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# *N'*-Acyl and *N'*-Nitroso Pyridine Alkaloids in Alkaloid Lines of Burley Tobacco during Growth and Air-Curing

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*N'*-Acyl and *N'*-nitroso pyridine alkaloids were quantified by GC in burley tobacco genotypes varying in accumulation of pyridine alkaloids. Leaves were sampled during field growth and air-curing. N'-Substituted alkaloid identities were confirmed by GC and GC–MS; *N'*-acetylanatabine was newly identified. Alkaloid derivatives in lamina were in the following order of decreasing content averaged over the sampling dates. Acylated compounds: formylnornicotine (FNN), *n*-octanoylnornicotine (ONN), *n*-hexanoylnornicotine (HNN), formylanatabine (FAT), acetylnornicotine (ANN), *n*-butanoylnornicotine (BNN), and acetylanatabine (AAT). Nitrosamines: nitrosoanatabine (NAT), nitrosonornicotine (NNN), and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Generally higher levels of N'-substituted pyridine alkaloids were found in lamina of the KY 78379 line (high nornicotine and total alkaloids) and B21 cultivar (high nicotine and total alkaloids) compared to LAB21 (low total alkaloids). *N'*-Acyl alkaloids occurred in green as well as cured leaves; *N'*-nitroso alkaloids were mainly present during curing. Thus, acylated alkaloids, unlike nitroso alkaloids, are apparent products of plant metabolism during late growth stages.

During investigations of the effects of controlled environmental air-curing and other postharvest processing procedures on tobacco-specific nitrosamines and minor alkaloids in burley tobacco leaf extracts (Andersen and Kemp, 1985; Andersen et al., 1987), several unidentified gas chromatographic peaks were observed that did not correspond to commonly encountered tobacco pyridine alkaloids such as nicotine, other commonly described pyridine alkaloids, or known tobacco-specific *N'*-nitroso pyridine alkaloids of burley leaf. Subsequently, it was determined that several of these components belonged to a series of nornicotine- and anatabine-related N'-acylated pyridine alkaloids differing in carbon chain length of the acyl substituent (Burton et al., 1988).

Although there is relatively little known about these *N'*-acyl compounds, several of this type were described earlier. Warfield et al. (1972) identified and quantified *N'*-formylnornicotine and *N'*-acetylnornicotine in aged, air-cured burley tobacco, and Bolt (1972) identified *N'*-*n*-hexanoylnornicotine and *N'*-*n*-octanoylnornicotine in flue-cured tobacco. Miyano et al. (1979, 1981) identified and determined quantities of *N'*-formylanatabine and *N'*-formylanabasine in aged air-cured burley tobacco and *N'*-(6-hydroxy-*n*-octanoyl)nornicotine in Japanese domestic tobacco. Matsushita et al. (1979) identified *N'*-*n*-butanoylnornicotine in flue-cured tobacco leaf, and later Matsushima et al. (1983) quantitatively analyzed *N'*-acyl pyridine alkaloids in tobacco lamina at the time of harvest

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and after leaves were air- or flue-cured. Recently, Severson et al. (1988) identified a group of N′-acylated pyridine alkaloids in fresh leaf of *Nicotiana*, section Repandae. The major component among these alkaloids was *N′*-(3-hydroxy-12-methyltetradecanoyl)nornicotine. It has structural similarity to previously identified *N′*-acyl pyridine alkaloids.

While *N′*-nitroso pyridine alkaloids have been implicated as possible human carcinogens in tobacco (Hecht et al., 1978), little has been said about the importance of *N′*-acyl pyridine alkaloids. The latter compounds may have biological properties that affect the smoker in an unknown manner, and they may play a role in plant–parasite interactions. Since the *N′*-acyl and *N′*-nitroso pyridine alkaloids presumably require the same precursor parent alkaloids for their biosynthesis, the relative amounts of specific compounds that accumulate may be dependent on various competing reactions that occur among these groups of compounds during plant growth and air-curing.

The purpose of this investigation was to quantify and compare *N′*-acyl and *N′*-nitroso pyridine alkaloids in alkaloid lines of burley tobacco during field growth and conventional air-curing.

## EXPERIMENTAL SECTION

**Plant Materials.** Three lines of burley tobacco (*Nicotiana tabacum* L.), viz. cv. B21, with typically high nicotine and total alkaloid contents, KY 78379 as a nornicotine converter breeding line with high nornicotine and total alkaloid contents, and LAB21 as a low alkaloid near-isoline of B21 cv. (MacKown et al., 1984), were grown at Spindletop Research Farm, Lexington, KY, in 1986. KY 78379 and LAB21 were developed for research purposes only and are not grown commercially. The experimental design was a randomized complete block with four replicates. Recommended practices for the culture, harvesting, and air-curing of burley tobacco were followed, and a contact sucker control chemical (fatty alcohol) was used to control axillary bud growth after topping (Atkinson et al., 1976; Chaplin, 1977). Three leaves from the middle stalk position of one plant of each replicate plot of a tobacco line selected at random were harvested at 2-week intervals beginning 6 weeks after transplanting. Three leaves from the middle stalk position of one plant from each replicate plot of a tobacco line were harvested during air-curing at 1-week intervals beginning from the start of curing for 8 weeks, except that the 7-week sampling was omitted. Leaf lamina and midveins from each sampled plant within the same replicate were separated, pooled, and then immediately frozen at −70 °C. Samples of pooled leaf parts were freeze-dried, ground to 100–200-mesh size, equilibrated overnight to ambient moisture content, and then stored at −70 °C prior to analysis.

**Reference Compounds.** Nornicotine and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were obtained from Chemsyn Science Laboratories, Lenexa, KS. Anatabine was synthesized by the procedures of Quan et al. (1965). *N′*-Nitrosonornicotine (NNN) and *N′*-nitrosoanatabine (NAT) were synthesized by nitrosation of nornicotine and anatabine, respectively, in a manner described for the nitrosation of morpholine (Lijinksy and Taylor, 1975). *N′*-Formylnornicotine (FNN), *N′*-formylanatabine (FAT), *N′*-acetylnornicotine (ANN), *N′*-*n*-butanoylnornicotine (BNN), *N′*-*n*-hexanoylnornicotine (HNN), and *N′*-*n*-octanoylnornicotine (ONN) were synthesized as described by Burton et al. (1988). *N′*-Acetylanatabine (AAT) and ANN (alternately) were prepared by reaction of anatabine and nornicotine, respectively, with a 4:1 v/v pyridine–acetic anhydride mixture in a sealed vial at 70 °C for 30 min. Excess reactants and unwanted products were removed from the acylated compounds by evaporation at 130 °C. The mass spectral fragmentation pattern (ion trap detector) for authentic AAT included the following [mass/charge (%)]: 202 (M, 13), 159 (65), 143 (32), 130 (32), 117 (31), 105 (43), 80 (50), 79 (35), 78 (71), 65 (39), 63 (36), 53 (51), 52 (37), 51 (100). Alternately, FNN and FAT were synthesized from nornicotine and anatabine, respectively, by reaction with *N*-formylimidazole according to the procedure described by Warfield et al. (1972).

**Extraction of Tobacco Samples for *N′*-Acyl and *N′*-Nitroso Pyridine Alkaloids.** The following procedure was used for identification (GC–MS and coelution with authentic compounds) and quantitative analysis. A 1-g sample of tobacco was extracted for 45 min with constant shaking at room temperature with 10 mL of citrate–phosphate buffer (0.025 M citrate–0.05 M phosphate), pH 4.5, containing 5 mM ascorbic acid. After extraction, the solution mixture was adjusted to pH 5.0 and partitioned with one 30-mL portion of ethyl acetate. After phase separation, a 20-mL aliquot of the ethyl acetate phase containing the N′-substituted pyridine alkaloids was partitioned three times with 5.0-mL portions of 1 N HCl. The combined aqueous phases were adjusted to pH 5.0 with 10 N NaOH in an ice bath and then partitioned three times with 5.0-mL portions of chloroform. Sodium sulfate was added to the combined chloroform phases, which were stored in sealed containers at 5 °C until analysis by GC or GC–MS procedures.

**GC and GC–MS Analyses.** The stored tobacco extract in chloroform was filtered through a coarse fritted-glass funnel at room temperature to remove sodium sulfate, and the sodium sulfate on the filter pad was washed with 1 mL of fresh chloroform. The combined chloroform filtrate and washing was taken to dryness at room temperature under nitrogen. The residue was dissolved in 1 mL of a chloroform solution containing 10 ng of azobenzene/μL as the internal standard. Aliquots of 0.5–4.0 μL were injected into a Hewlett-Packard Model 5880A GC under the following conditions: operation in splitless mode utilizing a 30 m × 0.25 mm fused silica DB-5 capillary column and NPD detector; inlet at 280 °C; inlet purge flow at 1.2 mL/min; purge time 1.0 min; helium carrier linear velocity 31 cm/s; column temperature programmed from 60 °C with an initial 1-min hold to 260 °C at 4 °C/min and then held at 260 °C for 5 min; detector temperature 280 °C. Quantitation was carried out by internal standardization with azobenzene after calibration of retention times and response factors with authentic N′-substituted pyridine alkaloid derivatives and correcting for the recoveries of these compounds carried through the entire extraction procedure. Relative GC-NPD detector responses of the individual pyridine alkaloid derivatives along with their recovery factors representing the fraction of compound recovered in the analytical procedure final extract are given in Table I. Results were subjected to a statistical analysis of variance.

Kovats indices were calculated for authentic N′-substituted pyridine alkaloid derivatives relative to hydrocarbon standards (Perry, 1981) under GC conditions similar to those used for the analysis of tobacco extracts except that detection was by FID (Table I). Determination of peak identities in tobacco extracts and confirmation or establishment of fragmentation patterns of authentic N′-substituted pyridine alkaloids were carried out by GC–mass spectrometry. A Finnegan 705 ion trap detector and a Hewlett-Packard Model 5985A system in electron

**Table I. GC Indices of N'-Substituted Pyridine Alkaloids Identified in Burley Tobacco**

| compound | Kovats index[a] | rel detector resp (RR)[b] | rec factor (RF)[c] |
|---|---|---|---|
| *N'*-nitrosonornicotine (NNN) | 1756 | 0.25 | 0.65 |
| *N'*-formylnornicotine (FNN) | 1783 | 1.02 | 0.23 |
| *N'*-nitrosoanatabine (NAT) | 1808 | 0.79 | 0.46 |
| *N'*-formylanatabine (FAT) | 1858 | 0.30 | 0.16 |
| *N'*-acetylnornicotine (ANN) | 1827 | 1.03 | 0.27 |
| *N'*-acetylanatabine (AAT) | 1902 | 0.31 | 0.19 |
| 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)butanone (NNK) | 1935 | 0.67 | 0.67 |
| *N'-n*-butanoylnornicotine (BNN) | 1961 | 0.98 | 0.71 |
| *N'-n*-hexanoylnornicotine (HNN) | 2159 | 0.75 | 0.71 |
| *N'-n*-octanoylnornicotine (ONN) | 2367 | 0.47 | 0.99 |

[a] Index for the DB-5 capillary column used with conditions described in the Experimental Section. [b] RR = relative GC-NPD response of compound to that of an equal weight of azobenzene, where azobenzene response is unity. [c] RF = fraction of compound recovered in the final extract of the analytical procedure.

impact mode at 70 eV were used interchangeably with GC conditions similar to those used for the analysis of tobacco extracts.

**Other Chemical Analyses.** Total alkaloids were determined spectrophotometrically at 460 nm after reaction with cyanogen bromide and buffered aniline (Harvey et al., 1969); the basis of the colorimetric reaction of cyanogen bromide and aniline with the pyridine moiety of pyridine alkaloids was described by Konig (1904). Nornicotine was determined by capillary gas chromatography (Severson et al., 1981). Calcium was determined as previously described (Andersen et al., 1982), except that a 0.25-g sample of tobacco and an Instrumentation Laboratory S11 atomic absorption spectrophotometer were used. Quantitative results for alkaloids and alkaloid derivatives in tobacco during air-curing were proportionally adjusted for Ca content changes to correspond to values per unit Ca contents at the time of harvest; this corrected for dry-weight changes during curing.

## RESULTS AND DISCUSSION

**N'-Acyl Pyridine Alkaloids Identified in Burley Tobacco.** In addition to three *N'*-nitroso pyridine alkaloids, seven *N'*-acyl pyridine alkaloids were present in lamina and midveins of each of the three lines of burley tobacco after air-curing. Kovats GC indices of the N'-substituted derivatives are shown (Table I). Most of the acyl-substituted alkaloids were also present in these tobacco lines at some time during field growth prior to curing. Structure confirmations were carried out by GC cochromatography with authentic compounds and GC–MS based on comparisons with previously reported electron impact mass ion fragmentation patterns for the N'-acylated pyridine alkaloids (Burton et al., 1988), FNN and ANN (Warfield et al., 1972), FAT (Miyano et al., 1979), BNN (Matsushita et al., 1979), and HNN and ONN (Bolt, 1972). Identification of AAT, which was not previously reported in burley tobacco, was carried out by GC cochromatography with authentic AAT and GC–MS based on comparisons with synthetic AAT.

**Effects of Growth Stage and Air-Curing Time on Nornicotine, Total Alkaloid, and N'-Substituted Pyridine Alkaloid Contents.** Nornicotine concentrations in lamina and midveins among the three burley tobacco lines during growth and air-curing are given in Table II. The results show that there are significantly higher levels of nornicotine in the lamina and midveins of the KY 78379 line than in the LAB21 and B21 lines at each sampling date. The only exception was midvein nornicotine contents at 6 weeks after transplant.

Variations of total pyridine alkaloid (as nicotine equivalent) contents in lamina among the three burley lines during growth and air-curing are given in Figure 1. In a preliminary experiment, authentic nornicotine samples analyzed by Harvey's cyanogen bromide procedure yielded 40% of the total alkaloids (expressed as nicotine equivalence) that were determined with the same amounts of authentic nicotine. The results in Table II show that the maximum amount of nornicotine determined by the capillary GC procedure occurred in the KY 78379 line at the completion of air-curing and was less than 4 mg/g. It seems probable that the values obtained by the cyanogen bromide procedure for total alkaloids (as nicotine) in this line (Figure 1) would not be underestimated by more than

**Table II. Nornicotine in Alkaloid Lines of Burley Tobacco during Growth and Air-Curing**

| weeks after transplant | tobacco line | nornicotine,[a] μg/g lamina | nornicotine,[a] μg/g midveins |
|---|---|---|---|
| 6 | LAB21 | <1* | 101* |
| | B21 | 12* | 92* |
| | KY 78379 | 42** | 73** |
| 14 (harvest/ start cure) | LAB21 | 7* | 33* |
| | B21 | 318* | 55* |
| | KY 78379 | 2210** | 96** |
| 22 (cure complete) | LAB21 | 27* | <1* |
| | B21 | 169* | 23* |
| | KY 78379 | 3895** | 367** |

[a] Mean values are from three to four replicated field-grown leaf samples from the middle leaf position on the stalk. All nornicotine values for air-cured samples are corrected for Ca content departures from those at harvest. The mean values in a column for a given sampling date followed by an asterisk are significantly different from values followed by two asterisks at the 0.05 level of probability.

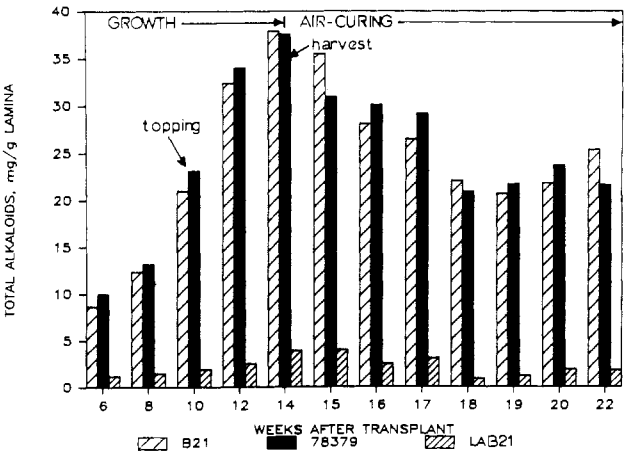


**Figure 1.** Total alkaloid concentrations (as nicotine equivalent) in leaf lamina of three burley tobacco lines during field growth and successive air-curing.

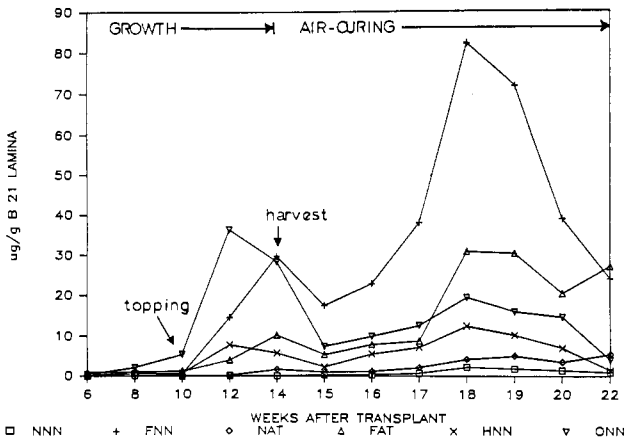


**Figure 2.** Variation of quantities of N′-substituted pyridine alkaloids in B21 lamina during field growth and successive air-curing.

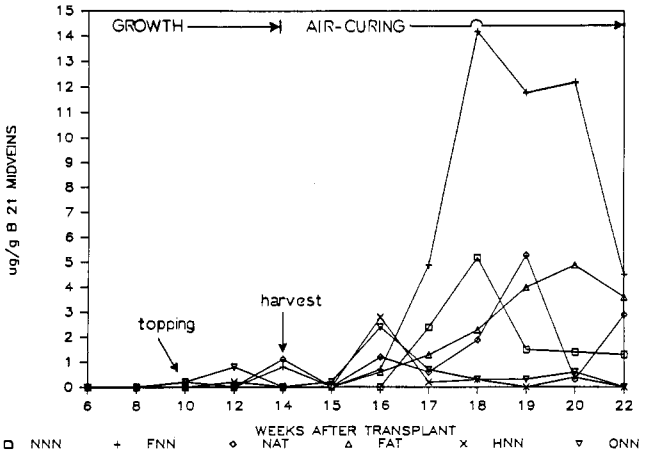


**Figure 3.** Variation of quantities of N′-substituted pyridine alkaloids in B21 midveins during field growth and successive air-curing.

5%. Contents of lamina total alkaloids were about 10-fold higher in B21 and KY 78379 tobacco than in LAB21 tobacco throughout the growth and air-curing periods. There were, however, only minor differences of total alkaloid contents between B21 and KY 78379 lamina. Concentrations of total alkaloids increased with time during field growth and reached maximum levels at the time of harvest. During the first 4 weeks of air-curing, total alkaloids generally decreased by about 20%, after which they remained about the same. Variations of total alkaloid concentrations in midveins of these tobacco lines were similar to those in lamina, except that midvein concentrations were only about 20% of those found in corresponding lamina. Calcium contents in lamina samples of the tobacco lines ranged from 37 mg/g at harvest to 54 mg/g during curing; corresponding contents in midveins ranged from 32 mg/g at harvest to 54 mg/g during curing. Calcium contents were used to adjust the total and N′-substituted pyridine alkaloid concentrations determined during air-curing to eliminate concentration changes caused by changes in leaf dry weights from those at harvest. Our results on the effects of plant maturity, genetic line, and air-curing on total alkaloid concentrations are in general agreement with those of previous studies (Andersen et al., 1977; Burton et al., 1983).

Concentrations of the four major *N′*-acyl and two *N′*-nitroso alkaloids in B21 lamina during growth and curing are presented in Figure 2. The concentration values for compounds in this line had about the same precision as the corresponding values for the KY 78379 and LAB21 lines given in Tables III and IV. In general, the compounds were present in the following order of decreasing concentrations: FNN, ONN, FAT, HNN, NAT, NNN. The results showed that *N′*-acyl derivative concentrations were relatively higher than their *N′*-nitroso counterparts. Although concentrations of some *N′*-acyl and *N′*-nitroso compounds generally increased as a function of time, only the *N′*-acyl derivatives were present in more than trace quantities (>0.5 μg/g) during field growth beginning 6 weeks after transplant. Maximum accumulations of the sum of *N′*-acyl compounds were reached after about 4 weeks of air-curing, and these levels then declined somewhat during the final weeks of curing. Maximum concentrations of the *N′*-nitroso compounds NNN and NAT occurred after 4–8 weeks of curing. The accumulation of these specific *N′*-acyl pyridine alkaloids in green leaves of *Nicotiana tabacum* during growth prior to harvest has not (to our knowledge) been described previously. Other reports described their presence at harvest and after curing (Bolt, 1972; Warfield et al., 1972; Miyano et al., 1979, 1981; Matsushita et al., 1979; Matsushima et al., 1983; Burton et al., 1988). Our results indicate that they are normal products of plant metabolism during late growth and postharvest air-curing, in contrast to *N′*-nitroso pyridine alkaloids, which are mainly formed in postharvest tobacco (Hecht et al., 1978; Andersen and Kasperbauer, 1984; Andersen and Kemp, 1985). The results of analysis of several tobacco samples as ethyl acetate (only) extracts with no further solvent partitioning showed the presence of *N′*-acyl and *N′*-nitroso pyridine alkaloids and provided evidence that these compounds were not artifacts of the complete analytical procedure. The structural characteristics of the various acyl substituents resemble compounds found during fatty acid biosynthesis as a result of the stepwise elongation of acetyl coenzyme A with two-carbon subunits (Goodwin and Mercer, 1983).

The four *N′*-acyl and two *N′*-nitroso alkaloids in B21 midveins during growth and air-curing were present in the following general order of decreasing concentrations: FNN, FAT, NAT, NNN, ONN, HNN (Figure 3). Except for FNN, the relative concentrations of the N′-substituted pyridine alkaloids in midveins were different from those in lamina. Maximum levels of the sum of *N′*-acyl and *N′*-nitroso compounds in midveins occurred after 2–6 weeks of air-curing.

Similar trends were found for variations of the N′-substituted pyridine alkaloids in LAB21 and KY 78379 burley tobaccos (Tables III and IV) except that concentrations were generally proportional to the total alkaloid contents of the tobacco lines.

**Comparisons of Accumulation Time Trends of *N′*-Acyl and *N′*-Nitroso Pyridine Alkaloids.** The effects of sampling date on accumulations of specific N′-substituted compounds in lamina of the three burley lines during the growth/air-curing time continuum are illustrated in Figures 4–6 for FNN, ONN, and NNN, respectively. These compounds, which represent a short carbon chain *N′*-acyl, a long carbon chain *N′*-acyl, and an *N′*-nitroso pyridine alkaloid, respectively, exhibited different trends in their accumulations. FNN began to accumulate during field growth at the time of topping and, thereafter, concentrations in the KY 78379 and B21 lines generally increased with time during late growth and subsequent curing until maximal FNN levels were reached after 4–5 weeks of air-curing (Figure 4). In the case of ONN, relatively high concentrations were present in the three lines at the time of topping, and in KY 78379, the ONN level was maximal at this time (Figure 5). The highest con-

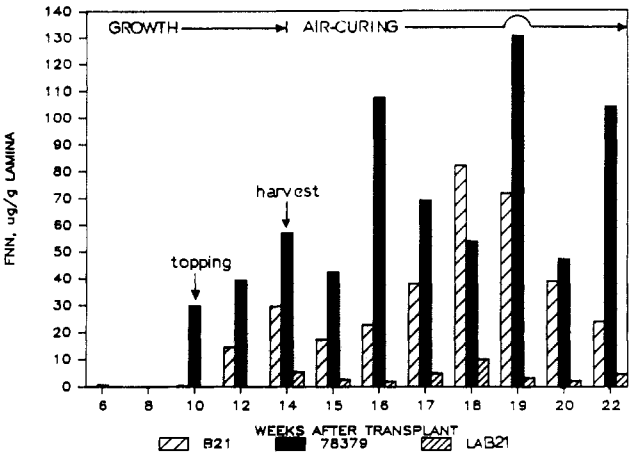


**Figure 4.** FNN concentrations in leaf lamina of three burley lines during field growth and successive air-curing.

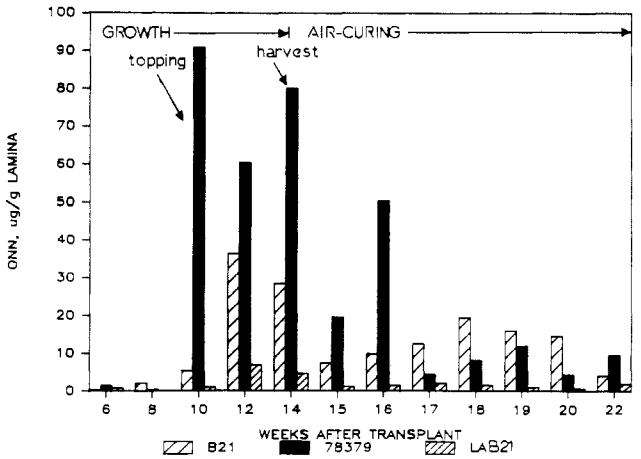


**Figure 5.** ONN concentrations in leaf lamina of three burley lines during field growth and successive air-curing.

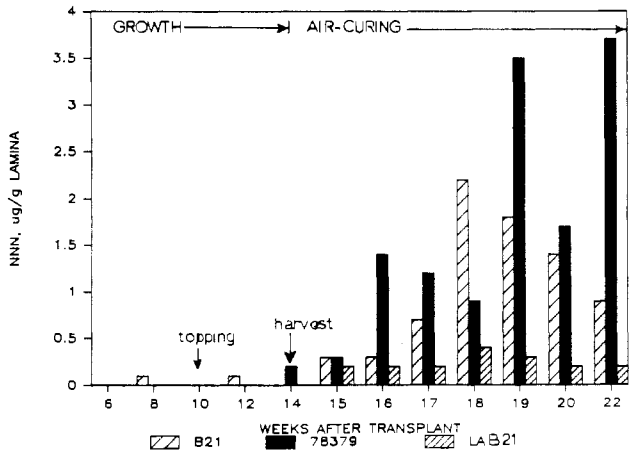


**Figure 6.** NNN concentrations in leaf lamina of three burley lines during field growth and successive air-curing.

centrations of ONN in B21 and LAB21 occurred 1 week after topping. A decline of contents occurred in the three lines throughout air-curing. Thus, maximal levels of ONN occurred much earlier in the growth/curing time continuum than was the case for FNN. The significance of earlier accumulation and concentration maxima of the long carbon chain acylated alkaloid (ONN) compared to short-chain FNN is not apparent at the present time. The metabolism or degradation of ONN during growth and curing might yield shorter chain *N'*-acyl substituted alkaloids. In contrast to FNN and especially to ONN, there were no appreciable (>1.0 μg/g) accumulations of NNN in leaf lamina during tobacco growth (Figure 6). Accumulations of NNN in the three lines began to occur 1 week after air-curing was started, and levels generally continued to increase until maximal levels were reached at a later time during curing. However, NNN concentrations did not exceed 4 μg/g at any time, indicating that some *N'*-acyl pyridine alkaloids were present at much higher concentrations than NNN in tobaccos grown and cured under our ambient conditions. Maximal levels of NNN were found after 4 weeks of curing for B21 tobacco, 8 weeks for KY 78379, and 4 weeks of LAB21. Our present results showed that NNN contents of burley tobacco after conventional air-curing are similar to those previously reported (Andersen and Kemp, 1985). Our earlier suggestion that nitrosamine biosynthesis and accumulation in tobacco leaves is directly related to both nitrite and pyridine alkaloid concentrations (Andersen et al., 1982; Andersen and Kemp, 1985) is consistent with our interpretation of the present results of leaf contents of NNN [and nitrite, unpublished results]. These compounds were low or absent during field growth and were elevated to varying degrees during air-curing.

**Comparison of Pyridine Alkaloid Derivatives in High-Alkaloid 78379 and Low-Alkaloid LAB21 Tobacco.** Lamina concentrations of *N'*-nitroso and *N'*-acyl pyridine alkaloids in the high total alkaloid–nornicotine line KY 78379 and the low total alkaloid line LAB21 during field growth and air-curing are given in Table III. Generally higher levels of all N'-substituted pyridine alkaloids were present in corresponding lamina samples of KY 78379 compared to LAB21 tobacco throughout growth and air-curing. Although there were relatively large standard deviations for the mean values of samples from four replicates, the differences were often highly significant and in the case of nitrosamines were in agreement with those reported by MacKown et al. (1984). The order of decreasing abundance for nitrosamines in both lines was NAT, NNN, and NNK; the order (approximate) of decreasing content of acylated derivatives in both lines was FNN, ONN, HNN, FAT, ANN, BNN, and AAT. The variations of NAT and NNK contents with time during growth and subsequent air-curing were similar to that previously described for NNN in lamina of the three lines (see discussion of Figure 6). However, only low levels of NNK were found in all the samples. The variations in contents of the acylated alkaloids, i.e., FAT, ANN, AAT, BNN, and HNN, with time during growth and curing were analogous to those previously described for FNN or ONN (see discussions of Figures 4 and 5) and were directly related to the similarity of the length of the *N'*-acyl carbon chain of the specific compound to that of FNN or ONN.

Midvein concentrations of *N'*-nitroso and *N'*-acyl pyridine alkaloids in the KY 78379 and LAB21 lines are given in Table IV. In most cases in which the two lines were compared, and when at least one of the N'-substituted compound's concentration was greater than 1 μg/g, the levels of N'-substituted alkaloids were higher in KY 78379 than in LAB21 tobacco. In the case of nitrosamines, the order of decreasing abundance in midveins of both lines was NAT, NNN, and NNK, although NNK was not detected in most samples. The order of decreasing overall abundance of acylated derivatives in both lines was ANN, FNN, FAT, ONN, AAT, HNN, and BNN. These specific N'-acylated alkaloids in midveins of the burley lines differed in overall abundances from those in lamina. In general, corresponding concentrations of acylated alkaloids in midvein were less than those in lamina. Variations of

**Table III. Pyridine Alkaloid Derivatives in High-Alkaloid KY 78379 versus Low-Alkaloid LAB21 Burley Tobacco Lamina during Growth and Air-Curing**

| weeks after transplant | tobacco line | *N'*-nitroso pyridine alkaloid[a,b] | | | *N'*-acyl pyridine alkaloid[a,b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NNN | NAT | NNK | FNN | FAT | ANN | AAT | BNN | HNN | ONN |
| 6 | KY 78379 | nd | nd | nd | 0.7 ± 0.9 | 1.4 ± 0.7* | nd | nd | nd | 0.2 ± 0.1 | 1.4 ± 0.8 |
| | LAB21 | nd | 0.1 ± 0.1 | nd | 0.1 ± 0.0 | nd | nd | nd | nd | 0.1 ± 0.1 | 0.8 ± 0.5 |
| 8 | KY 78379 | nd | nd | nd | nd | 0.8 ± 0.1** | nd | nd | nd | 0.1 ± 0.1 | 0.4 ± 0.3 |
| | LAB21 | nd | 0.2 ± 0.2 | nd | nd | nd | nd | nd | nd | nd | nd |
| 10 (topping) | KY 78379 | nd | nd | nd | 30.9 ± 19.2 | 2.7 ± 0.2** | 5.4 ± 3.7* | nd | 3.7 ± 3.1 | 51.1 ± 34.1* | 90.9 ± 55.8* |
| | LAB21 | nd | nd | nd | nd | nd | nd | nd | nd | 0.1 ± 0.1 | 1.0 ± 0.7 |
| 12 | KY 78379 | nd | 0.8 ± 0.8 | nd | 39.4 ± 15.9* | 4.1 ± 1.1** | 4.2 ± 1.3** | nd | 3.9 ± 3.0 | 34.4 ± 20.7* | 60.4 ± 33.7* |
| | LAB21 | nd | nd | nd | 0.1 ± 0.1 | 0.1 ± 0.2 | nd | nd | 1.1 ± 1.0 | 6.9 ± 5.2 | 0.1 ± 0.1 |
| 14 (harvest/ start cure) | KY 78379 | 0.2 ± 0.1* | 3.2 ± 2.2 | nd | 57.0 ± 41.3 | 6.6 ± 3.7* | 16.0 ± 12.9 | nd | 6.8 ± 6.7 | 42.8 ± 39.1 | 80.1 ± 65.4 |
| | LAB21 | nd | 0.7 ± 0.4 | nd | 5.5 ± 2.6 | 0.4 ± 0.1 | 0.3 ± 0.3 | nd | nd | 0.6 ± 0.2 | 4.6 ± 1.7 |
| 15 | KY 78379 | 0.3 ± 0* | 2.0 ± 0.6* | 0.1 ± 0.1 | 42.5 ± 20.8* | 4.8 ± 0.8** | 10.7 ± 5.4* | nd | 3.6 ± 3.6 | 19.3 ± 18.1 | 19.6 ± 17.5 |
| | LAB21 | 0.2 ± 0.1 | 0.3 ± 0.5 | nd | 2.7 ± 1.4 | 0.2 ± 0.2 | 0.2 ± 0.4 | nd | nd | 0.2 ± 0.1 | 1.1 ± 0.4 |
| 16 | KY 78379 | 1.4 ± 0.5 | 4.3 ± 1.6* | 1.2 ± 0.3* | 107.5 ± 14.8** | 4.4 ± 0.5** | 24.3 ± 6.0* | 0.4 ± 0.1* | 13.3 ± 2.6d* | 49.6 ± 2.0* | 50.5 ± 4.4* |
| | LAB21 | 0.2 ± 0.1 | 0.2 ± 0.2 | nd | 2.0 ± 0.5 | 0.3 ± 0.1 | 0.3 ± 0.1 | nd | nd | 0.4 ± 0.2 | 1.5 ± 0.5 |
| 17 | KY 78379 | 1.2 ± 0.6** | 3.4 ± 1.1** | 0.2 ± 0.1* | 69.1 ± 35.0* | 60.9 ± 1.5** | 22.1 ± 10.1** | 2.4 ± 0.7** | 1.0 ± 1.3 | 3.3 ± 3.6 | 4.4 ± 2.3 |
| | LAB21 | 0.2 ± 0 | 0.5 ± 0.4 | nd | 5.1 ± 4.0 | 0.8 ± 0.2 | 0.5 ± 0.5 | 0.5 ± 0.2 | nd | 1.0 ± 0.6 | 2.0 ± 1.0 |
| 18 | KY 78379 | 0.9 ± 0.4 | 4.4 ± 1.8 | nd | 53.9 ± 31.8 | 34.1 ± 1.9** | 12.7 ± 2.2 | 1.5 ± 1.2 | 0.4 ± 0.5 | 3.2 ± 2.4 | 8.2 ± 2.2* |
| | LAB21 | 0.4 ± 0.1 | 1.4 ± 0.4 | nd | 10.2 ± 4.8 | 7.3 ± 2.6 | 1.4 ± 0.9 | 1.0 ± 0.6 | 0.1 ± 0.1 | 0.8 ± 0.7 | 1.5 ± 0.9 |
| 19 | KY 78379 | 3.5 ± 1.4** | 7.8 ± 1.3** | 0.2 ± 0.1* | 130.3 ± 48.8** | 23.7 ± 14.1* | 25.4 ± 12.8* | 2.3 ± 0.8* | 2.8 ± 1.7* | 10.6 ± 5.5* | 11.9 ± 4.7** |
| | LAB21 | 0.3 ± 0.1 | 1.7 ± 0.7 | nd | 3.3 ± 2.0 | 3.1 ± 1.4 | 0.3 ± 0.5 | 0.6 ± 0.2 | nd | 0.5 ± 0.6 | 1.0 ± 1.0 |
| 20 | KY 78379 | 1.7 ± 1.0 | 3.6 ± 0.7** | 0.1 ± 0.1 | 47.3 ± 25.6* | 21.6 ± 5.5** | 14.9 ± 10.6 | 1.7 ± 0.5* | 0.6 ± 0.5 | 2.4 ± 2.0 | 4.3 ± 2.0* |
| | LAB21 | 0.2 ± 0 | 0.6 ± 0.4 | nd | 2.3 ± 0.8 | 2.6 ± 0.4 | 0.2 ± 0.1 | 0.6 ± 0.1 | nd | 0.3 ± 0.1 | 0.6 ± 0.3 |
| 22 | KY 78379 | 3.7 ± 1.5** | 5.8 ± 0.8** | 0.1 ± 0.1 | 103.9 ± 33.3* | 17.4 ± 5.1** | 28.4 ± 7.4** | 1.4 ± 0.7 | 1.2 ± 0.7* | 5.8 ± 3.8 | 9.6 ± 5.0* |
| | LAB21 | 0.2 ± 0.1 | nd | nd | 4.8 ± 1.4 | 1.6 ± 0.4 | 0.4 ± 0.2 | 0.6 ± 0.2 | nd | 0.6 ± 0.4 | 1.9 ± 1.2 |

[a] Mean values are from four replicated field-grown leaf samples from the middle leaf position on the stalk. Those mean values in a column for a given compound and sampling date followed by one or two asterisks are significantly different between the tobacco lines at the 0.05 and 0.01 levels of probability, respectively. All values for samples taken during air-curing are corrected for Ca content departures from those at harvest. Units: μg/g (±SD). [b] nd = not detected.

**Table IV. Pyridine Alkaloid Derivatives in High-Alkaloid KY 78379 versus Low-Alkaloid LAB21 Burley Tobacco Midvein during Growth and Air-Curing**

| weeks after transplant | tobacco line | *N'*-nitroso pyridine alkaloid[a,b] | | | *N'*-acyl pyridine alkaloid[a,b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NNN | NAT | NNK | FNN | FAT | ANN | AAT | BNN | HNN | ONN |
| 6 | KY 78379 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| | LAB21 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8 | KY 78379 | nd | nd | nd | nd | 0.1 ± 0.1* | nd | nd | nd | nd | nd |
| | LAB21 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 10 (topping) | KY 78379 | nd | 0.2 ± 0.2 | nd | nd | nd | nd | nd | nd | 1.5 ± 1.4 | 3.7 ± 2.58* |
| | LAB21 | nd | nd | nd | 0.1 ± 0.2 | nd | nd | nd | nd | nd | nd |
| 12 | KY 78379 | nd | 0.1 ± 0.2 | nd | nd | 0.2 ± 0.0 | nd | nd | nd | 0.4 ± 0.4 | 1.0 ± 0.5* |
| | LAB21 | nd | nd | nd | nd | nd | nd | nd | nd | nd | 0.1 ± 0.1 |
| 14 (harvest/ start cure) | KY 78379 | nd | 0.2 ± 0.3 | nd | 0.4 ± 0.2 | 1.1 ± 0.2 | nd | nd | nd | 0.1 ± 0.1 | 0.2 ± 0.2 |
| | LAB21 | 0.1 ± 0.1 | 0.4 ± 0.5 | nd | 0.3 ± 0.3 | 1.2 ± 0.3 | nd | nd | nd | nd | nd |
| 15 | KY 78379 | nd* | 0.5 ± 1.0 | 0.3 ± 0.0* | nd | 0.3 ± 0.2 | 0.2 ± 0.3 | nd** | 0.2 ± 0.1 | 0.5 ± 0.3 | 0.6 ± 0.4 |
| | LAB21 | 0.2 ± 0.1 | nd | nd | nd | 0.4 ± 0.4 | nd | 0.3 ± 0.1 | nd | nd | 0.2 ± 0.1 |
| 16 | KY 78379 | nd | 0.8 ± 0.1 | nd | nd | 0.2 ± 0.1 | 0.2 ± 0.2 | 1.0 ± 0.6 | nd | 0.6 ± 0.2 | 0.8 ± 0.2 |
| | LAB21 | nd | 0.3 ± 0.3 | nd | nd | 0.7 ± 0.5 | nd | 0.8 ± 0.7 | nd | 0.4 ± 0.4 | 0.5 ± 0.4 |
| 17 | KY 78379 | 0.3 ± 0.2 | 2.0 ± 0.3** | nd | 3.8 ± 3.0 | 2.7 ± 1.1* | 2.9 ± 1.9 | 0.3 ± 0.6 | 0.5 ± 0.5 | 0.6 ± 0.6 | 0.8 ± 0.2 |
| | LAB21 | 0.2 ± 0.3 | 0.2 ± 0.3 | nd | 0.4 ± 0.8 | 0.2 ± 0.3 | 0.2 ± 0.3 | 0.6 ± 0.4 | 0.3 ± 0.4 | 0.2 ± 0.2 | 0.7 ± 0.6 |
| 18 | KY 78379 | 6.2 ± 7.3 | 4.1 ± 6.7 | nd | 11.0 ± 10.3 | 3.9 ± 3.1 | 12.6 ± 7.3* | nd | 0.6 ± 0.4 | 0.2 ± 0.2 | 0.4 ± 0.3 |
| | LAB21 | 0.1 ± 0.2 | 0.8 ± 0.5 | nd | 0.7 ± 0.9 | 0.1 ± 0.3 | 0.1 ± 0.1 | 0.5 ± 0.5 | nd | 0.1 ± 0.2 | 0.2 ± 0.2 |
| 19 | KY 78379 | 2.8 ± 2.0* | 7.4 ± 4.2* | nd | 13.5 ± 10.7* | nd | 16.8 ± 7.8** | 2.4 ± 4.2 | 0.4 ± 0.3* | 0.1 ± 0.1* | 0.4 ± 0.1** |
| | LAB21 | nd | 0.1 ± 0.1 | nd | nd | nd | nd | nd | nd | nd | nd |
| 20 | KY 78379 | 1.9 ± 2.2 | nd* | nd | 12.7 ± 7.6* | 4.1 ± 2.4* | 18.7 ± 8.6** | 0.3 ± 0.3 | 0.5 ± 0.5 | 0.3 ± 0.4 | 0.4 ± 0.5 |
| | LAB21 | nd | 0.2 ± 0.1 | nd | 0.4 ± 0.1 | nd | 0.1 ± 0.1 | nd | nd | nd | 0.1 ± 0.1 |
| 22 | KY 78379 | 3.9 ± 1.6** | 8.7 ± 3.5** | nd | 13.6 ± 5.6** | 6.1 ± 6.6 | 17.9 ± 6.1** | nd | 0.2 ± 0.2* | 0.1 ± 0.1 | 0.4 ± 0.1 |
| | LAB21 | 0.1 ± 0.0 | 0.5 ± 0.5 | 0.1 ± 0.0 | nd | 0.1 ± 0.2 | nd | nd | nd | nd | 0.2 ± 0.2 |

[a] Mean values are from four replicated field-grown and air-cured leaf samples from the middle leaf position on the stalk. The mean values in a column for a given compound and sampling date followed by one or two asterisks are significantly different between the tobacco lines at the 0.05 ± 0.01 levels of probability, respectively. All values for samples taken during air-curing are corrected for Ca content departures from those at harvest. Units: μg/g (±SD). [b] nd = not detected.

midvein nitrosamine and N′-acyl alkaloid contents during late field growth and air-curing were similar to those observed for lamina.

**Possible Origin of N′-Acyl Pyridine Alkaloids.** In several plant biosynthetic processes the formyl group is known to derive from glyoxylate and to be transferred to its acceptor molecule enzymatically, with $N^{10}$-formyltetrahydrofolic acid serving as a carrier molecule (Goodwin and Mercer, 1983). This route to formylated pyridine alkaloids may operate in tobacco leaves during growth.

The acyl groups, i.e., acetyl, butanoyl, hexanoyl, and octanoyl, may be synthesized from the sequence of intermediates involved in the biosynthesis of common long-chain ($C_{16}$ and $C_{18}$) fatty acids or from degradation of fatty acids via the β-oxidation process (Goodwin and Mercer, 1983). The latter scheme involves the removal of two carbon units sequentially from the carboxyl end of $C_{16}$ and $C_{18}$ fatty acids to give even-numbered shorter chain acyl intermediates. Tobacco leaves contain a high proportion of unsaturated fatty acids formed as the last step of fatty acid synthesis. This unsaturation would be retained in degradation products produced by β-oxidation. Since the acyl moieties of the alkaloids isolated were saturated and the acyl moieties from fatty acid biosynthesis are more likely to be saturated than those from degradation, it seems more likely that the N′ moieties of the alkaloids are formed from intermediates arising during fatty acid biosynthesis prior to desaturation.

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