

Formation of NNK from pseudooxynicotine (PON)

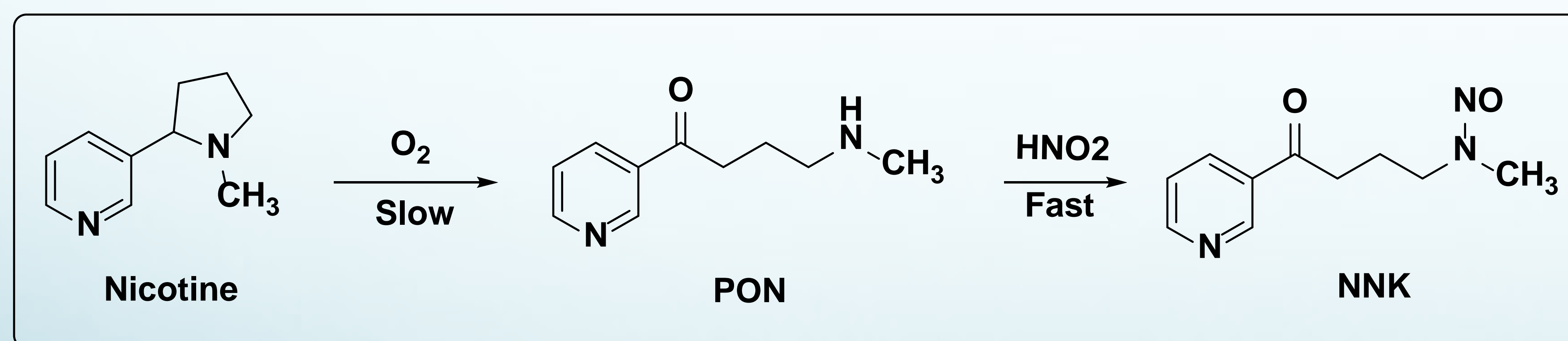
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Abstract

Oxidation of nicotine may result in three nitrosoamines being formed 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-1-butanal (NNA), nitrosornicotine (NNN), and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK generally is considered to be formed from the nitrosation of pseudooxynicotine (PON), an oxidation product of nicotine. PON was found in green tobacco but accumulation of NNK was not detected. The objective of this research was to determine the nitrosation rate of PON to NNK in different conditions. Kinetics of PON nitrosation to NNK was determined at pH levels of 3.0, 5.5, 7.5 at 20°C and 37°C. Measurement of PON and NNK were done by UPLC/MS/MS protocols. Reactions were done in citric acid (0.16M) and disodium phosphate (0.08M) buffers at the three different pH levels. Very little NNK was formed in the two higher pH levels in 24 h. At pH 3 and 20°C with an initial PON of 6.3 nmole ml⁻¹ and 200 μmole ml⁻¹ NO₂⁻ a very rapid formation of NNK was measured. Increased NO₂⁻ from 25 μmole ml⁻¹ to 250 μmole ml⁻¹ increased NNK about 5X with initial PON of 6.3 nmole ml⁻¹ in 30 min at 20°C. When PON was increased from 0.63 to 25.2 nmole ml⁻¹ NNK accumulation was linear over 30 min at 20°C with maximum NNK accumulation of 3.56 nmole ml⁻¹. The reaction was more rapid at 37°C with about 1.5X the NNK formed within 60 min. These results indicate that the generation of NNK is pH dependent and temperature can affect the reaction rate from PON to NNK. These conditions will greatly influence NNK formation and accumulation in the green leaf and during curing.

Introduction

Tobacco-specific nitrosamines (TSNA) are a class of nitrosamines that occur in a wide variety of tobacco-related products and are considered important carcinogens. One of these, NNK 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone is considered to be the most potent carcinogen of the TSNAs. The metabolism and degradation of nicotine during curing of tobacco can cause oxidation of the 2'- carbon, resulting in the formation of 2'-hydroxynicotine (pseudooxynicotine, PON). PON is a key intermediate for formation of NNK. Nicotine nitrosation of the tertiary amine is a very slow reaction to formation of NNK. However, oxidation of nicotine to PON and subsequent nitrosation to NNK may be much more rapid (Scheme 1). We have detected PON in green tobacco, but no accumulation of NNK in the tissue until curing. The objective of this research was to measure PON nitrosation to NNK in vitro to better understand conditions that may enhance NNK formation and accumulation in vivo.



Scheme 1: Formation of NNK from nicotine

Materials and Methods

Chemicals: Pseudooxynicotine (PON), NNK and NNK-d₄ were purchased from Toronto Research Chemicals Inc, Sodium nitrite (NaNO₂), sodium diphosphate (Na₂HPO₄) and citric acid were purchased from Sigma Chemical.

Instruments: Reactions and products were measured with an UPLC-MS/MS equipped with a C18 2.1 x 5 mm guard column with 2.5 μm particle size. Column was 2.1 x 50 mm C18 with 1.7 μm particles.

Procedure: Standard stock solutions were 126 μmole ml⁻¹ PON, 9.5 nmole ml⁻¹ NNK-d₄. PON, NNK-d₄ and NO₂⁻ were added in LC-MS vial with reaction buffer 0.8 M Na₂HPO₄: 0.16 M citric acid (1:1 pH 3, 5.5 and 7.5 by NaOH). Total volume was 1 ml. The reaction time and reagents concentration were variable.

Results and Discussion

For the determination of reactions from PON to NNK, nitrite concentrations ranged from 25 to 250 μmole ml⁻¹ and PON concentrations ranged from 0.63 to 25.2 nmole ml⁻¹ (Fig 1 and Fig 2). Very rapid formation of NNK and disappearance of PON were measured over the nitrite range at pH 3 for 30 min at 20°C. As expected, NNK concentration increased with increased PON concentration (Fig 2). 200 μmole ml⁻¹ nitrite and 6.3 nmole ml⁻¹ PON were selected for further testing. The reaction was rapid with 1.5X NNK formed within 30 min compared to the one min level at 20°C. At 6 h the NNK concentration was 4X the 1 min at 20°C (Fig 3). The reaction was more rapid at 37°C than 20°C for NNK formation. PON concentration decreased rapidly within 30 min in both 20°C and 37°C and was near zero after 6 h at 37°C (Fig 4).

Fig 1. PON loss and NNK formation with NO₂⁻ concentration during 30 min

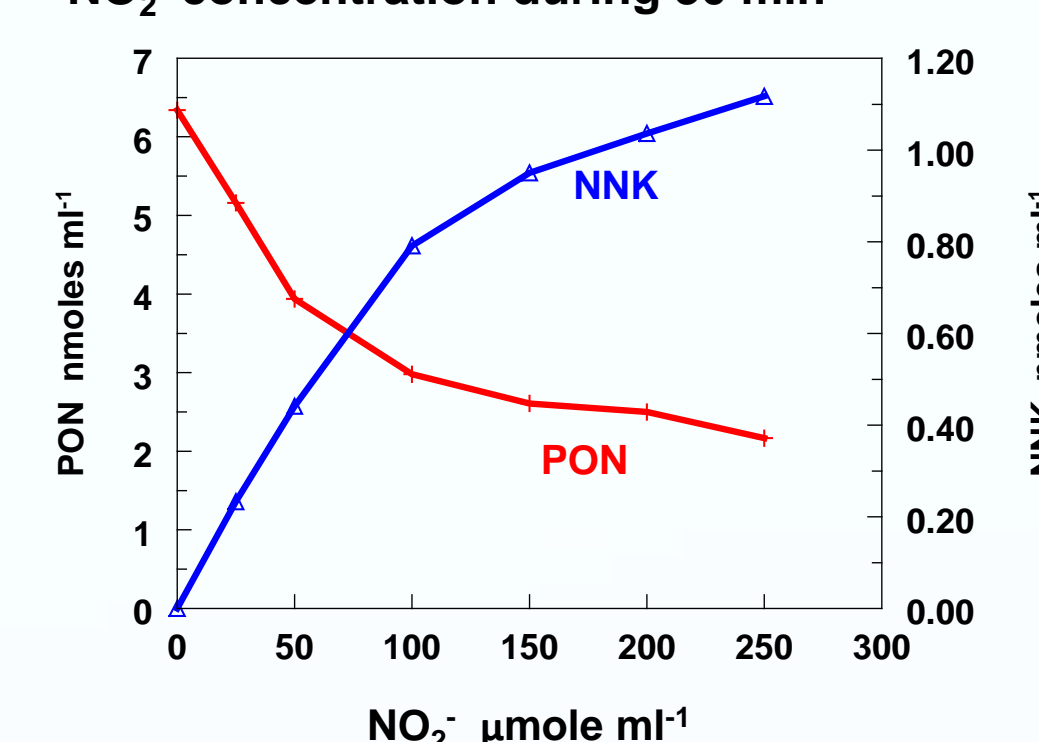


Fig 2. NNK formation from PON during 30 min

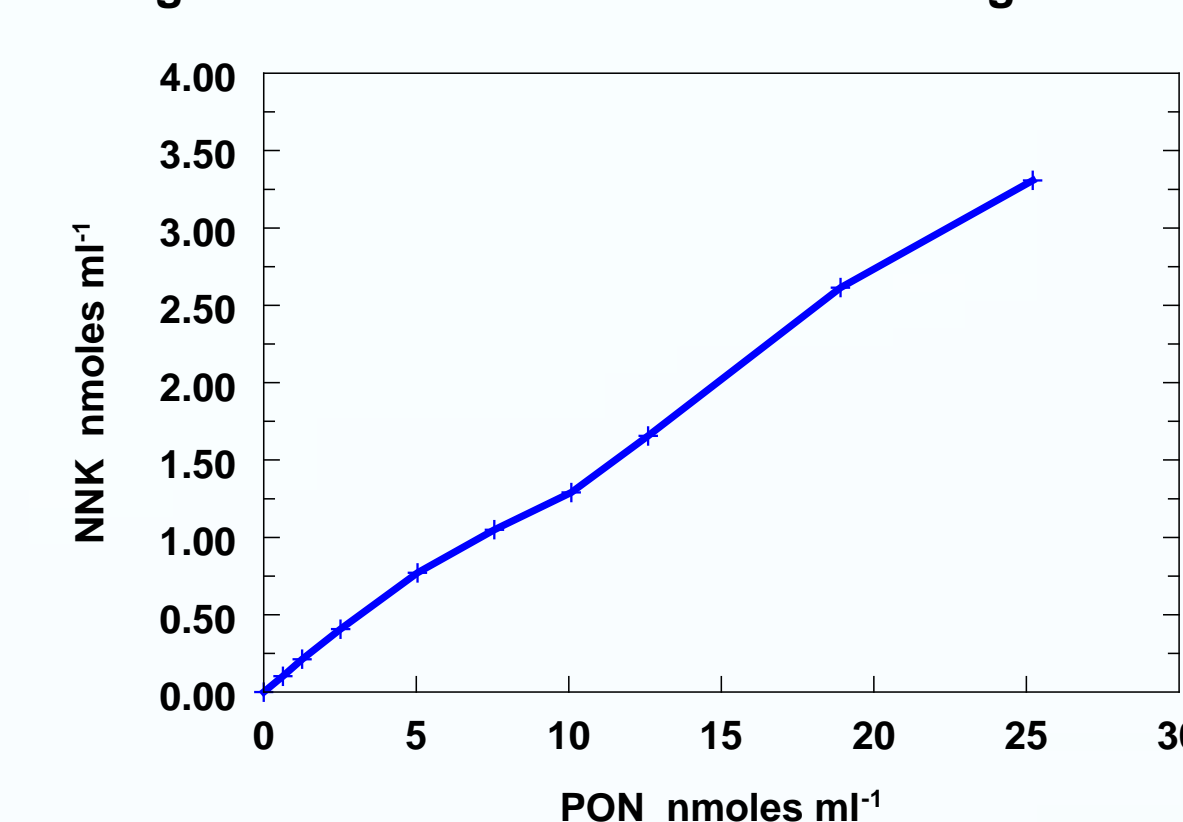


Fig 3. NNK formation at 37C and 20C Initial PON = 6.3 nmole ml⁻¹; NO₂⁻ = 200 μmole ml⁻¹

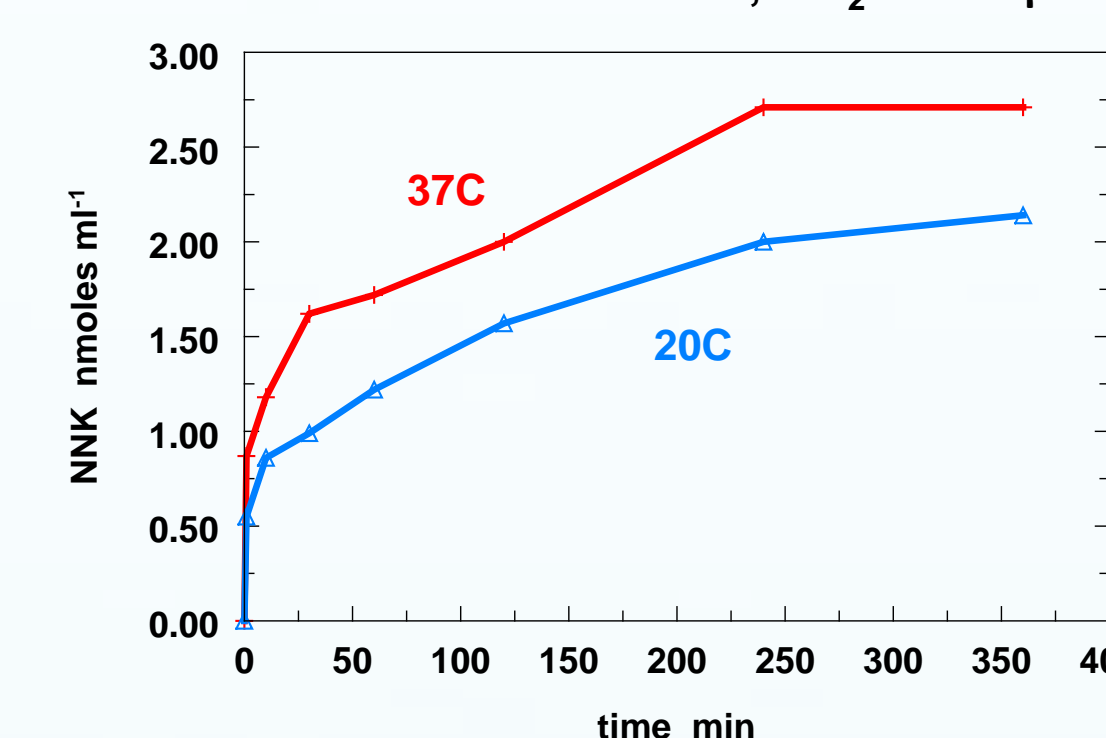


Fig 4. Loss of PON at 37C and 20C Initial PON = 6.3 nmole ml⁻¹; NO₂⁻ = 200 μmole ml⁻¹

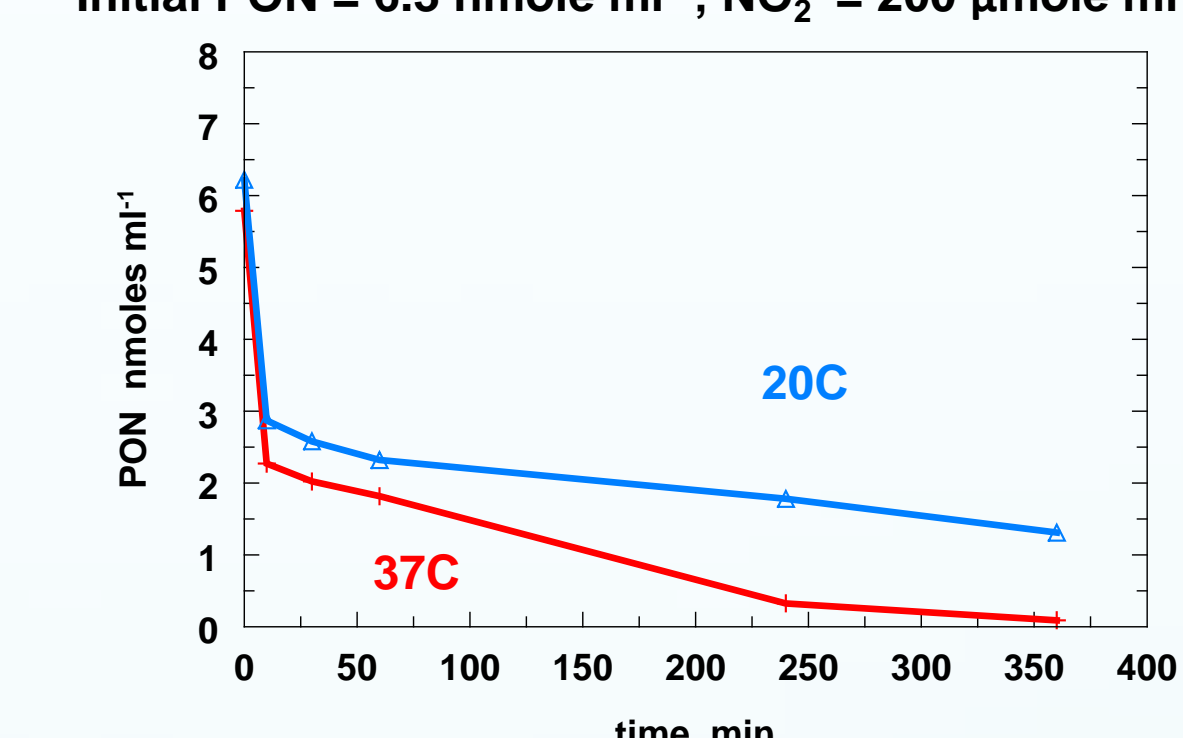


Table 1: NNK formation at 20°C and 37°C, pH 5.5 and 7.5

Time (mins)	NNK (nmole ml ⁻¹)	
	20 °C, pH 5.5	20 °C, pH 7.5
0	0	0.00
0.17	0.00	0.00
0.5	0.00	0.00
1	0.01	0.00
10	0.01	0.00
30	0.01	0.00
60	0.02	0.00
Overnight	0.28	0.02
	37 °C, pH 5.5	37 °C, pH 7.5
10	0.029	0.00
30	0.027	0.00
120	0.125	0.00

Results summarized in Table 1, Fig 1 and Fig 3 indicated that the optimal pH for NNK formation from PON was 3.0. We detected only 0.125 nmole ml⁻¹ NNK formed in pH 5.5 at 37°C and no NNK formation at pH 7.5 at 20°C and 37°C.

Conclusions

We determined nitrite concentration of 200 μmole ml⁻¹ was necessary to utilize all the PON when the PON concentration was fixed on 6.3 nmole ml⁻¹. The formation of NNK from pure PON is more rapid in 37°C than 20°C with 50% formation over 30 mins. The effect of pH on the nitrosation for maximum NNK formation occurred at pH 3. In weak basic condition, PON was not nitrosated at all. These results suggest that at physiological conditions NNK formation from PON may occur slowly.

Acknowledgements

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