Nitrogen Metabolism in Lignifying Pinus taeda Cell Cultures*

Pieter S. van Heerden, G. H. Neil Towers†, and Norman G. Lewis§

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

The primary metabolic fate of phenylalanine, following its deamination in plants, is conscription of its carbon skeleton for lignin, suberin, flavonoid, and related metabolite formation. Since this accounts for 30–40% of all organic carbon, an effective means of recycling the liberated ammonium ion must be operative. In order to establish how this occurs, the uptake and metabolism of various 15N-labeled precursors (15N-Phe, 15NH4Cl, 15N-Gin, and 15N-Glu) in lignifying Pinus taeda cell cultures was investigated, using a combination of high performance liquid chromatography, 15N NMR, and gas chromatography-mass spectrometry analyses. It was found that the ammonium ion released during active phenylpropanoid metabolism was not made available for general amino acid/protein synthesis. Rather it was rapidly recycled back to regenerate phenylalanine, thereby providing an effective means of maintaining active phenylpropanoid metabolism with no additional nitrogen requirement. These results strongly suggest that, in lignifying cells, ammonium ion reassimilation is tightly compartmentalized.

The primary metabolic fate of phenylalanine, following its deamination in plants, is conscription of its carbon skeleton for lignin, suberin, flavonoid, and related metabolite formation. Since this accounts for 30–40% of all organic carbon, an effective means of recycling the liberated ammonium ion must be operative. In order to establish how this occurs, the uptake and metabolism of various 15N-labeled precursors (15N-Phe, 15NH4Cl, 15N-Gin, and 15N-Glu) in lignifying Pinus taeda cell cultures was investigated, using a combination of high performance liquid chromatography, 15N NMR, and gas chromatography-mass spectrometry analyses. It was found that the ammonium ion released during active phenylpropanoid metabolism was not made available for general amino acid/protein synthesis. Rather it was rapidly recycled back to regenerate phenylalanine, thereby providing an effective means of maintaining active phenylpropanoid metabolism with no additional nitrogen requirement. These results strongly suggest that, in lignifying cells, ammonium ion reassimilation is tightly compartmentalized.

The colonization of land by vascular plants, from their aquatic forerunners, was in large measure due to elaboration of the phenylpropanoid/phenylpropanoid-acetate pathways. At this critical juncture in evolution, phenylalanine (tyrosine) became the portal entry of phenols into lignins, lignans, flavonoids, suberins, and proanthocyanidins. Vascular plants thus have a very high Phe/Tyr turnover, since 30–40% of all assimilated carbon in photosynthesis is of phenylpropanoid/phenylpropanoid-acetate origin (1–5).

Phenylpropanoid metabolism is not only a feature of normal development, but can also be induced. For example, when loblolly pine (Pinus taeda) cell suspension cultures are exposed to high levels of sucrose (6), there is an induction of lignin synthesis. Curiously, little attention has been paid to the issue of the relationship between phenylpropanoid and nitrogen metabolism (7).

In the development of allorganic carbon, an effective means of recycling the ammonium ion must be operative. In order to do this, the point whereby nitrogen is introduced (9–15). Second, when Phe/Tyr are committed to phenylpropanoid metabolism, rather than to protein or alkaloid synthesis, nitrogen (as the ammonium ion) is immediately removed via the appropriate lyase reaction (16–18) (Scheme 1). Third, for every mole of cinnamate (p-hydroxybenzylamine) formed, an equimolar amount of ammonium ion is generated. Consequently, an efficient means of nitrogen recycling must exist within cells undergoing active phenylpropanoid metabolism, otherwise severe nitrogen deficiency would result. A possible mechanism for recycling is shown in Scheme 2, where the ammonium ion released during lysis is metabolized via glutamine synthetase/glutamate synthase to generate glutamate (19–22), thereby permitting arogenate synthesis, and hence completion of the cycle.

The operation of such a phenylpropanoid-nitrogen cycle during lignification was established using actively lignifying P. taeda cell cultures. This was carried out by examining the uptake, metabolism, and product formation of several 15N-labeled precursors, in the presence and absence of specific enzyme inhibitors, as described below.

MATERIALS AND METHODS

Plant Materials—Suspension cultures of P. taeda (loblolly pine) were maintained on a modified Brown and Lawrence medium (23) containing 3% sucrose and 2,4-dichlorophenoxyacetic acid (11.3 μM) as auxin. Reagents—All chemicals were either reagent or tissue culture grade unless otherwise stated, whereas HPLC solvents were of liquid chromatographic grade. L-[15N]Phenylalanine, L-Phe (99% 15N), 15NH4Cl (99.9% 15N), L-[1-15N]glutamic acid, L-Glu (99% 15N), and L-[1-15N]glutamine, L-Gln (amide 15N, 99% 15N) were purchased from Isotec, Inc. (Miamisburg, OH). Pico-Tag diluent was obtained from Waters (Milford, MA). Phenylisothiocyanate, triethylamine, L-methionine-S-sulfoximine (MSO), azaserine (AZA), and pyridine were purchased from Sigma, while L-2-aminoxy-3-phenylpropanoic acid (L-AOPP) was obtained from Genosys Biotecnologies, Inc. (Woodlands, TX). Anhydrous sodium acetate was purchased from J. T. Baker (Phillipsburg, NJ), while N-methyl-N-(tert-butyl dimethylsilyl)-trifluoroacetamide (MTBSTFA) and acetic acid were obtained from Aldrich.

Instrumentation and Chromatography—15N NMR spectra were recorded at 30.42 MHz on a Bruker AMX 300 spectrometer using a 5-mm diameter broadband frequency probe head employing a Waltz-16 composite pulse sequence. Chemical shifts are quoted relative to the 15NH3 resonance at 0 ppm obtained using 15NH4Cl (100 mM) as an external standard. The resonances in each sample were assigned by comparison of their chemical shifts to authentic standards and published data (24–32). In order to obtain a good signal/noise ratio, it was necessary to accumulate 3000–5000 scans.

HPLC was performed on a Waters model 600E system controller.

The abbreviations used are: HPLC, high performance liquid chromatography; MSO, L-methionine-S-sulfoximine; AZA, azaserine; L-AOPP, L-2-aminoxy-3-phenylpropanoic acid; MTBSTFA, N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide; GC-MS, gas chromatography-mass spectroscopy; PAL, phenylalanine ammonia lyase; gfw, gram fresh weight; N-DMTBS, N(O)-dimethyl-tert-butylsilyl; TBDMS, tert-butyl-dimethylsilyl.
Nitrogen Metabolism in P. taeda

SCHEME 1. Biosynthetic routes leading to the formation of the aromatic amino acids, tyrosine (Tyr) and phenylalanine (Phe), from prephenate.

SCHEME 2. Proposed scheme for nitrogen recycling in P. taeda during active phenylpropanoid metabolism.
Administration and Metabolism of $^{15}$N-Labeled Substrates—In vivo $^{15}$N-labeling was performed by subculturing 7-day-old, 2,4-D treated P. taeda cells (2.5 ml packed cell volume in 25 ml of medium) with a sterile 8% sucrose medium supplemented with the individual $^{15}$N-labeled substrates, in the presence or absence of selected inhibitors (35–37). $^{15}$N-Phe, $^{15}$NH$_4$Cl, $^{15}$N-Gln, and $^{15}$N-Glu were administered at a final concentration of 10 mM at $t = 0$ h. For inhibitor studies, L-AOPP was administered at a final concentration of 0.1 mM at $t = 0$ h, whereas MSO and AZA were at 5 mM. In each experiment, the cells were incubated at 25 °C, over a time course of 24, 48, 72, and 96 h, on a Lab-Line (Melrose Park, IL) model 3520 orbital shaker (105 rpm) under continuous light provided by two fluorescent lights (40-watt, Philips, Cod White, 25–45 μmol s $^{-1} m^{-2}$).

Extraction of Amino Acids from Sucrose-treated Suspension Culture Cells—Following each incubation, suspension culture cells were harvested by filtration of medium on Miracloth, washed with distilled H$_2$O (50 ml), weighed, frozen (liquid N$_2$), and stored at $-80^\circ$C until needed. Frozen cells were ground in a chilled mortar, extracted with cold EtOH (5 ml), with the resulting slurry transferred, by means of two rinses (3 ml of 95%EtOH each) into a conical tube. The resulting suspension was centrifuged for 10 min (2,200 g, 4 °C) in a Beckman model TJ-6 centrifuge, the supernatant was decanted, and the pellet was collected, then resuspended in 95% EtOH (3 ml) and centrifuged for 10 min as before (two times). Supernatants were combined and evaporated to dryness under reduced pressure at 30 °C to give the crude amino acid extracts.

Preparation of Amino Acid Extracts for $^{15}$N NMR Spectroscopy—The dried amino acid extracts were individually resuspended in distilled H$_2$O (5 ml) and extracted with CHCl$_3$ (5 ml). The resulting aqueous phase from each experiment was vigorously agitated using a vortex mixer, centrifuged for 10 min (2,200 g, 4 °C), with the supernatant frozen (liquid N$_2$) and lyophilized. Each dry amino acid sample was dissolved in 0.1 M HCl (1 ml), containing D$_2$O (50 μl), and subjected to NMR spectroscopic analysis.

Derivatization with Phenylisothiocyanate and MTBSTFA—Each amino acid extract (initially used for $^{15}$N NMR analyses) was next derivatized, using the MTBSTFA (38) and Pico-Tag (39) methods described previously, with the resulting N-DMTBS and phenylisothiocyanate derivatives analyzed by GC-MS and HPLC, respectively.

RESULTS AND DISCUSSION

The apparent metabolic fate of the ammonium ion released during metabolism of $^{15}$N-Phe was examined, under conditions where cells were undergoing active lignin synthesis. Thus, 10 mM $^{15}$N-Phe (99.9 atom % $^{15}$N) was administered to lignifying P. taeda cell cultures, these then being allowed to metabolize over a 4-day period, with cells removed at 24-h intervals for $^{15}$N NMR spectroscopic analyses. At $t = 24$ h, three clearly resolved signals (data not shown) were observed at δ 91.1, 18.3, and 16.7 ppm. These resonances were assigned to the δ-amide nitrogen of Gln, α-amino nitrogen of Gln and Glu, and the amino group of Phe, respectively, based on chemical shifts of authentic standards and previously published data (24–32). By 96 h, additional resonances at δ 19.9 and 13.2 ppm were also evident (Fig. 1A); these were attributed to alanine and serine as pre-

![Representative $^{15}$N-NMR spectra of P. taeda cellular amino acid extracts obtained after administration of A, 10 mM $^{15}$N-Phe (96 h); B, 10 mM $^{15}$N-Phe and 0.1 mM L-AOPP (96 h); C, 10 mM $^{15}$N-Phe and 5 mM MSO (72 h); and D, 10 mM $^{15}$N-Phe and 5 mM AZA (72 h).](image-url)
viously assigned in nitrogen metabolism studies with white spruce (Picea glauca) buds (24). Importantly, no resonances at any stage corresponding to $^{15}\text{NH}_4^+$ were observed, in accordance with earlier observations, such as with potato (Solanum tuberosum) slices, where it did not reach detectable levels (7) during active phenylpropanoid-glutamine synthetase/glutamate synthase metabolism.

Subsequent GC-MS and HPLC analyses of the extracts confirmed and extended these observations (see Table I). Thus, both isotopic enrichment and total amounts (micrograms/g fresh weight) of each principal metabolite (Gln and Glu) were determined, following incubation of $^{15}\text{N}$-Phe (99.9 atom %, 10 mM) with P. taeda cell cultures, for periods up to 96 h. As can be seen, the phenylalanine present in the soluble pool in the cells was $\sim$90 atom % $^{15}\text{N}$-enriched at all intervals sampled (24, 48, 72, and 96 h). But its amount decreased from 677 $\mu$g/gfw ($t = 24$ h) to $\sim 25$ $\mu$g/gfw ($t = 96$ h) as a result of the utilization of its carbon skeleton for phenylpropanoid metabolism. However, the Gln/Glu pools were enriched by only $\sim 43$–53 atom % $^{15}\text{N}$, with both nitrogens of Gln labeled in relatively equal amount. In contrast to that of Phe pool sizes, the relative amounts (micrograms/gfw) of Gln/Glu dropped by only about 50% over the duration of the 96-h experiment.

To prove unambiguously that the glutamine synthetase/glutamate synthase pathway was assimilating the ammonium ion released during lignification, incubations of 10 mM $^{15}\text{N}$-Phe (99.9 atom % $^{15}\text{N}$) were repeated, but now in the presence of specific inhibitors of phenylalanine ammonia lyase, glutamine synthetase, and glutamate synthase, respectively. Thus, when incubations were conducted with lignifying P. taeda cell cultures in the presence of 0.1 mM L-AOPP (35), a known PAL inhibitor, the major resonance now observed was that of unmetabolized $^{15}\text{N}$-Phe (Fig. 1B). Small resonances were also noted at $\delta 18.3$ ppm, suggesting that phenylpropanoid metabolism was not completely inhibited by L-AOPP, in accordance with previous observations (35, 40). This was confirmed by quantitative measurements which revealed that the amounts of Phe ($\sim$90% 15N enriched) remained essentially constant (2926–2105 $\mu$g/gfw) throughout the 96-h duration of the experiment. Indeed, the employment of L-AOPP resulted in considerable PAL inhibition as evidenced by the 4- to 100-fold increase in Phe levels over the 24–96-h time frame examined (cf. Table I, Phe with and without L-AOPP). But PAL was not inhibited fully, as revealed by the small isotopic enhancement of both

![Representative $^{15}$N-NMR spectra of P. taeda cellular amino acid extracts obtained after administration of A, 10 mM $^{15}\text{NH}_4\text{Cl}$ (72 h); and B, 10 mM $^{15}\text{NH}_4\text{Cl}$ in the presence of 0.1 mM L-AOPP (96 h).](image)

**Table II**

Amino acid levels and isotopic ($^{15}$N) enrichment after 24–96-h treatment of lignifying P. taeda cell suspension cultures with (A) 10 mM $^{15}\text{NH}_4\text{Cl}$, (B) 10 mM $^{15}\text{NH}_4\text{Cl}$ and 0.1 mM L-AOPP, (C) 10 mM $^{15}$N-Gln, (D) 10 mM $^{15}$N-Gln and 0.1 mM L-AOPP, (E) 10 mM $^{15}$N-Glu, and (F) 10 mM $^{15}$N-Glu and 0.1 mM L-AOPP

| Precursor/Inhibitor | Sampling point | Gln | Glu | Phe |
|---------------------|----------------|-----|-----|-----|
|                     | Pool size | 15N Isotopic enrichment | 15N Total | Pool size | 15N | Pool size | 15N |
| A. NH$_4$Cl | | | | | | | |
| 24 | 495 | 40.1 | 38.4 | 78.5 | 307 | 60.5 | 5 | 26.8 |
| 48 | 203 | 40.8 | 33.2 | 74.0 | 159 | 56.8 | 9 | 36.9 |
| 72 | 199 | 44.9 | 18.8 | 63.7 | 137 | 43.7 | 16 | 29.9 |
| 96 | 98 | 37.7 | 13.8 | 51.5 | 89 | 33.3 | 13 | 19.0 |
| B. NH$_4$Cl/L-AOPP | | | | | | | |
| 24 | 1109 | 32.8 | 56.5 | 89.3 | 152 | 68.5 | 201 | 51.2 |
| 48 | 753 | 33.8 | 55.4 | 89.2 | 253 | 70.5 | 353 | 60.9 |
| 72 | 267 | 36.0 | 53.2 | 89.2 | 102 | 68.3 | 1301 | 63.6 |
| 96 | 55 | 40.5 | 27.2 | 67.7 | 59 | 48.0 | 809 | 59.3 |
| C. Gln | | | | | | | |
| 24 | 159 | 31.9 | 6.8 | 38.7 | 351 | 25.5 | 13 | 24.9 |
| 48 | 135 | 48.3 | 6.0 | 54.3 | 309 | 26.4 | 7 | 27.6 |
| 72 | 151 | 42.0 | 6.4 | 48.4 | 210 | 20.6 | 7 | 22.9 |
| 96 | 205 | 48.6 | 7.4 | 56.0 | 175 | 26.3 | 10 | 25.1 |
| D. Gln/L-AOPP | | | | | | | |
| 24 | 161 | 48.2 | 11.9 | 60.1 | 461 | 37.0 | 581 | 29.1 |
| 48 | 153 | 54.3 | 8.0 | 62.3 | 275 | 34.7 | 1282 | 33.7 |
| 72 | 205 | 58.0 | 15.7 | 73.7 | 400 | 36.5 | 970 | 35.6 |
| 96 | 280 | 58.6 | 16.0 | 74.6 | 325 | 36.9 | 1012 | 37.8 |
| E. Glu | | | | | | | |
| 24 | 150 | 19.7 | 7.1 | 26.8 | 304 | 39.0 | 10 | 47.3 |
| 48 | 123 | 19.5 | 6.9 | 26.4 | 250 | 37.9 | 12 | 42.6 |
| 72 | 100 | 38.0 | 18.9 | 56.9 | 263 | 51.2 | 6 | 43.2 |
| 96 | 134 | 42.4 | 22.4 | 64.8 | 295 | 53.1 | 10 | 41.8 |
| F. Glu/L-AOPP | | | | | | | |
| 24 | 291 | 36.6 | 35.2 | 71.8 | 341 | 66.5 | 228 | 55.7 |
| 48 | 125 | 41.1 | 34.9 | 76.0 | 274 | 63.4 | 432 | 52.3 |
| 72 | 123 | 38.1 | 36.2 | 74.3 | 324 | 72.3 | 1074 | 60.9 |
| 96 | 100 | 40.0 | 47.6 | 87.6 | 260 | 78.9 | 909 | 74.8 |

*Total = % of label in both.*
Gln (19–20%) and Glu (22–25%), respectively (Table I). Nevertheless, these results clearly showed that overall PAL inhibition by L-AOPP adversely affected metabolic flux into Gln/Glu. Interestingly, it had little effect upon the pool sizes of each amino acid relative to that observed previously during active lignification.

The effects of treating lignifying P. taeda cell cultures with 5 mM MSO (36), a glutamine synthase inhibitor, was investigated. The results in Fig. 1C show only resonances corresponding to 15N-Phe and 15NH₄⁺ at δ 16.7 and 0.0 ppm; signals due to either 15N-Gln, 15N-Glu, 15N-Ala, and 15N-Ser were absent, indicating that their metabolism from 15N-Phe was now inhibited. Quantification of both isotopic enrichment and total amounts (micrograms/g fresh weight) supported this conclusion (Table I). The levels of 15N-Phe (−90 atom % 15N) rapidly decreased from −2492 μg/gfw (at 24 h) down to −34 μg/gfw within 96 h, due to the action of PAL. However, as suggested by the NH₄⁺ resonance at 0.0 ppm, no significant incorporation into 15N-Gln occurred, as confirmed from its low isotopic enrichment (2–3%). Glutamine synthase inhibition had no apparent effect on the relative pool sizes of Glu which again remained comparable to those previously noted, although, by contrast, the Gln levels dropped to being near undetectable. Interestingly, the Glu isolated was −5–10% enriched, perhaps suggesting that a small amount of Glu formation might occur via transamination of Phe, as suggested earlier (41). In summary, this experiment revealed that inhibition of glutamine synthase prevented an effective assimilation of 15NH₄⁺, released during Phe deamination, into either Gln, Glu, or any other amino acid.

The effects of treating lignifying P. taeda cells with 15N-Phe, in the presence of AZA (37), an inhibitor of glutamate synthase, was also examined. The results, illustrated in Fig. 1D, show that the predominant resonances were due to 15N-Phe and 15N-Gln (δ-amide), with only a very small signal at δ 18.3 ppm, due to either H₂,N¹-15N-Gln, or 15N-Glu if incomplete inhibition occurred. This interpretation was confirmed by quantification, as shown in Table I; azaserine treatment had little effect upon carbon metabolism into cinnamate, as evidenced by rapid depletion of Phe from −1162 to 70 μg/gfw fresh weight over the 96-h duration. Glutamine levels were now higher (315–120 μg/gfw) than previously noted, due to glutamate synthase inhibition, with the 15N-Gln essentially being only singly labeled (at the δ-amide-nitrogen); this established that further cycling of the nitrogen through glutamate synthase to ultimately afford the corresponding (15N,15N) double-labeled species was greatly reduced. Significantly, the effect of AZA on Glu levels resulted in a 4–9-fold depletion from previous levels.

Taken together, these results indicated that the primary metabolic fate of the nitrogen of Phe, following its release as ammonium ion during active phenylpropanoid metabolism, was sequential assimilation into glutamine and then glutamate. There was no evidence for ammonium ion assimilation by glutamine dehydrogenase, since in the presence of either MSO or AZA, neither of which inhibits glutamine dehydrogenase, essentially no detectable incorporation of 15NH₄⁺ into Glu occurred.

While the above experiments provided convincing evidence for the metabolic sequence 15N-Phe → 15NH₂⁺ → 15N(δ-amide)-Gln → 15N-Glu, they did not establish that the ammonium ion, liberated during deamination, was recycled back to Phe during active phenylpropanoid metabolism. Consequently, the fate of exogenously provided 15NH₄Cl, 15N(δ-amide)-Gln, and 15N-Glu to lignifying P. taeda cell cultures was next investigated, to ascertain whether Phe would accumulate in a 15N-enriched form. Experiments were carried out in the presence and absence of the PAL inhibitor, L-AOPP, as before.

P. taeda cell cultures were first administered 10 mM 15NH₄Cl for 96 h, with samples removed at 24-h intervals and analyzed. As can be seen in Fig. 2A, 15N NMR spectroscopic analyses of the extracts were devoid of any signal corresponding to 15N-Phe. Instead, a range of resonances attributed to 15N-Gln, 15N-Glu, 15N-H₂Cl, the δ and ω,ω' nitrogens of arginine (δ 66.8 and 53.4 ppm), 15N-Ser, the ω-nitrogen of proline (δ 32.4 ppm), and the side chain amino groups of Lys (δ), Orn (δ), and γ-amino-nobutyric acid at δ 11.7 ppm were evident. Thus, when an ammonium source, such as NH₄Cl, was administered to lignifying P. taeda cell cultures, it was made available to general pools for amino acid/protein synthesis, i.e. its fate differed from that of 15N-Phe-derived ammonium ion which resulted in the enhancement of 15N signals only in Glu, Gln, Ala, and Ser. This strongly suggests that the ammonium ion generated during lignin synthesis is tightly compartmentalized, and not made available for general amino acid metabolism/protein synthesis.

Interestingly, as can be seen from Table II, administration of 15NH₄Cl (10 mM) to the lignifying P. taeda cell cultures resulted in a rapid 6-fold increase in Glu levels (495 μg/gfw) followed by its gradual decline to −98 μg/gfw over 96 h, with...
both nitrogens being enriched (79–52%). This was expected since an increased availability of NH₄⁺ stimulates glutamine synthetase, with a concomitant increase in Glu accumulation (42, 43). By contrast, both the amount and isotopic enrichment of Glu were only slightly elevated over previous levels (see Table I). As before, the ¹⁵N-Phe pool size was very small, although significantly it was now partially enriched (19–37% ¹⁵N), thus providing the first hint of evidence in support of the proposed phenylpropanoid-nitrogen cycle.

Incubation of the lignifying P. taeda cell cultures with ¹⁵N-H₄Cl (10 mM), in the presence of L-AOPP (0.1 mM), was next carried out. As can be seen in Fig. 2B and Table II, the most notable feature was the steady growth of ¹⁵N-Phe (201–1301 µg/gw), ultimately resulting in the dominant signal (at δ 16.7 ppm) in the NMR spectrum. Additionally, all three metabolites, Glu (48–70% ¹⁵N), Phe (51–64% ¹⁵N), and Gln (68–89% total ¹⁵N) were isotopically enriched. Thus, proof that NH₄Cl was normally destined for the phenylpropanoid pathway was in hand.

Determination of pool sizes and isotopic enrichment as before verified that Phe concentrations were low (7–13 µg/gw) but enriched (23–28% ¹⁵N), whereas ¹⁵N-Gln and ¹⁵N-Glu levels remained fairly constant (δ 18.3 ppm contains a minor contribution due to Asp(δ) and Asn(δ) (43)).

The resonances due to ¹⁵N-Gln, ¹⁵N-Glu, ¹⁵N-Ala, and ¹⁵N-Ser, and ¹⁵N-Asn (Fig. 3A) were readily detected (Fig. 4A). This was further verified by quantification which again revealed a small Phe pool size (6–10 µg/gw) of 42–47% ¹⁵N enrichment (Table II).

With the combined use of HPLC, ¹⁵N NMR, and GC-MS, the mechanism for recycling of ammonia during phenylpropanoid metabolism in lignifying P. taeda (loblolly pine) cell cultures was unambiguously established. The use of enzyme inhibitors was employed to elucidate this mechanism, with metabolite accumulations being consistent with the known mode of action of L-AOPP, MSO, and AZA; namely inhibition of PAL, glutamine synthetase, and glutamate synthase, respectively. The ammonia ion generated during phenylpropanoid biosynthesis is first incorporated into the α-amide of glutamine, followed in turn by the α-amino position of glutamate, which then acts as an amino donor for a range of transamination products, including the aromatic amino acids, argenenate, and phenylaniline. This nitrogen cycle explains how optimum use is made of the plant’s available nitrogen, so that active phenylpropanoid metabolism and lignification can continue, even at low nitrogen levels.

Acknowledgments—Thanks are extended to Dr. W. F. Siems for the GC-MS determinations, and to K. Sikes for maintenance of the P. taeda cell cultures.

REFERENCES

1. Floss, H. G. (1985) Recent Adv. Phytochem. 20, 13

2. Dieck, P. M. (1985) Nat. Prod. Rep. 2, 405.

3. Lewis, N. G., and Yamamoto, E. (1989) in Chemistry and Significance of Condensed Tannins (Hemingway, R. W., and Karchesy, J. J., eds) pp. 23–47, Plenum Press, New York

4. Nogalski, F. A., Ellis, C. M., and Conn, E. E. (1985) J. Biochem. 17, 79–89

5. Davin, L. B., and Lewis, N. G. (1992) Recent Adv. Phytochem. 26, 325

6. Nogalski, F. A., Ellis, C. M., and Conn, E. E. (1986) Planta 167, 233

7. Razal, R. A., Ellis, S. G., Singh, S., Lewis, N. G., and Towers, G. H. N. (1996) Phytochemistry 41, 31

8. Parkhurst, C. E., and Jones, W. T. (1979) J. Exp. Bot. 30, 1109

9. Byng, G., Whitaker, R., Flick, C., and Jensen, R. A. (1981) Phytochemistry 20, 1289

10. Connelly, J. A., and Conn, E. E. (1986) Z. Naturforsch. B. 41, 69

11. Davin, C. B., Byng, G. S., Whitaker, R. J., and J Jensen, R. A. (1982) Planta 156, 233

12. Jung, E., Zwin, L. O., and Jensen, R. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7231

13. Sihey, D. L., and Conn, E. E. (1988) Arch. Biochem. Biophys. 260, 822

14. Eberhardt, T. L., Conn, E. E., Conn, J. A., and Conn, E. E. (1986) Z Naturforsch. B, 41, 79

15. Jensen, R. A. (1985) Recent Adv. Phytochem. 20, 57

16. Hanson, K. R., and Havir, E. A. (1981) in Biochemistry of Plants: A Comprehensive Treatise (Conn, E. E., ed.) Vol 2, pp. 577–625, Academic Press, New York

17. Koulakoff, J. B., and Conn, E. E. (1961) J. Biol. Chem. 236, 2692

18. Gross, G. G. (1965) in Biosynthesis and Degradation of Wood Components (Higuchi, T., ed.) pp. 229–271, Academic Press, New York

19. Lea, P. J., and Miflin, B. J. (1974) Nature 241, 614

20. Dougall, K. D. (1980) in Tannins (Stumpf, P. K., and Conn, E. E., eds) Vol 2, pp. 627–642, Academic Press, New York

21. Durzan, D. J., and Steward, F. C. (1983) in Plant Physiology, A Treatise (Steward, F. C., ed.) Vol VIII, pp. 55–186, Academic Press, New York

22. Oaks, A., and Hile, B. (1985) Annu. Rev. Plant Physiol. 36, 345

23. Eberhardt, T. L., Bernard, A., He, L. D., Bostock, J. B., and Lewis, N. G. (1993) J. Biol. Chem. 268, 21088

24. Thorpe, T. A., Bagh, K., Cutler, A. J., Dunstan, D. M., McIntyre, D. D., and Vogel, H. J. (1989) Plant Physiol. 93, 193

25. Altenburger, R., Aburiza, C., Callies, R., Grimes, L. M., Mayer, A., and Leibfritz, D. (1991) Arch. Microbiol. 156, 471.

26. Kuesel, A. C., Kuhn, W., Sianoutidis, J., Grimes, L. M., Leibfritz, D., and Mayer, A. (1989) Arch. Microbiol. 151, 434

27. Martin, F. (1985) FEBS Lett. 182, 85

28. Martin, F., Stewart, G. R., Genet, I., and Le Tacon, F. (1986) New Phytol. 103, 129–132

29. Kamami, K., Weiss, L. L., and Roberts, J. D. (1988) J. Biol. Chem. 263, 2817

30. Monselise, E. B., Kost, D., Porath, D., and Tal, M. (1987) New Phytol. 107, 341

31. Callies, R., Altenburger, R., Abarzuza, S., Mayer, A., Grimes, L. M., and Leibfritz, D. (1992) Plant Physiol. 100, 1584

32. Robinson, S. A., Slade, A. P., Fox, G. G., Phillips, R. G., Ratcliffe, C. G., and Stewart, G. R. (1991) Plant Physiol. 95, 509

33. Hars, R. F., Augstin, G. E., and Tassini, P. (1993) Food Chem. 46, 319

34. McLeay, F. W., and Turecek, F. (1993) Interpretation of Mass Spectra, 4th Ed., University Science Books, Sausalito, CA

35. Amrhein, N., and Godeke, K. (1977) Plant Sci. Lett. 8, 313

36. Miflin, B. J., and Lea, P. J. (1975) Biochem. J. 149, 403

37. Ronzi, R. A., Rowe, W. B., and Meister, A. (1969) Biochemistry 8, 1066

38. Rhodes, D., Rich, P. J., and Brunk, D. G. (1989) Plant Physiol. 89, 1161

39. Razal, R. A., Lewis, N. G., and Towers, G. H. N. (1994) Phytochem. Anal. 5, 98

40. Maxwell, C. A., and Phillips, D. A. (1990) Plant Physiol. 93, 1552

41. Wightman, F., and Forest, J. C. (1978) Phytochemistry 17, 1455

42. Rhodes, D., Rendal, G. A., and Stewart, G. R. (1975) Planta 125, 201

43. Rhodes, D., Rendal, G. A., and Stewart, G. R. (1976) Planta 129, 203