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Mutagen levels in urine from snuff users, cigarette smokers and non tobacco users — A comparison

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Summary

The mutagenic activity of concentrates of urine from snuff users, cigarette smokers and non tobacco users has been investigated. A concentration procedure involving use of Sep-Pak C_{18} columns and elution with methylene chloride was used. The concentrates were assayed for mutagenicity towards strain TA98 of *Salmonella typhimurium*, both in the presence and absence of a metabolic activation system, the post-mitochondrial liver fraction (S9) from Aroclor 1254 induced rats.

The mean mutagenic activity of smokers' urine concentrates was 8.6×10^3 revertants per 24 h and significantly higher than the corresponding values for snuff users, abstinent snuff users and non tobacco users, which were $(1.3, 1.3 \text{ and } 0.9) \times 10^3$, respectively. No significant difference in mutagenic activity was found between urine from snuff users, whether using or abstaining from snuff, and urine from non tobacco users.

It could thus be concluded that the level of urinary mutagens, isolated by adsorption on Sep-Pak C_{18} columns, is not elevated by habitual usage of Swedish wet snuff.

Mutagens have been detected in urine from cigarette smokers (Yamasaki and Ames, 1977; Jaffe et al., 1983; Kriebel et al., 1985; Putzrath et al., 1981), certain occupational groups (Falk et al., 1979; Dolara et al., 1981), patients receiving chemotherapy (Speck et al., 1976) and individuals on specific diets (Baker et al., 1982; Sasson et al., 1985; Sousa et al., 1985). These findings indicate that a urinary mutagen assay is useful as a rapid screening method for detecting exposure to environmental mutagens (Kriebel et al., 1983). According to Conolly and coworkers (1986) snuff users might be at an increased risk of developing oral cancer. It was, therefore, of interest to investigate if the urine from snuff users, like that of smokers, exhibits elevated levels of mutagens. We have accordingly examined the urine from 8 voluntary snuff users when consuming their regular brand ad libitum and the urine from 6 of them after having been abstinent for 1 week. A modified version of the frequently used method by Yamasaki and Ames (1977) for trapping mutagenic compounds in urine, was used in this study,

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prior to the screening based on *Salmonella typhimurium*. On account of the altered concentration procedure and to allow comparison and correlation with earlier studies, 8 smokers and 6 non tobacco users were also included in the present investigation.

Materials and methods

Materials

The Sep-Pak C_{18} columns (0.8 ml; Millipore^R) were obtained from Waters Associates. Just before use, each column was eluated with acetone (50 ml) and distilled water (50 ml) at a flow rate of 2 ml/min. No mutagenic activity was found in the concentrate of 100 ml of methylene chloride (CH_2Cl_2) eluate from the Sep-Pak C_{18} columns. Acetone and CH_2Cl_2 were of H.P.L.C. grade and purchased from Fisons; dimethyl sulfoxide (DMSO) was of reagent grade and obtained from Merck.

Urine collection

Urine was quantitatively collected for 24 h from 22 male volunteers (21–30 years old, 63–86 kg in weight). The snuff users ($n = 8$) consumed 15–40 g of Swedish wet snuff per day and the smokers ($n = 8$) smoked 15–38 filter cigarettes per day, while the non tobacco users ($n = 6$) did not use any type of tobacco. 6 of the snuff users also collected their urine for 24 h after 1 week of abstinence from snuff.

All the subjects who consumed Swedish wet snuff, deposited the pinch of snuff between the gum and the buccal mucosa or between the gum and the upper lip.

The urine samples (0.9–2.7 l) collected from the subjects were adjusted to pH 7.0, filtered through glass filters, frozen and then stored at −20 °C. Prior to adjustment, the pH of the urine ranged from 5.3 to 6.8. Urinary nicotine and cotinine were determined by high-resolution capillary gas chromatography (Curvall et al., 1982).

Storage of urine samples from snuff users at −20 °C did not affect the mutagenicity of the material, since fresh and stored samples were found to exhibit the same mutagenic activity.

Sample preparation

The urine samples were thawed at room temperature and filtered through glass filters to remove sediments. The urine (1000 ml) was passed over two Sep-Pak C_{18} columns in series at a flow rate of 2 ml/min. Each column was washed with distilled water (100 ml) to remove trapped urine and histidine and CH_2Cl_2 (100 ml) was used to elute the adsorbed material. The CH_2Cl_2 fraction was then extracted with water (25 ml), the organic layer separated, the solvent distilled off at reduced pressure (10 mm Hg, 30 °C) and the residue stored at −20 °C. The urine concentrates were dissolved in DMSO (2.5 ml) just prior to being assayed.

Mutagenicity testing

The urine samples were assayed for mutagenicity using the standard Salmonella reverse mutagenicity test (Maron and Ames, 1983). The samples were examined for their ability to induce frameshift mutations in strain TA98 both with and without the addition of the postmitochondrial liver fraction (S9). Of the available *Salmonella typhimurium* strains, the strain TA98 was selected for two reasons. It has been found to be the one of highest sensitivity to cigarette smokers' urine (Yamasaki and Ames, 1977; Putzrath et al., 1981) and to cigarette smoke condensate (Sato et al., 1977). Moreover, a urine concentrate from a snuff user was assayed using both strain TA98 and TA100, with and without S9, and it did not exhibit any mutagenic activity in either strain. The S9 fraction was obtained from Sprague–Dawley rats pretreated with Aroclor 1254 and the S9 mix was prepared according to Ames (Ames et al., 1975). The urine concentrate samples were tested at 5 doses of urine equivalents (5, 10, 20, 30 and 40 ml) both with and without the addition of 50 µl of S9 per plate. Each sample was assayed in duplicate in two separate experiments. The dose–response data obtained were subjected to linear regression analysis. The significance was tested at 3 different levels, $p < 0.05$, 0.01 and 0.001. The mutagenicity was expressed as the mean value of the number of revertants per ml of urine or the number of revertants per 24-h urine as calculated from the linear part of the dose–response curves obtained from two experiments.

Students' *t*-test for paired observations was used for comparison of mutagenicity data from the 4 different categories of individuals.

Results and discussion

The extraction procedure described by Yamasaki and Ames (1977), coupled with the Salmonella mutagenicity assay, has been widely used to detect mutagens in the urine of cigarette smokers (Yamasaki and Ames, 1977; Jaffe et al., 1983; Kriebel et al., 1985; Putzrath et al., 1981). This method was applied in a preliminary set of experiments in which the mutagenicity of XAD-2 urine concentrates from 2 heavy smokers, 3 snuff users and 1 non tobacco user was tested. The mean number of revertants per 24-h urine was 15.0×10^3 for smokers, 1.8×10^3 for snuff users, whether using or abstaining from snuff, and 1.5×10^3 for the non tobacco user. However, we found this concentration procedure time-consuming, laborious and irreproducible. Moreover, high background levels of mutagenic activity were detected in XAD-2 concentrates of water samples. Therefore, a modified version of the method developed by De Raat and Van Ardenne (1984), in which urinary mutagens were trapped on Sep-Pak C_{18} columns, was introduced.

In order to test the applicability of the Sep-Pak C_{18} concentration procedure for screening of urinary mutagens, 3 concentrates derived from the pooled urine from 1 smoker were assayed. The amounts of organic material recovered from the Sep-Pak C_{18} columns were consistent for the 3 samples as were the number of induced revertants per ml of urine, i.e. 5.1, 5.3 and 4.9, respectively. Since these results indicated that this assay is well suited for screening of urinary mutagens, it was used throughout this study. Dose–response curves were obtained for urine samples from 8 users of Swedish wet snuff, during habitual usage and from 6 of them after 1 week of abstinence, and from 8 smokers and 6 non tobacco users.

Representative curves derived from data for one subject from each category of individuals are shown in Fig. 1(a–d).

The mutagenic activity was calculated from two independent experiments as the mean of the regression coefficients of the linear portion of the curves. The number of his^+ revertants of *Salmonella typhimurium* strain TA98 was expressed per 24-h urine to correct for differences in urinary flow between individuals. Mutagenic activity of the urine samples was found only in the presence of S9. Mutagenicity data for urine samples obtained from each subject are presented in Fig. 2. Mean mutagenicity values for the urine concentrates from the 4 groups of individuals expressed both as revertants per ml of urine and as revertants per 24-h urine are depicted in Table 1. Urine samples obtained from smokers all showed a significant ($p < 0.001$) mutagenic effect and they were within the range $(4.2–17.6) \times 10^3$ revertants per 24-h urine. This is in good agreement with the data reported by others (Yamasaki and Ames, 1977; Putzrath et al., 1981; Recio et al., 1982). A significant mutagenic effect was also detected in some of the urine sample concentrates from non tobacco users and from snuff users, both during habitual usage and after 1 week of abstinence. However, the mutagenic activities of these samples were far lower than those from smokers and were within the range $(0.2–1.5) \times 10^3$ revertants per 24-h urine. Since there was no difference in mutagenic activity between the urine from non tobacco users and snuff users, whether using or abstaining from snuff, the small effect found in some of the urine samples might be

TABLE 1

MUTAGENIC ACTIVITY OF URINE CONCENTRATES FROM SMOKERS, SNUFF USERS AND NON TOBACCO USERS TOWARDS SALMONELLA STRAIN TA98 WITH THE ADDITION OF S9

Subjects	Revertants per ml of urine			Revertants per 24 h (10^3)		
	Mean	S.D.	Range	Mean	S.D.	Range
Smokers ($n = 8$)	6.5	2.7	4.2–12.8	8.6	4.5	4.2–17.6
Snuff users ($n = 8$)	0.9	0.4	0.3– 1.5	1.3	0.8	0.3– 2.5
Abstinent snuff users ($n = 6$)	0.8	0.3	0.4– 1.1	1.3	0.7	0.5– 2.4
Non tobacco users ($n = 6$)	0.5	0.2	0.2– 0.9	0.9	0.7	0.4– 2.2

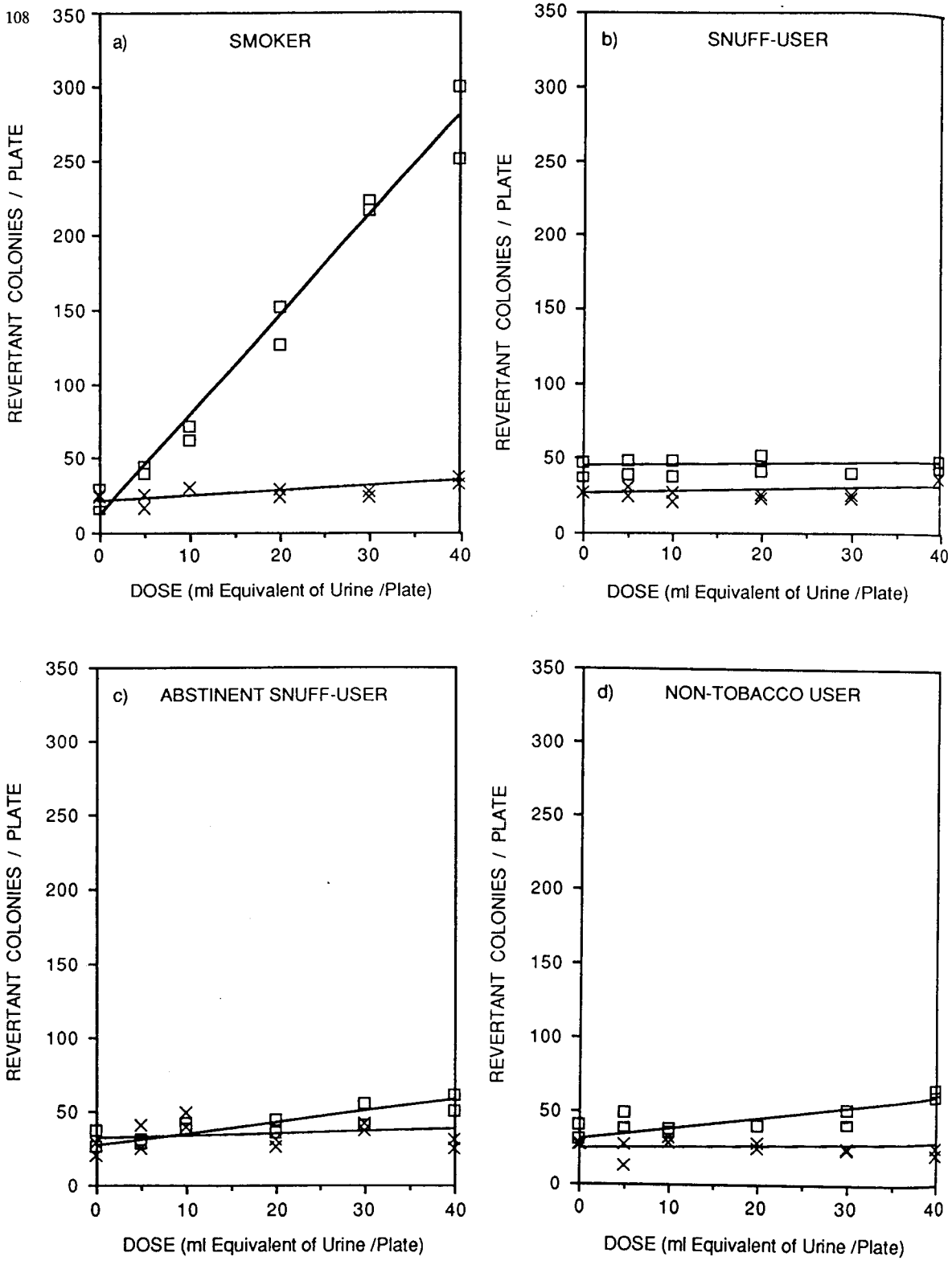

Fig. 1. Representative dose–response curves of urine concentrates from a smoker (a), a snuff user (b), an abstinent snuff user (c) and a non tobacco user (d), tested on *Salmonella typhimurium* strain TA98 both in the presence (□) and in the absence (×) of a metabolic activation system (S9).

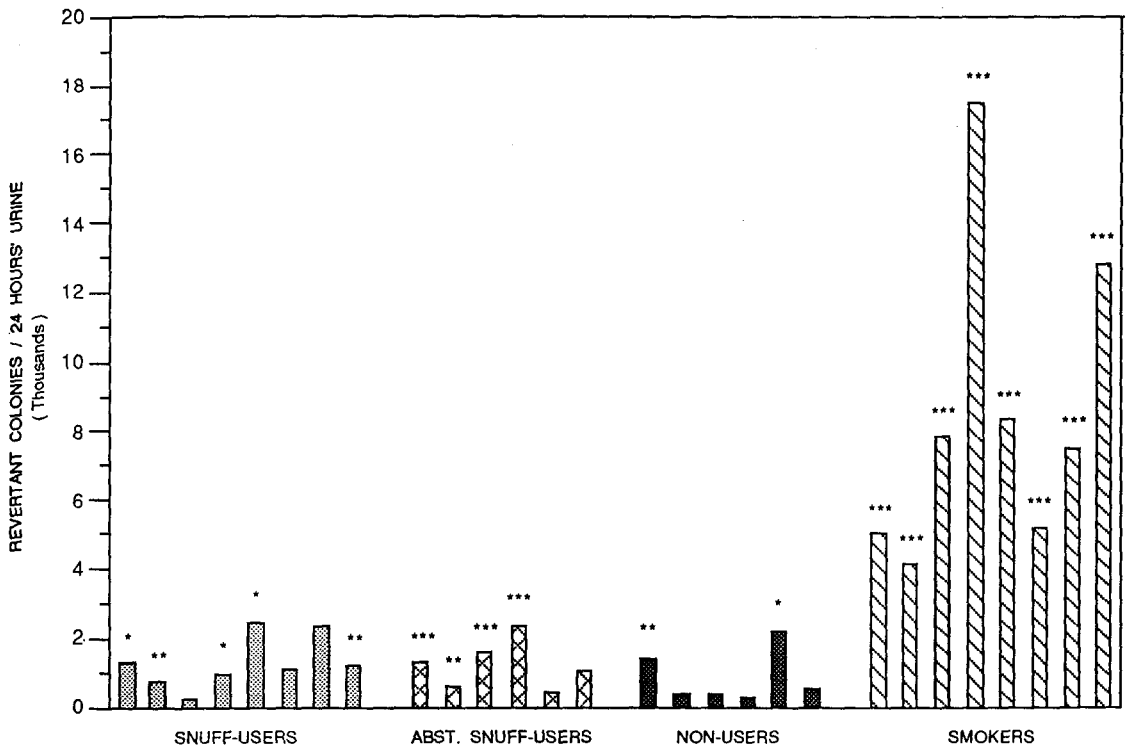

Fig. 2. Mutagenic activity of urine concentrates from 8 snuff users, 6 abstinent snuff users, 6 non tobacco users and 8 smokers, measured by using *Salmonella typhimurium* TA98 with metabolic activation. The 6 abstinent snuff users are identical to the subjects represented by the first 6 bars. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

attributed to other sources, such as dietary factors (Baker et al., 1982; Sasson et al., 1985).

The mean urinary levels of nicotine and cotinine in smokers, snuff users and non tobacco users are summarized in Table 2. There was no significant difference in urinary levels of nicotine and cotinine between snuff users and smokers or between abstinent snuff users and non tobacco users. As can be seen in Table 2, the amounts of nicotine and cotinine excreted during 24 h by abstinent snuff users and non tobacco users were less than one hundredth of the amount excreted by smokers and snuff users. It could thus be concluded, that in the case of smoking, normal consumer levels of nicotine are accompanied by elevated urinary levels of mutagens, while no such increase in urinary

TABLE 2

MEAN URINARY LEVELS OF NICOTINE AND COTININE IN SMOKERS, SNUFF USERS AND NON TOBACCO USERS

Subjects	Urinary nicotine		Urinary cotinine	
	mg/l	mg/24 h	mg/l	mg/24 h
Smokers ($n = 8$)	1.67 (1.04) *	2.04 (1.17)	2.44 (1.29)	3.15 (1.76)
Snuff users ($n = 8$)	1.39 (1.11)	2.00 (1.77)	1.46 (1.13)	2.12 (1.39)
Abstinent snuff users ($n = 6$)	0.009 (0.006)	0.015 (0.010)	0.014 (0.007)	0.020 (0.010)
Non tobacco users ($n = 6$)	0.005 (0.002)	0.008 (0.002)	0.006 (0.005)	0.008 (0.005)

* Mean (standard deviation).

mutagens is observed when the same nicotine levels are reached on consumption of Swedish wet snuff.

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