Carcinogenic Tobacco-specific N-Nitrosamines in Snuff and in the Saliva of Snuff Dippers

Dietrich Hoffmann and John D. Adams

Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, New York 10595

ABSTRACT

Human data indicate an increased risk for cancer of the oral cavity for snuff dippers. Popular snuff products from the United States, Germany, Sweden, and Denmark were analyzed for tobacco-specific N-nitrosamines (TSNA). These compounds are formed during tobacco processing from nicotine, nornicotine, and anatabine and represent the only known carcinogens that their concentrations in saliva can vary widely between individuals. Efforts should be made to reduce the TSNA in snuff by modifications of the production process and by wrapping individual snuff portions in airtight packets.

INTRODUCTION

A number of retrospective studies in the southern United States have linked oral cancer with chronic snuff use (3, 16, 22, 27). Recently, Winn et al. (29) published a case-control study from North Carolina involving 255 women with oral and pharyngeal cancer. The authors calculated a relative risk of 4.2 to be associated with snuff dipping among white nonsmokers (estimated at 23% of the adult female population in North Carolina), and among chronic snuff dippers the risk approached a 50-fold increase for cancer of the gum and buccal mucosa (29). In a retrospective study on oral cancer from Sweden, which included 33 cases of snuff users, Axell et al. (1) have shown that snuff dipping increases the risk of cancer at the site of direct contact with snuff 5 to 6 fold.

In 1980, Kirkland (14), Russell et al. (23), and Schievebein et al. (24) suggested snuff as a feasible alternative to cigarette smoking. Pindborg and Axelsen (20) agree, pointing out, however, that in Scandinavia and in the southern United States, where snuff is not commonly placed in the nostrils but in the mouth, an increased risk for oral cancer is indicated. This consideration gains importance in view of the increasing popularity of snuff dipping among young people in the United States, Sweden, Denmark, and possibly in other Western countries (4, 17, 21). It has been estimated that in 1975 2.5% of all men and 1.3% of all women in the United States, ages 21 years and older, consumed snuff (26).

Experimental data in support of the carcinogenic potential of snuff were thus far lacking. Bioassays for carcinogenicity were negative when snuff was implanted in hamster cheek pouches (18). Injection of alcohol extracts of snuff in the connective tissue of rats (25) and 20% of snuff in laboratory feed given to hamsters in a lifetime assay had also negative results (12). However, more recently, it has been demonstrated that nicotine, nornicotine, and anatabine give rise to 3 TSNA3 during processing of the tobacco and manufacture of the snuff product (11). These TSNA are NNN, NNK, and NAT (Chart 1). Laboratory evidence suggests that additional quantities of TSNA are also formed in the oral cavity during snuff dipping (7). NNN is a moderately active carcinogen that induces tumors in the lung of mice, carcinoma in the nasal cavity and esophagus of rats, and papilloma in the trachea of Syrian golden hamsters (6, 9, 10). When NNN is given to Fischer rats in the drinking water, it induces primarily tumors of the esophagus (11) but not nasal cavity tumors as upon s.c. injection (6). N'-Nitrosoanabasine, a minor tobacco nitrosamine in cigarette smoke, induces esophageal tumors when given to rats in the drinking water (2). NNN is a relatively strong carcinogen, producing carcinoma of the lung in mice, rats, and hamsters; carcinoma of the nasal cavity in rats and hamsters; and hepatocarcinoma in rats (6, 9, 10). NAT is currently being assayed in rats.

It was the purpose of this study to analyze popular types of snuff from the United States, Germany, Sweden, and Denmark in order to determine the levels of TSNA in these products. The saliva of snuff dippers was also examined for these nitrosamines.

MATERIALS AND METHODS

Snuff. Popular United States snuff was obtained on the open market in New York and in Tennessee. Bavarian snuff was brought in Munich. The leading Swedish snuff was purchased in Umeå, Uppsala, and Lund during the summer of 1980, and the leading Danish snuff was obtained in Copenhagen. The foreign samples were airmailed, and all samples were stored in a cold room (3°) prior to analysis.

Chemicals. NNN, NNK, and NAT were more than 99.5% pure, according to gas chromatography, HPLC, thin-layer chromatography, and mass spectrometry (6, 9). [2'-14C]NNN (18.4 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.) and purified by thin-layer chromatography on silica gel (E. Merck, Darmstadt, Germany) with elution by CHCl3:methanol (15:1).

Analyses. Earlier published methods were applied for the analyses.

1 This study is dedicated to Dr. Ernst L. Wynder, Founder of the American Health Foundation on the occasion of the 10th anniversary of the Naylor Dana Institute for Disease Prevention. This study was supported by Grant PO 1 CA-29560 from the Division of Cancer Cause and Prevention, National Cancer Institute. This is Paper 23 in the series "A Study of Tobacco Carcinogenesis."

2 To whom requests for reprints should be addressed.

Received May 5, 1981: accepted July 29, 1981.

3 The abbreviations used are: TSNA, tobacco-specific N-nitrosamines; NNN, N'-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanoine; NAT, N'-nitrosoanatabine; HPLC, high-performance liquid chromatography.
Specifically, these included the benzene distillation method for moisture determination (28) for which we used 3 g of a representative 100-g sample. The HPLC method for alkaloids (19) was done with a 7-g portion. For the TSNA analysis, we used 30 to 35 g of snuff, which was suspended in 200 to 250 ml of 5 mM ascorbic acid solution at pH 4.5 (citric acid:sodium phosphate buffer) containing 0.5 μg of [2'-14C]NNN (specific activity, 18.4 mCi/mmol) for determining the recovery rate with the isotope dilution method [recoveries of NNN and [2'-14C]NNN are identical (6)]. The analysis was carried out by the HPLC-thermal energy analyzer method (8).

Accuracy of the moisture determination was ±5% (27); the alkaloid determination was within ±5% for nicotine and ±10% for the minor alkaloids (19). TSNA values were determined within ±6% accuracy (8).

In earlier studies, we demonstrated that the extraction of tobacco with a pH 4.5 buffer solution containing 5 mM ascorbic acid prevents the artifactual formation of TSNA (8). In control experiments for this study, we increased nicotine levels 10-fold and nornicotine levels 8-fold by adding 500 mg of nicotine or 10 mg of nornicotine to 2.0 g each of snuff and 100 ml of buffered ascorbic acid solution. Extractions were completed after 24 hr. In another 24-hr experiment, we added 500 mg of nicotine, 10 mg of nornicotine, and 100 mg of sodium nitrite to 2.0 g of snuff in 100 ml of buffered ascorbic acid solution (50 mM ascorbic acid, pH 4.5).

In order to study the possible effect of aging on the TSNA content of snuff, we removed the airtight aluminum envelopes and left the individual snuff portions, in porous paper bags, exposed to open air in the laboratory. After 3, 8, and 14 days, the snuff samples were analyzed for alkaloids and TSNA.

Saliva Collection. Women in 2 furniture companies who have been snuff dippers for at least 10 years were asked to use their own brand of snuff and to place it in their gingival-buccal fold. This first study was begun about 10 a.m. Fifteen min later, the snuff dippers were given a dental plug (uncompressed, diameter =1 cm; length =2 cm), which they kept in the mouth for 1 min. The plug was then removed with forceps, placed in a vial, and weighed and, then =10 ml buffered ascorbic acid solution were added. The vial was then placed in a dry ice container and airmailed for analysis.

In a second study, women in another group volunteered to use a popular United States snuff with known alkaloid and TSNA content. They were asked to dip this product in their own customary manner. After, 15 min, saliva was collected as above. The entire procedure was repeated on the following day. The TSNA in saliva were confirmed by HPLC-thermal energy analyzer and by microreactions as suggested by Krull et al. (15).

Extraction of Alkaloids and TSNA from Snuff. In consideration of the possible differences in the rates of extraction of the alkaloids versus the TSNA from snuff, we conducted a model extraction. Five g of snuff tobacco were placed in 100 ml of a buffer solution (KH2PO4, plus NaOH; at pH 6.4) with 10 mmol of ascorbic acid and [14C]NNN (30,000 dpm) as an internal standard. This mixture was gently stirred by magnetic force, and samples were taken after 5, 10, 20, 30, and 60 min and also 24 hr later. After filtration through Celite, the samples were analyzed for alkaloids and for TSNA (8, 19).

RESULTS AND DISCUSSION

Table 1 lists the analytical data for alkaloids and tobacco-specific N-nitrosamines in popular snuff from the United States, Germany, Sweden, and Denmark. Whereas, in general, the concentrations of N-nitrosamines in environmental samples amount to less than 0.1 ppm (13), the carcinogenic TSNA in snuff ranged from 5.5 to 106 ppm, with 3.5 to 77 ppm of NNN, 0.6 to 7.0 ppm of NNK, and 0.8 to 44 ppm of NAT. These values for TSNA exceed those reported in cigarette tobacco (0.3 to 5 ppm) and cigarette smoke (0.3 to 5 μg/cigarette) (10). A recently introduced United States brand (III) contained only 6.6 ppm of total TSNA, which indicates that a change in manufacturing can lead to reduced TSNA yield. Table 1 also supports the earlier observation that the concentration of the alkaloids, especially of nicotine, is not a rate-determining factor for the TSNA yield in processed tobacco such as snuff (7). Except for the Bavarian snuff, all products analyzed in this study are manufactured and used primarily for snuff dipping.

Results from the 4 popular Swedish snuff brands, which were purchased at about the same time in the summer of 1980 in the north (Umeå), center (Uppsala), and the south of Sweden (Lund), underline the spread which can occur in the concentrations of the TSNA. In Swedish Snuff III, TSNA values range from 15 to 106 ppm. Since the accuracy of the method is ±8% for the individual TSNA (8), the spread reflects actual occurrences. This wide difference could result from deviations in the tobacco or manufacture from batch to batch. "Aging" (temperature, exposure to airborne bacteria, etc.) appears to play a lesser role for the overall variation of the TSNA values, although the increase as seen in Table 2 is statistically significant. Thus, a manufacturing process similar to the one used for United States III and Sweden V, with individual snuff portions wrapped in airtight aluminum foil, could result in a product with reduced levels of carcinogenic N-nitrosamines.

As we reported earlier, the addition of excessive amounts of nicotine, nornicotine, and sodium nitrite to snuff in buffered ascorbic acid, does not measurably increase the TSNA (8). In this study, the addition of the NNN and NNK precursors to the extraction mixture (see "Materials and Methods," under "Analysis") did not result in values different from those for the analysis of the genuine snuff (±8%).

Finally, our earlier studies had indicated that additional amounts of TSNA can be formed during snuff dipping (7). This led us to examine the saliva from women who had been long-term snuff dippers and who were employed in 2 furniture companies in the southern United States. Table 3 shows a wide range in the concentrations of TSNA in the saliva of individual snuff dippers and this indicates that during snuff use the TSNA are extracted from the tobacco plug at varying rates. During daytime, an adult produces about 60 ml of saliva per hr. Thus, using the data of Table 3, we calculated that the women were exposed to 1 to 20 μg of TSNA during 1 hr of snuff dipping.

In the second assay, we gave 4 long-time snuff dippers samples of a specific brand for which alkaloid and TSNA values were known. The NNN:nicotine ratio in this brand of snuff ranged from 1:890 to 1:1040 (we assigned a specific snuff-containing box to each woman). Fifteen min after the volunteers...
Table 1

Carcinogenic Nitrosamines in Snuff and Saliva

Values for NNN, NNK, and NAT are averages of 3 runs; other TSNA values are averages of 2 runs.

<table>
<thead>
<tr>
<th>Snuff origin</th>
<th>Type of packaging</th>
<th>% of alkaloids</th>
<th>Tobacco-specific N-nitrosamines (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>NN</td>
</tr>
<tr>
<td>United States</td>
<td>I</td>
<td>A</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>A</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>B</td>
<td>1.45</td>
</tr>
<tr>
<td>Germany</td>
<td>I Bavaria</td>
<td>C</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>II Bavaria</td>
<td>C</td>
<td>0.54</td>
</tr>
<tr>
<td>Sweden</td>
<td>I Umeå</td>
<td>A</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>II Umeå</td>
<td>A</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>III Umeå</td>
<td>A</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>III Uppsala</td>
<td>A</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>III Lund</td>
<td>A</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>III Umeå</td>
<td>A</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>III Uppsala</td>
<td>A</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>III Lund</td>
<td>A</td>
<td>0.67</td>
</tr>
<tr>
<td>Germany</td>
<td>IV Umeå</td>
<td>A</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>IV Uppsala</td>
<td>A</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>IV Lund</td>
<td>A</td>
<td>1.14</td>
</tr>
<tr>
<td>Sweden</td>
<td>V Umeå</td>
<td>D</td>
<td>2.17</td>
</tr>
</tbody>
</table>

A, waxed paper container with metallic lid (≈50-g package); B, individual portions packaged in paper with 25 portions/plastic container (≈11 g); C, plastic foil-lined aluminum bags containing 100 g; D, individual snuff portion in paper bag packaged in crimped airtight aluminum envelope. There are 10 envelopes in a plastic bag amounting to ≈10 g; E, hard-plastic container.

Table 2

Aging of snuff on open air and formation of tobacco-specific N-nitrosamines

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>NNN (ppm)</th>
<th>NNK (ppm)</th>
<th>NAT (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.73</td>
<td>1.28</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>6.08</td>
<td>1.77</td>
<td>1.10</td>
</tr>
<tr>
<td>8</td>
<td>6.34</td>
<td>1.90</td>
<td>1.17</td>
</tr>
<tr>
<td>14</td>
<td>6.11</td>
<td>2.02</td>
<td>1.47</td>
</tr>
</tbody>
</table>

Table 3

Determination of tobacco-specific N-nitrosamines in saliva of snuff-dipping women

<table>
<thead>
<tr>
<th>Snuff dipper No.</th>
<th>Age</th>
<th>Saliva collected (mg)</th>
<th>Tobacco-specific N-nitrosamines (ng/g saliva)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NNN</td>
<td>NNK</td>
<td>NAT</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
<td>840</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>900</td>
<td>27.1</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>1500</td>
<td>21.7</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>1600</td>
<td>125</td>
</tr>
</tbody>
</table>

* Values are based on dry weight.

had started snuff dipping, we collected the saliva and determined nicotine, NNN, NNK, and NAT. On the following day, the same women were asked to dip the same type of snuff, and the saliva was again collected after 15 min. As Table 4 shows and as one may have expected, we observed not only significant differences in the nicotine and TSNA content of the saliva of several individuals, but also variations in the saliva after an individual's snuff use on different days. The great variations in the TSNA values in saliva are at least partially explained by the differences in the intensities with which individuals practice snuff dipping at different times; perhaps, they are also due to varying rates of salivation. One could have studied these effects in detail. However, the present analyses had the primary goal to demonstrate, with a reproducible method, that the saliva of snuff dippers contains significant amounts of carcinogenic TSNA and that their concentrations can vary widely. We had not expected the decrease but rather anticipated an increase of TSNA in saliva relative to nicotine because of the TSNA formation during snuff dipping. According to a model study, nicotine and TSNA are extracted from the snuff within 5 min. The buffer solution (pH 6.4) extracted from the snuff within 5 min contained 2.8% nicotine, 0.09% nornicotine, 0.03% anatabine, 46 ppm NNN, 9.0 ppm NNK, and 57 ppm NAT. Continued extraction (10, 20, 30, and 60 min and 24 hr) did not change the amount of extracted agents. Therefore, the relative decrease of the TSNA in the saliva is conceivably due to their more rapid absorption from the oral cavity and/or faster degradation during snuff dipping compared to that of nicotine.
Presently, this phenomenon is under detailed study.

In summary, this study presents experimental support for the correlation of snuff dipping with cancer of the gum and buccal mucosa. It has been shown that commercial snuff, as used for snuff dipping in the United States, Sweden, and Denmark, contains relatively high levels of carcinogenic N-nitrosamines (5.5 to 106 ppm). Snuff dipping cannot be exactly duplicated in an animal model. However, this analytical investigation has suggested several bioassays which can measure the relative carcinogenic potential of various snuff products and their TSNA concentrates when given by the oral route. NNN and N'-nitrosoanabasine induce esophageal tumors in rats when given in (5.5 to 106 ppm). Snuff dipping cannot be exactly duplicated in an animal model. However, this analytical investigation has suggested several bioassays which can measure the relative carcinogenic potential of various snuff products and their TSNA concentrates when given by the oral route. NNN and N'-nitrosoanabasine induce esophageal tumors in rats when given in the drinking water (2, 11) and a low incidence of carcinoma of the salivary glands in mice upon s.c. injection of NNN (5). Although snuff dipping does not represent a risk for cancer of the lung as does cigarette smoking, it increases the risk of cancer at the site of direct contact in the oral cavity. New manufacturing methods and airtight wrapping of individual portions appear to offer promising approaches towards reducing the concentrations of tobacco-specific nitrosamines, the only known carcinogens in snuff. It is hoped that additional efforts will be made to reduce those carcinogens in a product that is now increasingly being used by young people and which is recommended by some physicians as a possible alternative to cigarette smoking.

REFERENCES

Carcinogenic Tobacco-specific N-Nitrosamines in Snuff and in the Saliva of Snuff Dippers

Dietrich Hoffmann and John D. Adams


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/41/11_Part_1/4305

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/41/11_Part_1/4305.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.