers of cheesecloth to remove undisrupted tissue, and the filtrate was centrifuged for 20 min at 10000*g* to remove cellular debris. The supernatant was subjected to further centrifugation at 100000*g* for 60 min to pellet the microsomal fraction. The microsomal pellet was resuspended with the aid of a tissue homogenizer in 3 volumes of HEPES buffer (50 mM) supplemented with glycerol (30% v/v) and was stored in aliquots of 200 μL at −80 °C.

Nicotine Demethylation Assay. Nicotine [*pyrrolidine*-2-^{14}C] was obtained from NEN Research Products and had a specific activity of 42.4 mCi/mmol. Routine enzyme assay consisted of approximately 80 μg of resuspended microsomal protein, 20 μmol of NADPH, and 10 μmol of nicotine and was made up to 40 μL with 50 mM HEPES buffer of pH 7.5. NADPH was omitted for control incubations. The reaction was carried out at 30 °C for 30 min and was stopped with addition of 40 μL of 95% methanol, which also contained 50 mM nornicotine and nicotine to increase the recovery of [^{14}C]nornicotine and [^{14}C]nicotine. The amount of demethylation was measured using the thin-layer chromatography procedure of Fannin and Bush (1992).

Cytochrome P-450 Reductase Assay. The cytochrome P-450 reductase activity was determined by observing the reduction of cytochrome *c* with NADPH at 550 nm (Dohn and Krieger, 1984).

Oxygen Exclusion. Two treatments were carried out to determine if nicotine demethylation had a requirement for oxygen. The reaction components were dispensed into six 2.5-mL vials which were then hermetically sealed. Four vials were flushed with nitrogen (60 mL/vial), and two of these were then flushed with air (60 mL/vial). The two control vials were not flushed with either gas.

Solubilization. A number of detergents were screened for their ability to solubilize active nicotine demethylase from the microsomal fraction. About 100 μL of microsomal extract was incubated with 33.3 μL of various concentrations of detergent. The control had no detergent. The detergent–protein mixture was incubated at room temperature for 20 min and then centrifuged for 60 min at 100000*g*. An aliquot of the supernatant was carefully removed and assayed for nicotine demethylase activity. The supernatant was then discarded, and the pellet was resuspended in buffer with the aid of an automatic dispensing pipet and then assayed for enzyme activity.

RESULTS AND DISCUSSION

Powdering excised leaf tissue in liquid nitrogen and then extracting the soluble proteins with buffer (Frear *et al.*, 1969) resulted in yields of active microsomal preparations that varied greatly between preparations. Omitting the liquid nitrogen step and disrupting tissue in 8 volumes of extraction buffer increased the yield and reduced the variability. Browning of the crude extract could not be prevented by the addition of 0.2 g/g PVPP or by a similar concentration of Amberlite XAD-4. As 15 mM mercaptoethanol did prevent browning, the extraction was performed in the presence of mercaptoethanol.

A small amount of demethylation was detected in the supernatant from the 100000*g* centrifugation. The su-

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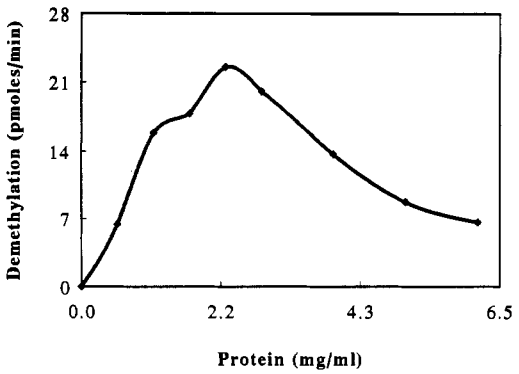

Figure 1. Relationship between microsomal protein concentration and demethylation. Protein concentration was measured using Coomassie protein assay reagent.

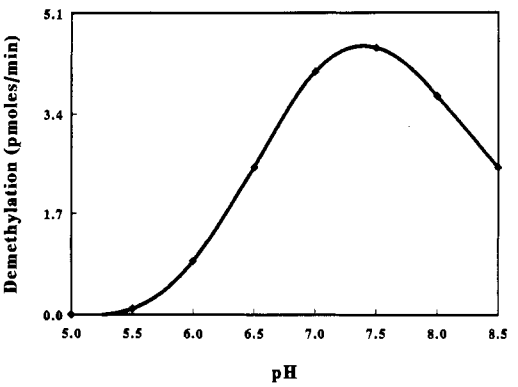

Figure 2. pH profile of nicotine demethylase. pH was varied using MES (pH 5.0–6.5) and HEPES (pH 7.0–8.5) buffers. Data are shown with a spline curve fit.

pernatant activity was not diminished by incubating the microsomal preparation in the absence of NADPH, at 4 or 55 °C, or by boiling the extract for 2 min prior to assaying. Hence, it was concluded that the demethylation associated with the supernatant was not enzymatic and was not characterized further. Although Schröter (1966) did not present any data on the *in vitro* characteristics of the demethylation of nicotine, the activity he measured may have been the nonenzymatic activity we observed in the 100000*g* supernatant. Schröter's activity too was located in the supernatant.

Microsomes stored for up to 5 months at −80 °C were still able to catalyze nicotine demethylation. Unlike the enzymes catalyzing the *N*-demethylation of substituted 3-(phenyl)-1-methylureas in cotton (Frear *et al.*, 1969), nicotine demethylase was not inactivated after one freeze–thaw cycle, but instead increased by up to 80%.

The level of microsomal *N*-demethylase in *N. otophora* [10–50 pmol min^{-1} (mg of protein)$^{-1}$], as measured by its capacity to demethylate nicotine, was considerably higher than *N*-demethylases from 12 species of higher plants. The levels of these *N*-demethylases [<8 pmol min^{-1} (mg of protein)$^{-1}$] were measured by their ability to demethylate substituted 3-(phenyl)-1-methylureas (Frear *et al.*, 1969). However, the rate of *N*-demethylation of aminopyrine by *Helianthus tuberosus* microsomes [186 pmol min^{-1} (mg of protein)$^{-1}$] (Fonne-Pfister *et al.*, 1988) was considerably higher than the observed rate of nicotine demethylation in *N. otophora*.

A comparison of the level of NADPH-dependent cytochrome P-450 reductase in our *N. otophora* microsomes with the level in higher plant microsomes prepared by other laboratories might indicate whether our procedure for microsomal preparation needed to be modified so as to optimize the *in vitro* yield of nicotine demethylation activity. The reductase activity in *N. otophora* was approximately 370 nmol min^{-1} (mg of protein)$^{-1}$, which was over 3-fold higher than the rate in microsomes from aging *H. tuberosus* tubers (Fonne-Pfister *et al.*, 1988), suggesting that our protocol was adequate for microsome preparation.

Nicotine demethylation activity was dependent on microsomal protein concentration in the assay (Figure 1). Increased protein, up to 2 mg/mL, resulted in increased nicotine demethylation, but higher concentrations resulted in decreased activity; consequently, the concentration of microsomal protein used in assays was always less than 2 mg/mL. Inhibition by high microsomal protein concentrations was also observed in the demethylation of *p*-chloro-*N*-methylaniline (PCMA) in *Persea americana* (Dohn and Krieger, 1984).

The effect of temperature on nicotine demethylation was observed by incubating aliquots of the assay at various temperatures between 15 and 40 °C. The temperature profile showed a maximum at 30 °C and a 60% loss in activity by 40 °C. All subsequent assays were conducted at the optimum of 30 °C, which was also the optimum temperature reported by Dohn and Krieger (1984) for the demethylation of PCMA.

The pH profile, determined with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and HEPES buffers, indicated that very little demethylation occurred below pH 6.0 (Figure 2). Optimum pH was between 7.0 and 7.5, which was very close to the pH 7.5 value reported by Fonne-Pfister *et al.* (1988) for aminopyrine *N*-demethylase in phosphate buffer from *H. tuberosus*. Routine assays for nicotine demethylation were performed in 50 mM HEPES of pH 7.5.

Initially a phosphate assay buffer (100 mM, pH 7.0) was utilized since it was effective for the *N*-demethylation of substituted 3-(phenyl)-1-methylureas (Frear *et al.*, 1969). When nicotine demethylation was measured in 50 mM HEPES buffer during the pH study, the rate of demethylation at pH 7.0 was over 3-fold higher than with phosphate. Magnesium too was found to be inhibitory, with a concentration of 10 mM resulting in a 50% reduction in activity. Hence, demethylation was measured in HEPES without added magnesium.

A number of treatments were carried out on the 100000*g* pellet in an attempt to solubilize active nicotine demethylase. The detergents tested were CHAPS, glycodeoxycholate, cholate, octyl β-glucopyranoside, octyl β-thioglucopyranoside, sodium lauryl sulfate, and Triton X-100. High salt (300 mM KCl) and pH (pH 10) were also tried. Only Triton X-100 appeared to be successful in solubilizing nicotine demethylase as activity was observed in the supernatant after the detergent–protein mixture was centrifuged at 100000*g* for 60 min (Figure 3).

Nicotine demethylation required the presence of a reduced pyridine nucleotide. The rate of demethylation was approximately 15-fold faster with NADPH than with NADH. At low concentrations of NADPH, the rate of demethylation was greatly enhanced by NADH (Figure 4). Neither 10 mM NAD nor 10 mM NADP supported any demethylation, and in the presence of NADPH they were significant inhibitors (54% and 15% of control, respectively). In the absence of NADH, competing reactions might utilize NADPH, resulting in limiting concentrations of NADPH for nicotine demethylation. However, NADH might be preferentially oxidized in the presence of NADPH and thus spare NADPH for the demethylation reaction.

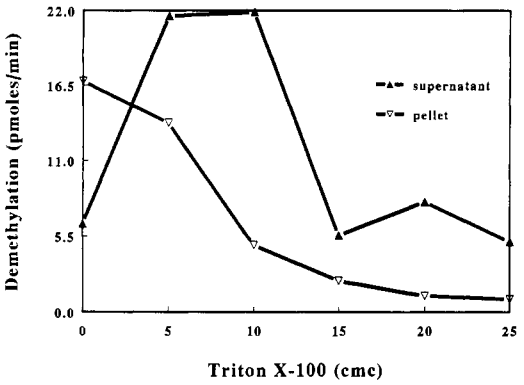

Figure 3. Solubilization of nicotine demethylase from the microsomal pellet with Triton X-100.

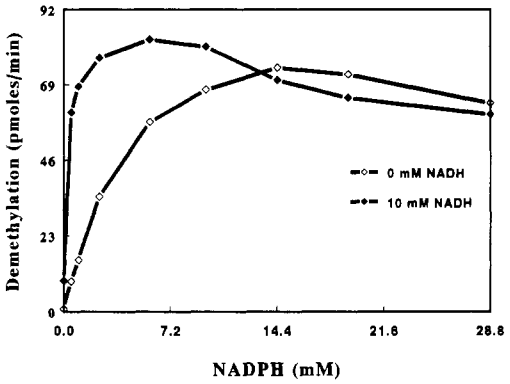

Figure 4. Effect of NADPH and NADH on nicotine demethylation.

Since the rate of demethylation was linear for only the first 30 min, assays were stopped after 30 min. Beyond 30 min the reaction rate decreased rapidly and was nearly zero after 4 h. In an attempt to find the cause for this decrease in the rate of demethylation with time, various combinations of the assay components were incubated at 30 °C for 1 h before further combinations of assay components were added and the reactions stopped after another 1-h incubation. Control was the incubation of all three components (NADPH, nicotine, and microsomes) for 2 h with extra buffer added after 1 h (Figure 5a). Incubating only microsomes for 1 h before adding NADPH and nicotine (Figure 5b) resulted in nornicotine production of approximately half that of the control (Figure 5a). Incubation of microsomes in the presence of NADPH for 1 h did not result in any demethylation in the second hour after addition of nicotine (Figure 5c). If nicotine was added with extra NADPH after 1 h of incubation of microsomes and NADPH, demethylation of nicotine was observed (Figure 5d), indicating that when the microsomes were incubated with only NADPH, the NADPH was probably consumed by competing reactions during the first hour and some nicotine demethylase denatured. Incubating microsomes and nicotine for 1 h before adding NADPH (Figure 5e) produced one-third of the nornicotine of the control (Figure 5a) but significantly more than when the microsomes were incubated with NADPH (Figure 5c). Incubating NADPH and nicotine together prior to adding microsomes (Figure 5f) yielded nornicotine levels comparable to the control (Figure 5a). Extra microsomes added after 1 h did not result in further demethylation because NADPH and probably nicotine were limiting (data not shown). Also, extra nicotine or nicotine and microsomes did not enhance the production of nornicotine over the control. Addition of extra NADPH or NADPH and microsomes resulted in 18% and 26%, respectively,

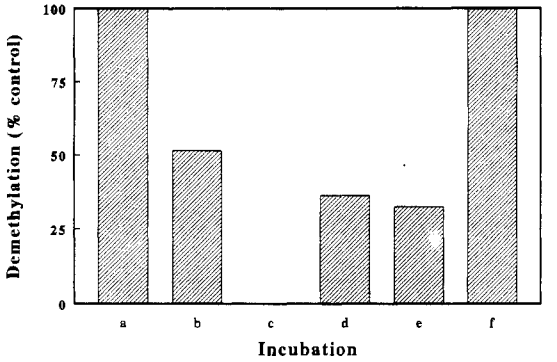

Figure 5. Stability of nicotine demethylase at 30 °C. Different combinations of microsomes, NADPH, and nicotine were incubated for 1 h. Further combinations of assay components were then added and the reactions allowed to proceed for an additional 1 h. The final volume for all incubations was 40 μL. The initial (first hour) and final (second hour) incubations were as follows: (a) (first) microsomes, NADPH, and nicotine; (second) extra buffer; (b) (first) microsomes; (second) NADPH and nicotine; (c) (first) microsomes and NADPH; (second) nicotine; (d) (first) microsomes and NADPH; (second) NADPH and nicotine; (e) (first) microsomes and nicotine; (second) NADPH; (f) (first) NADPH and nicotine; (second) microsomes.

more enzyme activity over the 2-h period. Addition of all three components after the first hour resulted in 74% additional production of nornicotine in the second hour compared to the first hour. These results suggest that at 30 °C the stability of nicotine demethylation was increased by the presence of both nicotine and NADPH.

Increased concentration of nornicotine (up to 50 mM) in the assay decreased the rate of nicotine demethylation. The concentrations of nornicotine encountered in routine assays ranged from 12.5 to 100 μM, and at these concentrations nornicotine did not inhibit nicotine demethylation. To determine if the nornicotine inhibition was a general alkaloid effect, an experiment was carried out with various concentrations of nicotine and nornicotine but with the total concentration at 2 mM. Nornicotine was a more effective inhibitor of demethylation than identical concentrations of nicotine. Hence, this inhibition was more likely a type of product inhibition.

Nicotine concentration was varied from 0 to 2 mM with the NADPH concentration kept constant (10 mM), to estimate K_m and V_{max}. From a Hanes plot of the resulting rates of demethylation, the apparent K_m for nicotine and V_{max} were estimated to be 51 μM and 24 pmol min^{-1} (mg of protein)$^{-1}$, respectively. The kinetic study was repeated with a different batch of microsomes, keeping the concentration of nicotine constant (0.33 mM) while varying the NADPH concentration from 0 to 4.8 mM. From a Hanes plot of the resulting rates, the apparent K_m for NADPH and V_{max} were estimated to be 5.75 mM and 105 pmol min^{-1} (mg of protein)$^{-1}$, respectively. The comparatively high apparent K_m for nicotine is mitigated by the high concentration of nicotine encountered in tobacco. At least one other *N*-demethylase, that demethylating PCMA in *P. americana*, has a K_m for PCMA (200 μM) (O'Keefe and Leto, 1989) significantly higher than the nicotine demethylase K_m for nicotine.

The involvement of cytochromes P-450 in the oxidative demethylation of several substrates has been demonstrated in higher plants (Reichhart *et al.*, 1980; Higashi *et al.*, 1985), and a number of tests were carried out to determine if this is true for nicotine demethylation. The heme containing cytochrome P-450 can also use hydroperoxides to catalyze demethylation (Estabrooke *et al.*, 1984; Hollenberg *et al.*, 1985). Neither hydrogen peroxide nor

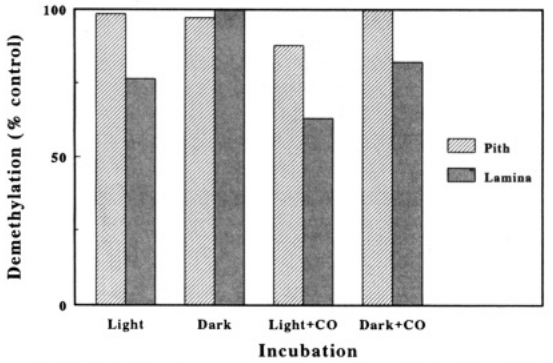

Figure 6. Effect of carbon monoxide on demethylation. Nicotine demethylase activity was measured after the assay mixtures were subjected to (a) light, (b) darkness, (c) carbon monoxide and light, and (d) carbon monoxide and darkness. The microsomal preparation, NADPH solution, and assay buffer were sparged with 99.5% carbon monoxide for 45 s prior to incubation. The light source was a tungsten–halogen lamp, producing 2000 μEinstein m^{-2} s^{-1}.

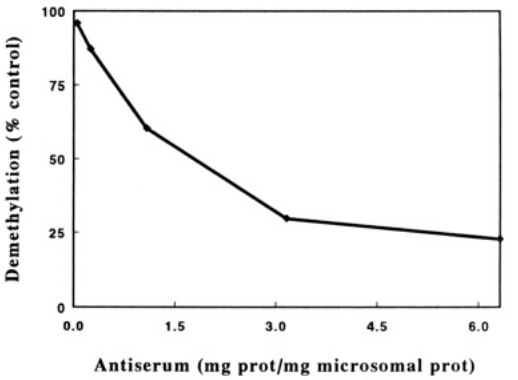

Figure 7. Effect of an antibody raised against *H. tuberosus* cytochrome P-450 reductase on the demethylation of nicotine.

cumene hydroperoxide could substitute for NADPH and oxygen in nicotine demethylation, and in the presence of NADPH and oxygen, both cumene hydroperoxide (>0.15 mM) and hydrogen peroxide (>4 mM) actually inhibited demethylation.

Flushing vials with nitrogen completely inhibited the demethylation of nicotine, but if the nitrogen were immediately removed by flushing with air, activities comparable to the control values were obtained. These results indicate that the inhibition by nitrogen was purely due to oxygen depletion.

Tetcyclasis, an inhibitor of cytochrome P-450 type enzymes (Canivenc *et al.*, 1989), at 60 μM inhibited nicotine demethylation by 50%. However, in wheat microsomes a concentration of only 10 μM resulted in an inhibition of over 80% of cytochrome P-450 dependent aryl hydroxylase activity (McFadden *et al.*, 1989).

Carbon monoxide also inhibits P-450 enzymes, and this inhibition can be reversed by light of 450 nm (Donaldson and Luster, 1991). Sparging the reaction components with carbon monoxide for 60 s (Omura and Sato, 1967) caused a reduction in nicotine demethylation activity of only 20% in lamina microsomes and no reduction in pith microsomes (Figure 6). White light (2000 μEinstein m^{-2} s^{-1}) from a tungsten–halogen lamp did not reverse the slight inhibition observed in lamina microsomes, but further inhibited activity, possibly by interacting with the pigments present, resulting in the production of inhibitors to nicotine demethylation.

Various concentrations of a polyclonal antiserum raised in a rabbit against *H. tuberosus* NADPH–cytochrome P-450 reductase were added to the routine nicotine demethylase assay. The control was the incubation in the presence of a corresponding concentration of nonimmunized serum. Increased concentrations of antiserum increased inhibition of nicotine demethylation (Figure 7), suggesting a role for cytochrome P-450 reductase in the demethylation reaction. On the basis of their inhibition studies with the antiserum to cytochrome P-450 reductase, Benveniste *et al.* (1989) have concluded that cytochrome P-450 reductase is involved in reactions catalyzed by cytochromes P-450 in higher plants. Thus, if cytochrome P-450 reductase is involved in nicotine demethylation, this is a strong argument for the involvement of cytochrome P-450 as well.

Cytochrome P-450 dependent monooxygenases have previously been reported to be involved in higher plant alkaloid metabolism (Madyastha *et al.*, 1976) and may also be involved in the demethylation of nicotine in *N. otophora*. Nicotine demethylation, like cytochrome P-450 mediated reactions, occurs in the microsomal fraction and is dependent upon molecular oxygen and reducing equivalents provided preferentially by NADPH and to a lesser extent by NADH. The stimulation of nicotine demethylation at subsaturating concentrations of NADPH by NADH is a characteristic of a number of cytochromes P-450 (West, 1980).

Although only partial inhibition by tetcyclasis and carbon monoxide suggests that cytochrome P-450 may not be involved in nicotine demethylation, these results do not rule out cytochrome P-450 involvement. Previous studies have indicated that the association between carbon monoxide and different cytochromes P-450 varies and can depend on the presence or absence of substrates (Tuckey and Kamin, 1983; Schröder and Diehl, 1987). It is also possible that tetcyclasis could have been bound to microsomal lipid, lowering the free concentration of tetcyclasis. At least one cytochrome P-450 dependent *N*-demethylase in wheat (Mougin *et al.*, 1991) is known to be less sensitive to tetcyclasis than nicotine demethylase. The ability of peroxides to support demethylation in the absence of NADPH is not cited as a higher plant cytochrome P-450 characteristic by West (1980) but was considered in our study as certain cytochromes P-450 have been shown to be able to utilize peroxy compounds instead of NADPH (Estabrooke *et al.*, 1984; Hollenberg *et al.*, 1985). More cytochrome P-450 tests have to be carried out on the demethylation of nicotine before a definitive statement can be made on the role of cytochrome P-450, as not all of the primary criteria are satisfied by any one system (West, 1980).

Senescent leaves are characterized by a degradation of chlorophyll, carotenoids, starch, and chloroplast proteins (Long and Weybrew, 1981). Yet, the level of nornicotine in the leaves of numerous *Nicotiana* species rises during the last stages of curing (Wada, 1956). Other *N*-demethylases have also been reported to become more active during senescence (Fonne-Pfister *et al.*, 1988). *In vivo* results from our laboratory have demonstrated greater levels of nicotine demethylation activity in leaves of *N. sylvestris* that were treated with ethylene to hasten senescence (Fannin and Bush, 1992). This suggests that the enzyme responsible for nornicotine synthesis is not degraded during early senescence but that the enzyme activity may be induced or activated during senescence.

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