Accumulation and Distribution of Acylated Nornicotine Derivatives in Flue-Cured Tobacco Alkaloid Isolines

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Acylnornicotine derivatives were analyzed and quantified in several alkaloid isolines of NC 95 flue-cured tobacco during growth and curing. N′-Formyl-, N′-acetyl-, N′-(n-butanoyl)-, N′-hexanoyl-, N′-octanoyl-, and N′-(hydroxyoctanoyl)nornicotines were determined by extraction and chromatographic procedures frequently used for tobacco-specific nitrosamines. Detection was with a thermionic N–P detector. Formyl-, (hydroxyoctanoyl)-, and octanoylnornicotine were present in greatest amounts in these flue-cured tobaccos. Mature green lamina of NC 95 contained 39–92 $\mu g\ g^{-1}$ of acylnornicotines, and the amount increased during curing (56–328 $\mu g\ g^{-1}$). Cured lamina from the alkaloid isoline tobaccos contained from 41 to 1182 $\mu g\ g^{-1}$ of N′-acylnornicotines. The highest amounts of acylated nornicotine derivatives were in the lamina from the upper stalk positions. The accumulation of acylnornicotines in the different alkaloid isolines was correlated positively ($r = 0.50$) with the alkaloid precursor nornicotine. Correlation coefficients of the individual acylnornicotines with N′-nitrosonornicotine (NNN) were highly significant ($r = 0.86$–0.98) in cured lamina from the third priming of the alkaloid isoline tobaccos.

There has been increased interest lately in formation and accumulation of acylated derivatives of secondary amine alkaloids in tobacco during growth and curing. While tobacco alkaloids have been thought to have an important role in the formation of smoke flavor (Matsushima et al., 1983), some acyl derivatives of nornicotine, anatabine, and anabasine have a marked effect on the taste of the smoke (Enzell and Wahlberg, 1980). Enzell et al. (1977) suggested that many of secondary amine alkaloid derivatives may be associated with the major tobacco alkaloid nicotine, which readily loses the N′-methyl group by transmethylation during postharvest treatment of tobacco, i.e., curing, fermentation, and aging, yielding nornicotine. Subsequently, nornicotine may be acylated to yield the various derivatives. The occurrence of N′-acyl alkaloids in tobacco and smoke was discussed by Snook et al. (1984). Burton et al. (1988) identified and quantified seven N′-acyl derivatives of nornicotine and anatabine in lamina of both green and air-cured burley tobacco. Andersen et al. (1989) reported that N′-formylnornicotine (FNN) appeared to be the most abundant acyl derivative, followed in decreasing order by N′-(n-octanoyl)nornicotine (ONN), N′-(n-hexanoyl)nornicotine (HNN), N′-formylanatabine (FAT), N′-acetylnornicotine (ANN), N′-(n-butanoyl)nornicotine (BNN), and N′-acetylanatabine (AAT) in burley tobaccos. Burton et al. (1988) found that plant maturity had more influence on accumulation of acylated secondary amine alkaloids in burley tobacco than curing temperature. A greater amount of the total acylnornicotines than total acylanatabines in tobacco was, according to the authors, due to the specificity for accumulation of acylnornicotines vs acylanatabines even though the concentrations of nornicotine and anatabine were equivalent. In another study, Burton et al. (1987) reported that there was an increased concentration of the acyl alkaloids with addition of nitrogen fertilizer to soil with low available nitrogen. Miyano et al. (1981) identified two hydroxy derivatives of ONN [1′-(6-hydroxyoctanoyl)nornicotine and 1′-(7-hydroxyoctanoyl)nornicotine] in air-cured Japanese tobacco. Although Matsushima et al. (1983) did not report the presence of 1′-(6-hydroxyoctanoyl)- and 1′-(7-hydroxyoctanoyl)nornicotine in green tobacco leaf lamina, they did report that HNN, ONN, and both (hydroxyoctanoyl)nornicotines (HYONN) increased during curing in both air-cured and flue-cured varieties, although the levels of most alkaloids decreased. The major acylnornicotine found in their air-cured, flue-cured, and sun-cured tobaccos was FNN.

Zador and Jones (1986) found N′-(hydroxyacyl)nornicotine (isomers and homologues were not distinguished) in green tissue of *Nicotiana stocktonii*. They demonstrated that N′-(hydroxyacyl)nornicotine formed and accumulated in trichome exudate, but it was not present inside trichomes or other internal parts of plants. A significantly greater portion of radioactivity was recovered as N′-(hydroxyacyl)nornicotine in tobacco leaves fed [2′-^{14}C]nornicotine compared with leaves fed [2′-^{14}C]nicotine, suggesting that nornicotine is the direct precursor for N′-(hydroxyacyl)nornicotine biosynthesis.

The objectives of this study were as follows: (a) to determine the accumulation pattern of N′-acylnornicotines in near-isogenic flue-cured tobacco genotypes different for total alkaloid content at harvest and during curing; (b) to determine N′-acylnornicotines accumulation and distribution within the tobacco plant; (c) to calculate correlation between N′-acylnornicotines and total and individual tobacco alkaloids; (d) to determine relationships between N′-acylnornicotines, N′-nitrosonornicotine (NNN), and their alkaloid precursor nornicotine.

MATERIALS AND METHODS

Materials. Six of the flue-cured tobacco genotypes chosen for this study were NC 95 isolines with typical alkaloid content ranging from 3 to 36 mg g^{-1}, and NC 95 was the commercial flue-cured "control" genotype (Chaplin and Burk, 1984). Tobacco was grown and cured under recommended conditions for flue-cured tobacco in Oxford, NC, in 1983, except that an additional 69 kg of N fertilizer ha^{-1} was applied. Samples were taken at harvest (mature leaves), during flue-curing at end-of-yellowing, and after curing was complete. Leaves were harvested at three primings (first = lower stalk position; second = middle stalk position; third = upper stalk position), with the interval between each priming about 2 weeks. A 10-cm strip was cut from the widest part of each leaf, midrib removed, and lamina frozen. Lamina tissue remained frozen until freeze-

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Table I. Alkaloid Content (mg/g) in Flue-Cured Lamina from the Third Priming (Leaves from Upper Stalk Position) of Alkaloid Isolines of NC 95

	alkaloid[a]		
genotype	nicotine	nornicotine	total alkaloid
LAFC 53	6.52	0.14	6.85
TA 1.0	30.64	0.39	32.56
TA 2.0	43.69	0.43	45.72
TA 3.1	39.64	6.47	49.28
TA 3.5	49.64	0.86	52.99
TA 3.9	54.19	0.66	56.20
NC 95	60.40	1.20	64.70

[a] Adapted from Djordjevic et al. (1989).

Table II. Alkaloid Content (mg/g) in Flue-Cured Lamina of NC 95 from Three Primings at Harvest, End-of-Yellowing during the Curing Process, and in the Cured Lamina

		alkaloid[a]		
sample	priming	nicotine	nornicotine	total alkaloid
harvest	1	22.63	0.56	24.50
	2	32.82	0.48	34.62
	3	47.33	0.87	50.45
end-of-yellowing	1	36.81	0.46	38.72
	2	46.34	0.45	48.38
	3	59.23	1.03	63.19
cured	1	37.37	0.57	39.58
	2	48.98	0.55	51.46
	3	60.40	1.20	64.70
		Sample Means		
harvest		34.26	0.64	36.16
end-of-yellowing		47.46	0.65	49.93
cured		48.92	0.77	51.65

[a] Adapted from Djordjevic et al. (1989).

dried and then ground to pass a 40-mesh screen prior to chemical analysis.

Methods. Nicotine and nornicotine were determined by using the method developed by Severson et al. (1981) and modified by Madsen et al. (1985). Summation of individual alkaloids—nicotine, nornicotine, anabasine, and anatabine—was used to calculate total alkaloid values.

N'-Acylnornicotine derivatives were determined by the same extraction and gas chromatographic procedure as described for tobacco-specific nitrosamine determination (Djordjevic et al., 1989) except that injector and detector temperatures were maintained at 280 °C and column temperature was programmed from 140 °C, with an initial 3-min hold, to 245 °C at 2 °C min^{-1} and then held at 245 °C for 5 min.

Quantification was carried out by internal standardization with azobenzene after calibration of retention times and response factors with authentic acylated derivatives of nornicotine (FNN, ANN, BNN, HNN, ONN) which were synthesized as described by Burton et al. (1988). The 1'-(6- and 1'-(7-hydroxy-*n*-octanoyl)nornicotine isomers were not resolved, and for quantification they were treated as one compound, *N'*-(hydroxy-*n*-octanoyl)nornicotine (HYONN). The response factor of ONN was used since authentic hydroxy derivative was not available for calibration.

Peak identification in some samples was verified by analysis in a gas chromatography–mass spectrometry computer system (a Varian 3700 GC equipped with an on-column injector and a Finnigan Model 705 ion trap detector).

RESULTS AND DISCUSSION

Alkaloids. Nicotine, nornicotine, and total alkaloid accumulation in lamina of the third priming increased among the isolines from LAFC 53 to NC 95 as expected on the basis of previous history (Chaplin and Burk, 1984), with the exception of genotype TA 3.1, which had higher than expected nornicotine content [Table I, adapted from Djordjevic et al. (1989)]. Overall alkaloid values are somewhat higher than those described by Chaplin and Burk (1984) because 1983 was a very dry year and additional N was applied. The low-alkaloid genotype LAFC 53 contained 6.5 and 6.8 mg g^{-1} of nicotine and total alkaloids, respectively, while the commercial genotype NC 95 contained approximately 10 times more nicotine and total alkaloids (60.4 and 64.6 mg g^{-1}, respectively). TA 3.1 contained 5–50 times more nornicotine than the other genotypes. Nornicotine, a secondary amine alkaloid, was 0.6–2% of the total alkaloid fraction except in line TA 3.1 where it was over 13% of the total alkaloid fraction.

Nicotine and total alkaloid concentrations in lamina of NC 95 increased from harvest through end-of-yellowing to cured tobacco (Table II). Nornicotine accumulation increased during curing but was significant only at $p = 0.31$, with the fully cured leaf having greater nornicotine content than the other two sample times.

At all sample times, nicotine and total alkaloid content of NC 95 lamina increased in leaf from lower (priming 1) to upper (priming 3) stalk positions, a trend that is well recognized in the literature (Tso, 1972). Nornicotine content in the leaves from lower and midstalk positions was approximately the same, and it was about half that in the leaves from the third priming or upper stalk position.

Acyl Derivatives of Nornicotine. Burton et al. (1988) showed that their method resulted in quantitative recovery of the more lipophilic HNN and ONN derivatives (100 and 108%, respectively). However, recovery of the

Table III. *N'*-Acylnornicotine Contents (μg/g) in Flue-Cured Lamina from the Third Priming of Alkaloid Isolines of NC 95

	N'-acylnornicotine[a]						
genotype	FNN	ANN	BNN	HNN	ONN	HYONN	MEAN
LAFC 53	21.6	6.4	0.08	2.0	7.3	3.90	6.9
TA 1.0	67.4	14.3	0.73	11.0	30.5	27.8	25.3
TA 2.0	116.2	23.6	1.68	25.6	57.7	74.3	49.9
TA 3.1	550.2	91.1	13.31	124.0	190.1	213.0	197.0
TA 3.5	96.3	17.8	1.11	23.3	61.2	96.9	49.4
TA 3.9	147.6	21.7	0.54	11.4	18.7	47.3	41.2
NC 95	138.2	24.1	1.08	22.6	52.9	89.2	54.7
LSD (0.05)[b]	230.2	34.4	5.38	34.1	33.4	40.2	35.9

[a] *N'*-Acylnornicotine abbreviations: FNN, *N'*-formylnornicotine; ANN, *N'*-acetylnornicotine; BNN, *N'*-(*n*-butanoyl)nornicotine; HNN, *N'*-(*n*-hexanoyl)nornicotine; ONN, *N'*-(*n*-octanoyl)nornicotine; HYONN, 1'-(6- + 1'-(7-hydroxyoctanoyl)nornicotine. [b] LSD (0.05) = least significant difference ($P = 0.05$).

Table IV. Order of Abundance of *N'*-Acylnornicotines in Flue-Cured Lamina from the Third Priming of Alkaloid Isolines of NC 95

	rank					
genotype	1	2	3	4	5	6
LAFC 53	FNN[a,b]	ONN	ANN	HYONN	HNN	BNN
TA 1.0	FNN	ONN	HYONN	ANN	HNN	BNN
TA 2.0	FNN	HYONN	ONN	HNN	ANN	BNN
TA 3.1	FNN	HYONN	ONN	HNN	ANN	BNN
TA 3.5	FNN	HYONN	ONN	HNN	ANN	BNN
TA 3.9	FNN	HYONN	ANN	ONN	HNN	BNN
NC 95	FNN	HYONN	ONN	HNN	ANN	BNN
mean	FNN	HYONN	ONN	HNN	ANN	BNN

[a] Abbreviations: see footnote on Table III. [b] Any acylnornicotines underlined with the same line are not significantly different at $P \leq 0.05$.

Table V. *N'*-Acylnornicotine Contents (μg/g) in Flue-Cured Lamina of NC 95 from Three Primings at Harvest, End-of-Yellowing during the Curing Process, and in the Cured Lamina

		N'-acylnornicotine[a]						
sample	priming	FNN	ANN	BNN	HNN	ONN	HYONN	TOTAL
harvest	1	35.1	8.6	0.22	0.9	2.7	0.3	47.8
	2	30.3	6.6	0.16	0.4	1.3	0.1	39.0
	3	48.6	10.9	0.40	5.8	20.7	5.3	91.8
	LSD (0.05)[b]	ns	3.8	0.15	1.9	5.2	2.2	35.1
end-of-yellowing	1	39.6	12.9	0.18	2.6	7.1	2.5	65.0
	2	36.6	11.0	0.12	1.7	4.7	2.4	56.4
	3	45.1	11.2	0.48	8.9	25.6	16.4	107.7
	LSD (0.05)	ns	ns	0.22	2.9	6.6	9.2	32.6
cured	1	47.4	12.7	0.10	2.8	8.9	3.5	75.3
	2	87.5	15.8	0.23	3.7	9.6	14.6	131.5
	3	138.2	24.1	1.08	22.6	52.9	89.2	328.1
	LSD (0.05)	42.7	4.7	0.50	3.4	9.2	13.4	54.3
		Sample Means[c]						
harvest		38.1[c]	8.7[d]	0.26[e]	2.4[e]	8.2[d]	1.9[e]	59.5
end-of-yellowing		40.5[c]	11.7[d]	0.26[f]	4.4[e]	12.5[d]	7.1[e]	76.4
cured		91.0[c]	17.5[e]	0.47[g]	9.7[f]	23.8[e]	35.8[d]	178.3
LSD (0.05)		11.6	2.2	0.14	1.2	3.4	4.4	17.0

[a] *N'*-Acylnornicotine abbreviations: see footnote on Table III. [b] LSD (0.05) = least significant difference ($P = 0.05$). [c] (c–g) numbers followed by different letters within a row are different at $P \leq 0.05$.

Table VI. Correlation Coefficients (*r*) of Acylnornicotines with Nicotine, Nornicotine, Total Alkaloids, and *N'*-Nitrosonornicotine in NC 95 Flue-Cured Tobacco during Curing from Three Primings and in Flue-Cured Leaf of Seven NC 95 Alkaloid Isolines

	priming and curing samples of NC 95				alkaloid isolines			
acylnornicotine[a]	NIC[b]	NNIC	TA	NNN	NIC	NNIC	TA	NNN
FNN	0.50[d]	0.42[c]	0.51[d]	0.94[e]	ns	ns	ns	0.99[e]
ANN	0.40[c]	0.39[c]	0.42[c]	0.85[e]	ns	ns	ns	0.98[e]
BNN	0.52[d]	0.58[d]	0.54[d]	0.82[e]	ns	0.42[c]	ns	0.97[e]
HNN	0.60[e]	0.65[e]	0.62[e]	0.87[e]	ns	0.56[d]	ns	0.97[e]
ONN	0.60[e]	0.72[e]	0.63[e]	0.80[e]	ns	0.71[e]	ns	0.88[e]
HYONN	0.51[d]	0.61[e]	0.53[d]	0.90[e]	ns	0.62[d]	ns	0.88[e]
total	0.55[d]	0.57[d]	0.57[d]	0.94[e]	ns	0.49[c]	ns	0.98[e]

[a] Abbreviations: see footnote *a* of Table III. [b] Abbreviations: NIC, nicotine; NNIC, nornicotine; TA, total alkaloids; NNN, nitrosonornicotine. [c] (c) $P \leq 0.05$; (d) $P \leq 0.01$; (e) $P \leq 0.0001$.

more hydrophilic acylated nornicotines such as FNN, ANN, and BNN was much less (24, 27, and 71%, respectively). The values for individual *N'*-acylnornicotines in different flue-cured tobacco alkaloid isolines as shown in Table III were calculated with use of recovery factors.

Genotype TA 3.1 contained the highest amount of each *N'*-acylnornicotine (Table III) as it did of nornicotine (Table II). The amounts of individual *N'*-acylnornicotines in genotypes of the lower alkaloid range increased in the series from LA 53 to TA 2.0 as did nornicotine. The amounts of nornicotine derivatives in isolines with higher alkaloid content were not as high as might be expected from the nornicotine levels in these tobaccos which were at least 2 times higher than in low-alkaloid isolines (Table I).

FNN was found in greatest amounts in all genotypes except TA 3.5 (Table IV). Comparison of the means indicated HYONN was present in amounts greater than HNN, ANN, and BNN. Generally, the shorter chain acyl derivatives—HNN, ANN, and BNN—were present in the lowest quantities. The relative great abundance of HYONN in the flue-cured tobacco was not expected because Matsushima et al. (1983) found HYONN to be less than 3% of the acylated nornicotine derivatives, whereas we found HYONN to be 25% of these compounds. Burton et al. (1988) and Andersen et al. (1989) did not report the presence of this alkaloid in burley tobacco. In cured burley tobaccos from the recommended harvest time, FNN was present in greatest amounts followed in decreasing order by ONN, HNN, ANN, and BNN. This is the same rank order as we found for these flue-cured tobaccos with the exception of HYONN.

In the NC 95 genotype *N'*-acylnornicotines increased from the time of harvest to the cured tobacco stage (Table V). Greatest amounts of acylated nornicotine derivatives were measured in the leaves from upper stalk positions (third priming). There was little difference in acylnornicotine contents of leaves from the first and second primings. Nornicotine content was also greatest in leaves from the third priming, whereas nornicotine contents of leaves from the first and second primings were similar and lower as were the acylated nornicotines. In this study nicotine content of the leaves from the second priming tended to be intermediate between those of the first and third primings.

FNN was the most abundant acyl derivative of nornicotine at all stages of flue-cured tobacco production and at all three primings (Table V). The composition of individual acylnornicotines in the leaves from the lower and middle stalk positions (first and second priming) was the same at all three sampling dates. The ranked order of abundance was FNN > ANN > ONN > HNN > HYONN > BNN. In lamina from the third priming the most abundant acylnornicotine following FNN was either ONN at harvest and at end-of-yellowing or HYONN in cured tobacco. The greater amount of FNN suggests either that the formyl group is present in the largest quantity in tobacco or that the reaction(s) for FNN formation occurs more readily. Degradative reactions of the curing pro-

cess are probably the source of one-carbon units, with tetrahydrofolate serving as the acceptor and carrier for formyl addition to nornicotine (Stryer, 1988). The other acyl groups could result from intermediates in fatty acid synthesis. However, very little, if any, tobacco fatty acid synthesis would occur during curing; consequently, smaller amounts of these derivatives were found. The one-carbon moieties that arise from the many degradative steps during curing would be in great abundance, and consequently FNN was the most abundant acylated nornicotine.

Correlations coefficients (r) of the acylnornicotines with nicotine, nornicotine, total alkaloids, or N'-nitrosonornicotine were positive and significant for the NC 95 priming and curing samples (Table VI). In the alkaloid isoline samples the only significant correlation coefficients of the acylnornicotines with alkaloid precursors were with nornicotine. In all associations of acylnornicotines with alkaloids, the values of r were low but tended to be higher for association with nornicotine than with nicotine. These results would not preclude nicotine being an immediate precursor of the acylnornicotines but suggest that nornicotine is the more likely immediate precursor with nicotine involved indirectly as the precursor of nornicotine. Correlation coefficients of the acylnornicotines with NNN were highly significant and likely reflect the availability of nornicotine for reaction with the acyl chains to form acylnornicotines or with oxides of nitrogen to form NNN.

CONCLUSIONS

In these flue-cured tobaccos the most abundant acylated nornicotine found was FNN as was reported for air-cured burley tobacco (Burton et al., 1988; Andersen et al., 1989) and sun-cured tobacco (Matsushima et al., 1983). HYONN was found in these green and cured lamina of flue-cured tobaccos, whereas it was not reported in the air-cured burley tobacco (Burton et al., 1988; Andersen et al., 1989). HYONN was very abundant in leaves from the upper stalk positions, and its accumulation increased with time from leaf harvest through the curing process. The relative abundance of HYONN among the acylated nornicotines increased with each priming, suggesting that there are different biochemical reactions occurring in the leaves from the upper portion of the plant other than just accumulation of alkaloids from the roots.

The rate of acylated nornicotine accumulation increased during curing, especially between the end-of-yellowing and the fully cured leaf stages. A similar rate change for accumulation of tobacco-specific nitrosamines was observed in these tobaccos (Djordjevic et al., 1989). This change in rate of accumulation during curing could be the result of chemical reactions that occur more readily as the leaf cell membranes change permeability during curing or it could be associated with microbial activity as in tobacco-specific nitrosamine formation (Parsons et al., 1986; MacKown et al., 1988).

On the basis of observations in this report and the previous report of Djordjevic et al. (1989) on tobacco-specific nitrosamines, the importance of nornicotine in the formation of acylated nornicotines and N-nitrosonornicotine is evident. The patterns of formation of each of the nornicotine derivatives are similar, and it is apparent the nornicotine available per se is more important than the potential of nicotine demethylation for nornicotine formation for subsequent reactions. In these experiments, correlations between the individual acylated nornicotines and NNN were between 0.80 and 0.99, with correlations of total acylnornicotines with NNN 0.94 and 0.98.

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Registry No. Nicotine, 54-11-5; nornicotine, 494-97-3; N'-formylnornicotine, 38840-03-8; N'-acetylnornicotine, 5979-94-2; N'-(n-butanoyl)nornicotine, 69730-91-2; N'-hexanoylnornicotine, 38854-09-0; N'-octanoylnornicotine, 38854-10-3; N'-(hydroxyoctanoyl)nornicotine, 123676-95-9.

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