Mutation Research, 261 (1991) 101–115

ADONIS 016512189100137A

MUTGEN 01699

Genotoxicity testing of extracts of a Swedish moist oral snuff

Tommy Jansson [1], Lennart Romert [1,2], Jan Magnusson [2] and Dag Jenssen [1,2]

[1] *Genotox AB, S-104 62 Stockholm and* [2] *Department of Genetic and Cellular Toxicology, Wallenberg Laboratory, Stockholm University, S-106 91 Stockholm (Sweden)*

(Received 26 November 1990)
(Revision received 25 March 1991)
(Accepted 5 April 1991)

Keywords: Snuff; Genotoxicity

Summary

The present study was designed to investigate the potential genotoxicity of aqueous and methylene chloride extracts of Swedish moist oral snuff. The test systems were selected to provide optimal data for the prediction of carcinogenicity in rodents and included assays for the induction of mutation in bacteria, sister-chromatid exchanges (SCE) in human lymphocytes, of chromosome aberrations and gene mutations in V79 Chinese hamster cells and of micronuclei in mouse bone marrow cells. In addition, the methylene chloride extract was tested for the induction of sex-linked recessive lethal mutations in *Drosophila melanogaster*.

The aqueous extract of 'Snus' induced SCE in human lymphocytes and chromosome aberrations in V79 cells, the latter effect being observed both with and without metabolic activation. No induction of point mutations was detected with the Ames test or in V79 cells and the micronucleus test in mice was negative. It was demonstrated that the induction of chromosome aberrations without metabolic activation may be due to a high salt concentration, indicating that the clastogenic agent(s) in this extract required metabolic activation. The methylene chloride extract showed genotoxicity in the Ames test, the SCE test and the chromosome aberration test, whereas no induction of gene mutations in V79 cells was observed. Once again, the results suggested that metabolism is required for genotoxicity. The methylene chloride extract did not cause induction of micronuclei in mice or of sex-linked recessive lethal mutations in *Drosophila melanogaster*.

These combined data on genotoxicity were analyzed using various models for the prediction of carcinogenicity. In a sequential testing model, the probabilities that the aqueous and methylene chloride extracts of 'Snus' are carcinogenic due to a genotoxic mechanism were both predicted to be low. Using carcinogenicity prediction by battery selection (CPBS), the probabilities of the methylene chloride and aqueous extracts being correctly identified as non-carcinogens are 71 and 77%, respectively. Up to date, the CPBS approach has been validated primarily for individual compounds, so some caution should at present be exercised in interpreting the results using this method.

Based on these results, the carcinogenic potential of Swedish 'Snus' should be considered to be low, a conclusion in agreement with the low incidence of oral cancer in Sweden compared to other countries.

Correspondence: Dr. T. Jansson, Genotox AB, Box 17051, S-104 62 Stockholm (Sweden).

Many different kinds of snuff are used around the world. The potential health impact of certain of these has been discussed, primarily in terms of their suggested carcinogenic properties (US Department of Health and Human Services, 1986; International Agency for Research on Cancer, 1985; World Health Organization, 1988). However, it is essential to distinguish between the different types of products. The present study concerns a non-fermented Swedish moist snuff called 'Snus', which is usually placed between the upper gum and the oral mucosa.

Although this is the first investigation on the genotoxicity of Swedish 'Snus', several other types of snuff have already been investigated in this respect. Aqueous extracts of American moist snuff have been shown to induce mutations in the Ames test (Guttenplan, 1987). Whong and coworkers (1984) found that an organic solvent extract of American dipping tobacco was mutagenic in the Ames test, but only when the pH of the extract was adjusted to 3. Increased frequencies of micronuclei in cells exposed to aqueous and saliva extracts of various smokeless tobacco products have been reported (Stich, 1986). Extracts of masheri, a pyrolysed tobacco product used in India which contains high levels of *N*-nitrosamines and polycyclic aromatic hydrocarbons (PAH), were positive in the Ames test and induced gene mutations in the HPRT locus of V79 Chinese hamster cells and micronuclei in mice (Bhide et al., 1987).

In the present study a combination of test systems for genotoxicity was chosen to provide optimal data for the prediction of carcinogenicity in rodents. The use of 'Snus' may lead to exposure to hydrophilic compounds extracted by the saliva and to local exposure to hydrophobic compounds dissolved in the oral mucosa by direct contact with 'Snus'. Therefore, the Swedish 'Snus' was extracted both with water and with methylene chloride, and these extracts were tested for induction of point mutations in the Ames test, of sister-chromatid exchanges (SCE) in human lymphocytes, of chromosome aberrations and gene mutations in V79 Chinese hamster cells and of micronuclei in mouse bone marrow cells. In addition, the methylene chloride extract was tested for the induction of sex-linked recessive lethal mutations in *Drosophila melanogaster*.

Materials and methods

Preparation of extracts of Swedish 'Snus'

Extraction with methylene chloride. Portions (50 g) of Swedish 'Snus' were extracted with 300 ml methylene chloride (FSA Laboratory Supplies, HPLC grade) in a Soxhlet extraction apparatus for 4 h. Extraction of 6 separate portions was performed. The efficiency and reproducibility of the extraction procedure were evaluated by measuring the nicotine content in an aliquot of each extract. After extraction, the solvent was distilled off at reduced pressure (10 mm Hg, 25°C). The absence of CH_2Cl_2 was confirmed by ^{1}H-NMR. For biological testing of the methylene chloride extract, 2 stock solutions were prepared in ethanol (100 mg/ml and 200 mg/ml). These were further diluted as described for each test system.

Aqueous extraction. A slurry of 100 g Swedish 'Snus' in 300 ml water (pH 8.4) was agitated for 1 h in an ultrasonic bath and then centrifuged for 30 min (4500 rpm). Extraction of 5 separate samples was performed and these were then pooled into a single batch. The efficiency and reproducibility of the extraction procedure were evaluated by measuring the nicotine content of each extract. The solution was then concentrated 6.7-fold under reduced pressure in a Speed Vac Concentrator (Savant Instruments Inc.). The pH of this concentrate was 7.9. The concentrate was further diluted as described for each test system.

Aqueous extraction of non-salted snuff. A sample of 'Snus' from a batch without any content of sodium chloride was prepared using a method identical to that used for the extraction with water described above. This non-salted aqueous extract of 'Snus' was diluted as described below in connection with the chromosome aberration test.

Chemical analysis

The contents of tobacco-specific nitrosamines in the various extracts were determined by gas chromatography with TEA detection, using a modification of the procedure by Hoffmann et al. (1987). The nicotine contents of the snuff samples

were determined spectrophotometrically after enrichment by steam distillation according to the standard ISO procedure (ISO, 1977). The nicotine concentrations of the extracts were determined as follows: an appropriate quantity of *N*-methylanabasine was added as an internal standard to 20 μl methylene chloride extract and nicotine was subsequently quantitated using a gas chromatograph (Varian Model 3400) equipped with a fused silica capillary column (DB-1, J&W Scientific, Inc., 60 m) and a flame ionization detector. Plotting of the chromatograms and integration of the peak areas were performed by a Shimatzu Model C-R3A plotter/ integrator (Curvall et al., 1982).

Ames test

Strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* (Ames et al., 1975; Maron and Ames, 1984) were grown in Oxoid nutrient broth. Revertants were scored on glucose minimal-salt medium. The experimental procedure was as described by Maron and Ames (1984) and followed OECD (1981) guidelines as detailed in Protocol 471. The methylene chloride extract (stock solution containing 100 mg residue/ml) was diluted with ethanol and the aqueous extract diluted with phosphate buffer and added in a quantity of 0.1 ml. 2-Aminoanthracene (all strains and S9 mix), quercitine (TA98 without S9 mix), sodium azide (TA100 and TA1535 without S9 mix) and 9-aminoacridine (TA1537 without S9 mix) were used as positive controls in these experiments. Linear regression analysis was employed for statistical analysis of the data obtained. A positive slope of the dose–response curve at a significance level of $p < 0.05$ in any of the tester strains and in 2 independent experiments was used as the criterion for genotoxicity of the extracts.

Chromosome aberration test

The experimental procedure for the chromosome aberration test followed OECD (1981) guidelines as described in Protocol 473. V79 Chinese hamster cells were seeded at a density of 10^4 cells/cm^2 and propagated at 37 °C in Eagle's modified minimum essential medium with Hanks' salt (HMEM) supplemented with 2 mM L-glutamine, 88 μg/ml streptomycin, 88 IU/ml penicillin and 9% fetal bovine serum (FBS). The cells were cultured for 24 h before addition of the extracts. Each extract was tested in the presence and absence of S9 mix for 2 h. Prior to addition to the cell cultures, the stock solution of the methylene chloride extract (100 mg/ml) was diluted in ethanol. For dilution of the aqueous extract, Hanks' balanced salt solution (HBSS) was used. The S9 mix, containing 10 mM $MgCl_2$, 30 mM KCl, 5 mM glucose-6-phosphate and 4 mM NADP in phosphate buffer (pH 7.8) and 10% S9 from rats induced with Aroclor 1254, was diluted 5-fold in HBSS. The S9 was prepared as described by Ames et al. (1975). After treatment, the cells were washed with HBSS and cultured as described above for another 18 h. Metaphases were collected after 4 h of colchicine treatment (50 ng/ml) and the cells were consecutively hypotonized, fixed, prepared on slides and stained as previously described (Jansson et al., 1986; Jansson and Zech, 1987). The frequencies of chromatid- and chromosome-type aberrations as well as of gaps were scored and recorded from coded slides. 100 cells were analyzed for each set of experimental conditions. The frequency of aberrant cells, gaps excluded, was used for subsequent evaluation. Methyl methanesulfonate (MMS) and cyclophosphamide were used as positive controls in experiments without and with metabolic activation by S9 mix, respectively. A significantly increasing slope of the dose–response curve ($p < 0.05$), as determined by linear or quadratic regression analysis, or a significant increase in the frequency of aberrant cells in both replicates at any of the test concentrations as determined by a chi-square analysis, was used as the criterion for genotoxicity.

Sister-chromatid exchange test

The SCE test followed in principle the UKEMS guidelines and was performed according to Jansson et al. (1986), with the modification that 72 IU penicillin/ml and 72 μg streptomycin/ml were added to the cultures. Prior to addition to the cell cultures, the stock solution of the methylene chloride extract (100 mg/ml) was diluted in ethanol. For dilution of the stock solution of the aqueous extract, HBSS was used. 25 cells were

analyzed for each experimental condition. Styrene-7,8-oxide was used as a direct-acting positive control, while (−)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene (BP-7,8-diol) was used to investigate metabolic activation. A significant ($p < 0.05$) increase in the slope of the dose–response curve, as determined by linear regression analysis, was the criterion for genotoxicity.

V79 / HPRT gene mutation test

The experimental procedure for testing for mutations in the HPRT locus followed OECD guidelines (Protocol 476). V79 Chinese hamster cells were seeded at a density of 2×10^4 cells/cm^2 and propagated at 37 °C in HMEM supplemented with 2 mM L-glutamine, 88 μg streptomycin/ml, 88 IU penicillin/ml and 9% FBS. After 24 h, the cells were incubated for 4 h in the absence or presence of S9 mix. Prior to addition to the cell cultures, the stock solution of the methylene chloride extract (200 mg/ml) was diluted in ethanol. For dilution of the stock solution of the aqueous extract, HBSS was used. The S9 mix, containing 10 mM $MgCl_2$, 30 mM KCl, 5 mM glucose-6-phosphate and 4 mM NADP in phosphate buffer (pH 7.8) and 10% S9 from rats induced with Aroclor 1254, was diluted 5-fold in HBSS. The S9 was prepared as described by Ames et al. (1975). The cells were washed and cultured to allow recovery for another 24 h before being reseeded for expression followed by selection of the mutant phenotype and determination of cytotoxicity according to Jenssen (1984). Dimethylnitrosamine (DMN) and ethyl methanesulfonate (EMS) were used as positive controls in experiments with and without activation by S9, respectively. Linear regression analysis was used for the statistical analysis of the results. A positive result was indicated by a significant ($p < 0.05$) increase in the slope of the dose–response curve in two independent experiments.

Micronucleus test in mice

The experimental procedure for the micronucleus test in mice followed OECD guidelines (Protocol 474). The method has been described by Jenssen (1982). CBA mice of both sexes were treated with the extracts by the oral route. The stock solution of the methylene chloride extract (100 mg/ml) was diluted with ethanol/water (2.4%) and the stock solution of the aqueous extract with water. Sublethal doses, as determined from a preliminary toxicity test, were administered. Five animals of each sex were sampled 24, 48 and 72 h after each treatment. EMS was used as a positive control. Bone marrow from the femur was prepared and suspended in equal parts of phosphate buffer (pH 7.0) and FBS. The cell suspensions were smeared on slides, which were air-dried and fixed in methanol. The slides were subsequently stained with Giemsa–May Grünwald and mounted in Euparal. 1000 polychromatic erythrocytes per animal were analyzed for micronuclei and the ratio of polychromatic to normochromatic erythrocytes was determined on the basis of 200 cells. Linear regression analysis was used for statistical evaluation of the results. A positive result was indicated by a significant ($p < 0.05$) increase in the slope of the dose–response curve.

Sex-linked recessive lethal mutation test in Drosophila melanogaster

The experimental procedure for the sex-linked recessive lethal mutation test followed OECD guidelines (Protocol 477). The test was performed as described in detail by Würgler et al. (1984). Wild-type male flies of the Karsnäs 60 strain were fed 1000 and 2000 ppm of the methylene chloride extract dissolved in 5% sucrose in water. These doses were non-lethal to the flies, whereas no males survived 4000 ppm. The procedure to analyze recessive lethal mutations followed in principle the description by Magnusson and Ramel (1978). The total number of chromosomes tested was 1728 in the treated groups and 1536 in the control. MMS was used as a positive control. Fisher's exact probability test was used for the statistical evaluation and a significant ($p < 0.05$) increase in recessive lethal mutations under any of the experimental conditions compared to the controls was considered to be a positive effect.

Results

Chemical analysis

The average yield of material per gram of Swedish 'Snus' obtained by methylene chloride

extraction was 28.4 mg (SD = 1.9) and the average yield of nicotine was 9.1 mg (SD = 1.1), which corresponds to an extraction efficiency of 88% for nicotine. The concentrations of extracted material, nicotine and tobacco-specific *N*-nitrosamines (TSNA) in the 2 stock solutions were 100/200 mg, 32/64 mg and 30.8/61.6 μg per ml ethanol, respectively.

The average concentration of nicotine in the aqueous extracts was 2.4 (SD = 0.25) mg/ml, which corresponds to an extraction efficiency of 57% for nicotine. 9% of the nicotine was lost during the concentration step. The stock solutions obtained after concentration contained 14.2 mg nicotine/ml and 16.5 μg TSNA/ml.

Genotoxicity testing

The results of the different tests with the aqueous and methylene chloride extracts are summarized in Tables 1 and 2, respectively. The

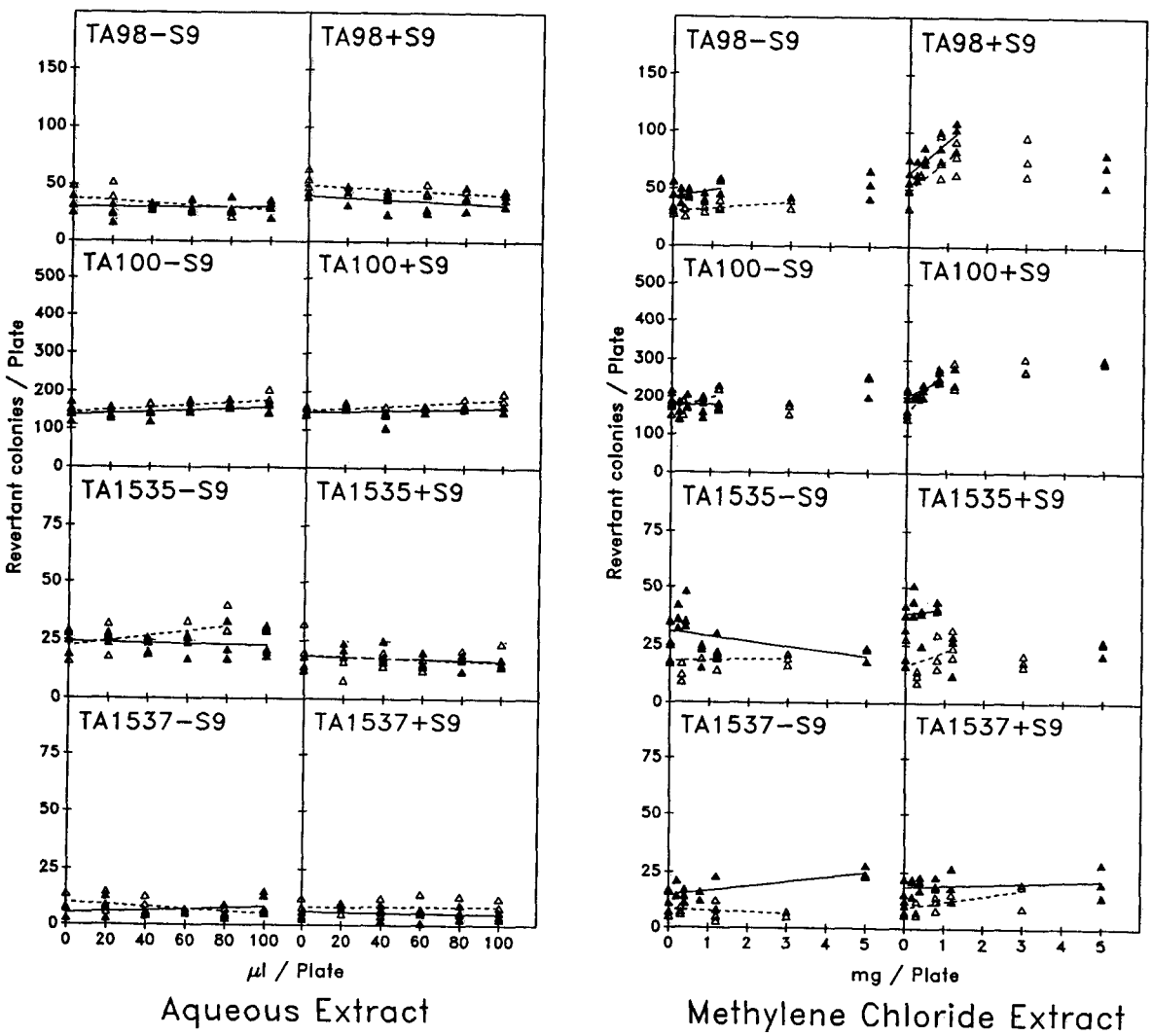

Fig. 1. Linear regression analysis of the dose–response data of an aqueous extract or a methylene chloride extract of 'Snus' on Salmonella strains TA98, TA100, TA1535 and TA1537 in the absence and presence of S9 mix. The broken line and open symbols represent experiment 1 and the solid lines and symbols experiment 2.

tables also include the results of the statistical analyses performed as described under Materials and methods. Figs. 1–6 present graphs of the data. The results are considered in greater detail in the sections describing the different test assays below. All tests were extended up to toxic con-

TABLE 1

STATISTICAL DATA FROM SHORT-TERM TESTS OF AQUEOUS EXTRACTS OF 'SNUS'

Test system	Statistical method [a]	t (χ^2, F)	$d.f.$	Significance [b]	Correlation coefficient
Ames test					
Expt. 1					
TA98 − S9	lin reg	−0.01	16	NS	0.00
TA98 + S9	lin reg	−1.55	16	NS	−0.36
TA100 − S9	lin reg	1.93	16	NS	0.43
TA100 + S9	lin reg	1.16	16	NS	0.28
TA1535 − S9	lin reg	−0.40	16	NS	−0.10
TA1535 + S9	lin reg	−0.81	16	NS	−0.20
TA1537 − S9	lin reg	1.02	16	NS	0.25
TA1537 + S9	lin reg	−0.58	16	NS	−0.14
Expt. 2					
TA98 − S9	lin reg	−1.94	16	NS	−0.44
TA98 + S9	lin reg	−2.17	16	NS	−0.48
TA100 − S9	lin reg	3.62	16	**	0.67
TA100 + S9	lin reg	3.44	16	**	0.65
TA1535 − S9	lin reg	2.14	13	NS	0.51
TA1535 + S9	lin reg	−0.74	16	NS	−0.18
TA1537 − S9	lin reg	−2.75	16	NS	−0.57
TA1537 + S9	lin reg	0.08	16	NS	0.02
HPRT test					
Expt. 1					
− S9	lin reg	1.63	10	NS	0.46
+ S9	lin reg	0.85	10	NS	0.26
Expt. 2					
− S9	lin reg	1.44	10	NS	0.41
+ S9	lin reg	−1.41	10	NS	−0.41
Chromosome aberration test					
− S9	lin reg	3.14	9	*	0.72
	chi sq	$\chi^2 = 5.29$/ 13.15	1/1	*/*** [c]	
+ S9	lin reg	4.89	8	**	0.87
	chi sq	$\chi^2 = 12.58$/ 22.75	1/1	***/*** [c]	
Chromosome aberration test using extract without salt					
− S9	lin reg	2.10	8	NS	0.60
	chi sq	$\chi^2 = 4.30$/ 0.16	1/1	*/NS [c]	
+ S9	lin reg	2.14	8	NS	0.60
	chi sq	$\chi^2 = 27.08$/ 1.65	1/1	***/NS [c]	
	quad reg	$F = 4.85$	2/7	*	0.76

TABLE 1 (continued)

Test system	Statistical method [a]	t (χ^2, F)	$d.f.$	Significance [b]	Correlation coefficient
SCE test	lin reg	3.37	10	**	0.77
Micronucleus test					
24 h					
males	lin reg	1.18	18	NS	0.27
females	lin reg	−0.88	18	NS	−0.20
48 h					
males	lin reg	−0.66	18	NS	−0.15
females	lin reg	−0.62	18	NS	−0.14
72 h					
males	lin reg	0.00	18	NS	0.00
females	lin reg	−0.96	18	NS	−0.22

[a] lin reg, linear regression analysis; chi sq, chi square analysis; quad reg, quadratic regression analysis.
[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[c] Significance of the 2 replicate points showing the largest effect.

centrations, with the exception of the Ames test employing the aqueous extract. Estimation of toxicity in the tests for induction of chromosome aberrations and SCE was based on measurement of the mitotic index, whereas in the test for gene mutations in V79 Chinese hamster cells toxicity was monitored as a decrease in cell survival compared to control groups. Preliminary tests for lethality were performed prior to the selection of appropriate doses for the in vivo assays. The positive control groups in the different experiments invariably exhibited positive results (data not shown).

Ames test. 100 μl of the undiluted aqueous extract was non-toxic to the Salmonella bacteria. This was the highest dose that could be tested due to limitations in the procedure for concentrating this extract. 100 μl of the undiluted extract per plate corresponds to 0.22 g 'Snus'. Negative results were obtained in all 4 tester strains used (Fig. 1 and Table 1).

With the methylene chloride extract the tests were performed using doses approaching toxic levels. As indicated in Fig. 1 and Table 2, a positive result was obtained in both experiments using strains TA98 and TA100, but only in the presence of a metabolizing system. A positive effect was obtained in strain TA1537 in 1 of the 2 experiments (Table 2), indicating that the mutagenicity of the extract was close to the level of detection using this strain. These results demonstrated a clear mutagenic effect of the methylene chloride extract in the Ames Salmonella/microsome assay.

Induction of chromosome aberrations in V79 Chinese hamster cells. The aqueous extract induced chromosome aberrations in V79 Chinese hamster cells in the presence as well as the absence of S9 mix (Fig. 2, Table 1). Since high osmolality or ionic strength has been shown to induce chromosome aberrations (Ashby and Ishidate, 1986; Galloway et al., 1987), the influence of the salt content in the 'Snus' extract was investigated. The result of the test on an aqueous extract from 'Snus' without added salt demonstrated that the positive effect obtained without metabolic activation could be explained by the presence of salt in 'Snus', whereas the activity arising from metabolic activation by S9 mix was also present in the extract without salt (Fig. 2 and Table 1).

The methylene chloride extract induced a low, but significant level of chromosome aberrations, but only in the presence of S9 mix (Fig. 2, Table 2).

Induction of sister-chromatid exchanges in human lymphocytes. Fig. 3 illustrates a highly significant (Tables 1 and 2) and dose-related increase in sister-chromatid exchange in human

lymphocytes in the presence of both the aqueous and the methylene chloride extracts of 'Snus'.

Induction of mutations in the HPRT locus of V79 Chinese hamster cells. The methylene chloride extract was approximately 3 times less cytotoxic in the presence than in the absence of S9 mix. Consequently, this extract could be administered in 3-fold higher doses in the presence of S9 mix. Only doses demonstrating a survival rate of up to 50% were analyzed as suggested by Jenssen (1984). Neither the aqueous nor the methylene chloride extract induced gene mutations in the HPRT locus of Chinese hamster cells, either in the presence or in the absence of S9 mix (Fig. 4, Tables 1 and 2).

Micronucleus test. In the micronucleus test the highest doses of both extracts tested were about 80% of the lethal dose. However, these

TABLE 2

STATISTICAL DATA FROM SHORT-TERM TESTS OF THE METHYLENE CHLORIDE EXTRACT OF 'SNUS'

Test system	Statistical method [a]	t (χ^2, p)	$d.f.$	Significance [b]	Correlation coefficient
Ames test					
Expt. 1					
TA98 − S9	lin reg	1.47	13	NS	0.38
TA98 + S9	lin reg	5.02	13	***	0.81
TA100 − S9	lin reg	0.04	13	NS	0.01
TA100 + S9	lin reg	3.61	10	**	0.75
TA1535 − S9	lin reg	−2.03	16	NS	−0.45
TA1535 + S9	lin reg	0.34	10	NS	−0.11
TA1537 − S9	lin reg	3.75	16	**	0.68
TA1537 + S9	lin reg	0.82	16	NS	−0.20
Expt. 2					
TA98 − S9	lin reg	3.32	13	**	0.68
TA98 + S9	lin reg	3.51	10	**	0.74
TA100 − S9	lin reg	2.76	10	*	0.66
TA100 + S9	lin reg	11.13	7	***	0.97
TA1535 − S9	lin reg	0.32	13	NS	0.09
TA1535 + S9	lin reg	1.44	10	NS	0.42
TA1537 − S9	lin reg	0.83	16	NS	0.20
TA1537 + S9	lin reg	4.14	16	***	0.72
HPRT test					
Expt. 1					
− S9	lin reg	1.59	10	NS	0.45
+ S9	lin reg	2.48	8	*	0.66
Expt. 2					
− S9	lin reg	−0.58	8	NS	−0.20
+ S9	lin reg	0.83	10	NS	0.25
Chromosome aberration test					
− S9	lin reg	−1.34	10	NS	−0.39
	chi sq	$\chi^2 = 0.05/1.04$	1/1	NS/NS [c]	
+ S9	lin reg	2.34	10	*	0.60
	chi sq	$\chi^2 = 1.67/1.67$	1/1	NS/NS [c]	
SCE test	lin reg	9.43	12	***	0.95

TABLE 2 (continued)

Test system	Statistical method [a]	t (χ^2, p)	$d.f.$	Significance [b]	Correlation coefficient
Micronucleus test					
24 h					
males	lin reg	−1.23	18	NS	−0.28
females	lin reg	−1.36	18	NS	−0.31
48 h					
males	lin reg	1.48	17	NS	0.34
females	lin reg	0.34	18	NS	0.08
72 h					
males	lin reg	−0.74	17	NS	−0.18
females	lin reg	−1.74	18	NS	−0.38
Drosophila test					
brood 1–3	Fisher	$p = 0.69$/ 0.37 [d]		NS/NS	
brood 4–6	Fisher	$p = 1.00$/ 1.00		NS/NS	
total	Fisher	$p = 1.00$/ 0.34		NS/NS	

[a] lin reg, linear regression analysis; chi sq, chi square analysis; Fisher, Fisher's exact probability test.
[b] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
[c] Significance of the 2 replicate points showing the largest effect.
[d] Probability in Fisher's exact test for the 2 doses tested.

doses did not cause any bone marrow toxicity, as determined from the ratio of polychromatic to normochromatic erythrocytes. No increase in the frequencies of micronuclei was observed in the bone marrow of male or female mice 24, 48 and 72 h after administration of the aqueous or methylene chloride extract (Fig. 5, Tables 1 and 2, respectively).

The induction of sex-linked recessive lethal mutations in Drosophila melanogaster. No significant increases in the recessive lethal mutation frequencies in Drosophila were caused by the methylene chloride extract, either in the different broods or the total material (Fig. 6, Table 2).

Discussion

The 2 extracts of 'Snus' have been investigated for their genotoxicity using both microbial and mammalian assays, the latter in somatic cells both in vitro and in vivo. Furthermore, the methylene chloride extract has also been tested in an assay utilizing germ cells. The aim of the investigation was to characterize 'Snus' in terms of genotoxicity and to make a prediction of its carcinogenicity.

The aqueous extract induces SCE in human lymphocytes in vitro and chromosome aberrations in vitro, both with and without metabolic activation by S9 mix. No induction of point mutations was detected in either Salmonella or V79 cells. The chromosome aberrations observed without activation may be due to the high salt concentration, whereas other components in this extract are metabolized to clastogenic agent(s). However, the clastogenic effect was not found in an in vivo assay, i.e., the test for induction of micronuclei in mouse bone marrow cells.

In the case of the methylene chloride extract, positive results were obtained using the Ames test, induction of SCE and chromosome aberrations. In contrast, no induction of mutations in mammalian cells in vitro was observed. Again, the results indicate activation by a metabolizing system(s). However, the test results in vitro could not be verified in the in vivo tests. No effects on either the induction of micronuclei or recessive lethal mutations in Drosophila were found.

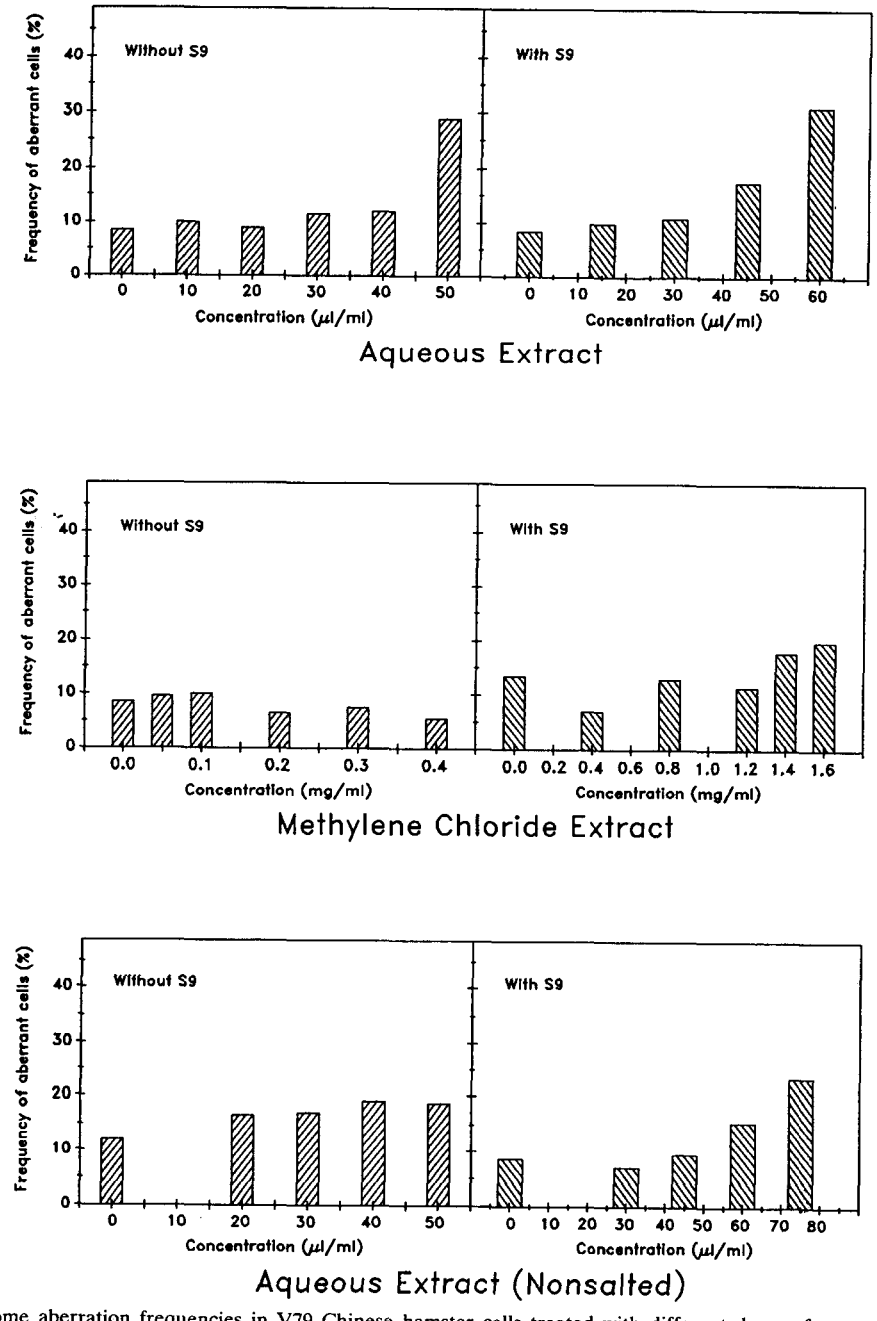

Fig. 2. Chromosome aberration frequencies in V79 Chinese hamster cells treated with different doses of an aqueous extract, a methylene chloride extract or an unsalted aqueous extract of 'Snus' in the absence and presence of S9 mix.

When evaluating the test results for complex mixtures, possible interactions between different compounds should be taken into consideration, as they may influence the findings. Both types of 'Snus' extracts contained nicotine. Since the basic mechanism for nicotine-mediated toxicity in vivo

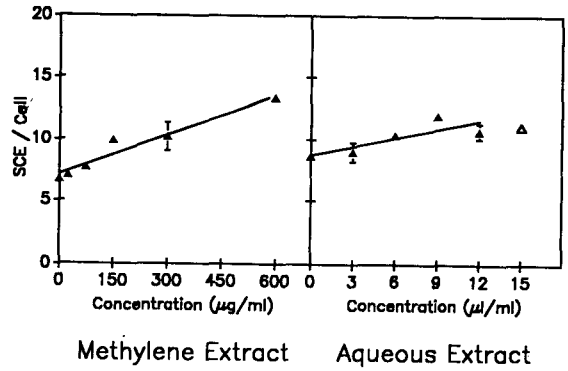

Fig. 3. Frequencies of sister-chromatid exchanges in PHA-stimulated human lymphocytes treated with different doses of a methylene chloride extract or an aqueous extract of 'Snus'.

involves receptors, the nicotine in 'Snus' would not be expected to influence in vitro toxicity. Thus, it was estimated that the nicotine present in the extracts could be responsible for all the toxicity in vivo in mice, but contributed only to a minor extent to the cytotoxicity in vitro. It may then be argued that the presence of nicotine in the extract may prevent the use of relevant doses in the in vivo micronucleus test (because of toxicity) and, accordingly, the prediction of carcinogenicity may not be correctly performed.

Prediction of carcinogenic properties of chemicals from short-term test data may be performed by 2 very different approaches, both of which are widely accepted. One approach uses a sequential testing strategy, such as described by Bridges (1976), Sobels (1977), Ashby (1986), Richold (1987) and in guidelines for mutagenicity testing (OECD, 1981). The other approach uses the mathematical model, carcinogenicity prediction by battery selection (CPBS), described by Chankong et al. (1985).

A sequential testing method usually includes an in vitro test as the first step, most often the Ames test. A negative result should then be confirmed in one or more additional in vitro tests to warrant the prediction of non-carcinogenicity With a positive result in any of the in vitro tests, the test material should be subjected to further testing using in vivo short-term tests. If the first in vivo assay gives a negative result, one or more additional in vivo tests with negative results are required in order for the test material to be predicted to be non-carcinogenic. With a positive result in any of the in vivo tests, the test material is suspected to be a mammalian carcinogen. Using this approach, the probability of both the aqueous and the methylene chloride extracts of Swedish 'Snus' being carcinogenic due to a genotoxic mechanism is low.

The CPBS method is based on correlation studies between short-term test data, obtained from the Gene-Tox Program data base, and results from long-term carcinogenicity studies (Palajda and Rosenkranz, 1985; Pet-Edwards et al., 1985; Chankong et al., 1985). The Bayesian

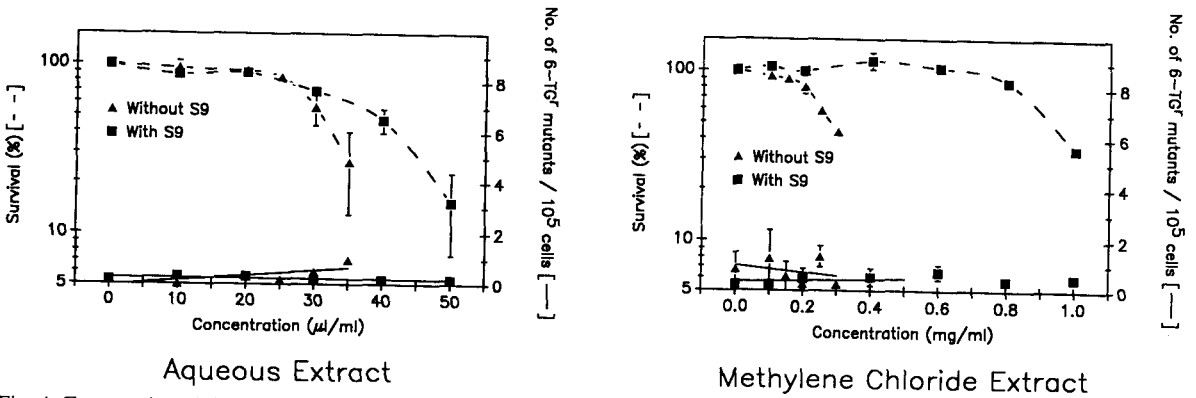

Fig. 4. Frequencies of 6-thioguanine-resistant mutants in V79 Chinese hamster cells treated with different doses of an aqueous extract or a methylene chloride extract of 'Snus' in the absence and presence of S9 mix.

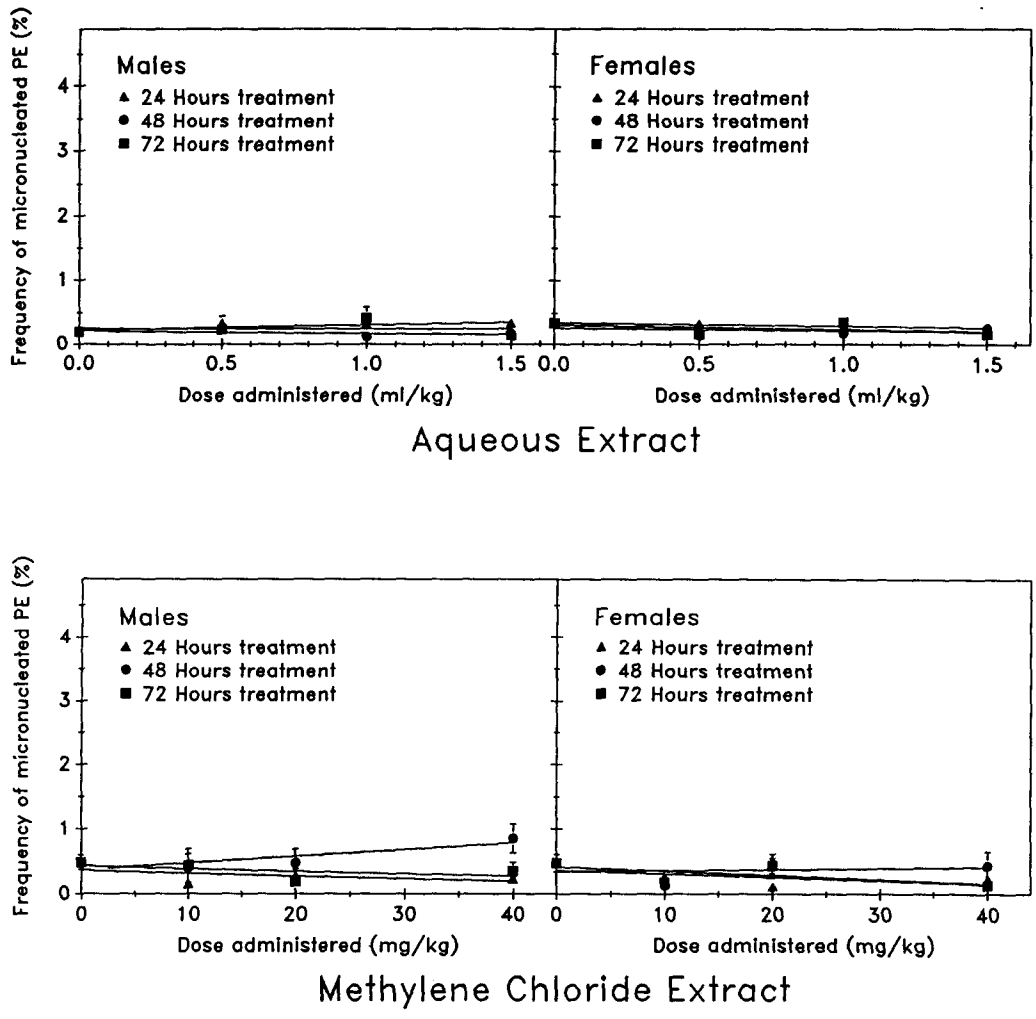

Fig. 5. Frequencies of micronucleated polychromatic erythrocytes isolated from female and male mice treated for different periods with 3 doses of an aqueous extract or a methylene chloride extract of 'Snus'.

theory is used to combine results obtained in a battery of short-term tests in order to obtain a prediction value for carcinogenicity (Pet-Edwards et al., 1985; Chankong et al., 1985). Applying this model to the test results obtained with the 2 'Snus' extracts, the probability of the methylene chloride and aqueous extracts of 'Snus' being correctly identified as non-carcinogens is 71% and 77%, respectively. Since the CPBS approach has mostly been evaluated for pure compounds, and rarely for complex mixtures, some caution should at present be exercised in interpreting results on the latter.

In certain reports, the use of smokeless tobacco (e.g., snuff and chewing tobacco) has been suggested to be associated with cancer in the human oral cavity and pharynx (Winn et al., 1981b; Cullen et al., 1986; Council on Scientific Affairs, 1986). These conclusions have been based mainly on epidemiological studies in India, where tobacco is chewed together with betel nuts and other ingredients (Jussawalla and Deshpande,

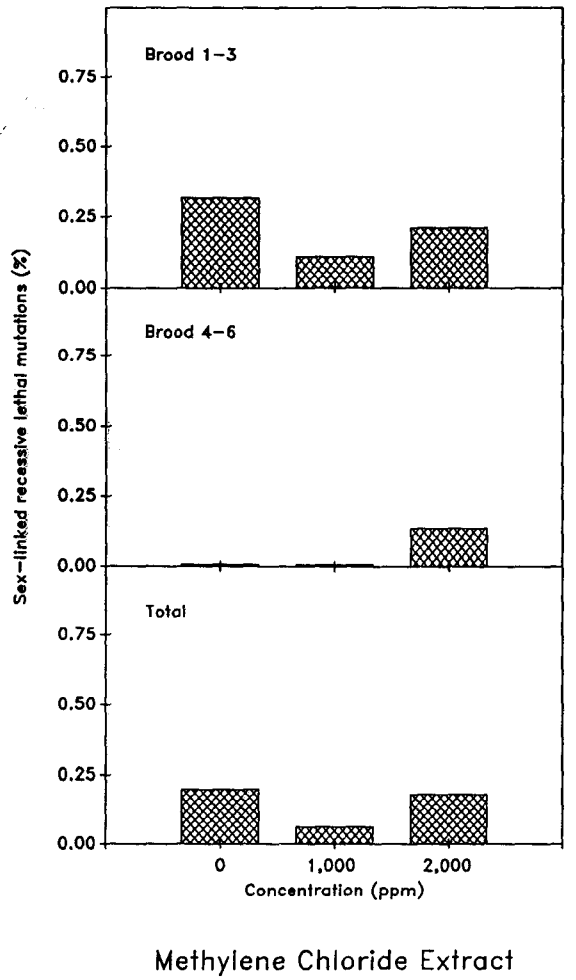

Fig 6. Percentage of sex-linked recessive lethal mutations in *Drosophila melanogaster* in broods 1–3 and broods 4–6 treated with 1000 or 2000 ppm of an aqueous extract (in 5% sucrose in water) or a methylene chloride extract of 'Snus'.

1971) and on a study in the southern U.S.A. (Winn et al., 1981a), where dry snuff is chewed. Some contradictory reports also exist (Smith et al., 1970; Smith, 1975). The risks ascribed to the use of smokeless tobacco may not be generally relevant because oral lesions induced in this manner may vary with the type of tobacco product, the oral hygiene of the user and the manner in which the product is used. Snuff placed by surgery in the oral cavity of F344 rats seems to induce tumors and a carcinogenic effect of snuff extracts has also been indicated (Hecht et al., 1986).

It is quite clear that different types of moist snuff may contain different amounts of well-known genotoxic and carcinogenic compounds, such as benzo[*a*]pyrene, certain volatile aldehydes, polonium-210, and nitrosamines, both tobacco-specific (TSNA) and others (Hoffmann et al., 1984, 1987; Hecht and Hoffmann, 1988). The concentrations of these compounds in different brands of snuff may vary greatly, in some cases several hundred-fold (Hoffmann et al., 1986, 1987). It can be assumed that the genotoxic and carcinogenic effect of extracts of these products may vary accordingly. Studies by Brunnemann et al. (1987) on the tumorigenic activity of TSNA indicate that snuff extracts may inhibit the carcinogenicity of these compounds. This finding is supported by the fact that extracts of 'Snus' also exhibited antimutagenic properties (manuscript in preparation).

At present, the results of this investigation on Swedish 'Snus' indicate that the carcinogenic hazards of snuff dipping in North America (Cullen et al., 1986) may not be directly relevant to Swedish moist snuff. Further support for this suggestion comes from the fact that different areas in the U.S.A. have an incidence of oral cancer 2–6 times that observed in Sweden. Only Colombia and parts of California have approximately the same rate as Sweden (4.4 new cases annually per 100,000, age-adjusted) (Waterhouse et al., 1982).

References

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347–364.

Ashby, J. (1986) The prospects for a simplified and internationally harmonized approach to the detection of possible human carcinogens and mutagens, Mutagenesis, 1, 3–16.

Ashby, J., and M. Ishidate (1986) Clastogenicity in vitro of the Na, K, Ca, and Mg salts of saccharin, and of magnesium chloride; consideration of significance, Mutation Res., 163, 63–73.

Bhide, S.V., J. Kulkarni, U.J. Nair, B. Spiegelhalder and R. Preussmann (1987) Mutagenicity and carcinogenicity of masheri, a pyrolysed tobacco product, and its content of tobacco-specific nitrosamines, IARC Sci. Publ., 84, 460–462.

Bridges, B. (1976) Evaluation of mutagenicity and carcinogenicity using a three-tier system, Mutation Res., 41, 71–72.

Brunnemann, K.D., A. Rivenson, J.D. Adams, S.S. Hecht and D. Hoffmann (1987) A study of snuff carcinogenesis, IARC Sci. Publ., 84, 456–459.

Chankong, V., Y.Y. Haimes, H.S. Rosenkranz and J. Pet-Edwards (1985) The carcinogenicity prediction and battery selection (CPBS) method: a Bayesian approach, Mutation Res., 153, 135–166.

Council on Scientific Affairs (1986) Health effects of smokeless tobacco, J. Am. Med. Ass., 28, 255, 1038–1044.

Cullen, J.W., W. Blot, J. Henningfield, G. Boyd, R. Mecklenburg and M.M. Massey (1986) Health consequences of using smokeless tobacco: summary of the advisory committee's report to the Surgeon General, Public Health Rep., 101, 355–373.

Curvall, M., E. Kazemi-Vala and C.R. Enzell (1982) Simultaneous determination of nicotine and cotinine in plasma using capillary column gas chromatography with nitrogen-sensitive detection, J. Chromatogr., 232, 283–293.

Galloway, S.M., D.A. Deasy, C.L. Bean, A.R. Kraynak, M.J. Armstrong and M.O. Bradely (1987) Effects of high osmotic strength on chromosome aberrations, sister-chromatid exchanges and DNA strand breaks, and the relation to toxicity, Mutation Res., 189, 15–25.

Guttenplan, J.B. (1987) Mutagenic activity in smokeless tobacco products sold in the USA, Carcinogenesis, 8, 741–743.

Hecht, S.S., and D. Hoffmann (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke, Carcinogenesis, 9, 875–884.

Hecht, S.S., A. Rivenson, J. Braley, J. DiBello, J.D. Adams and D. Hoffmann (1986) Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff, Cancer Res., 4162–4166.

Hoffmann, D., E.J. LaVoie and S.S. Hecht (1984) Nicotine: a precursor for carcinogens, Cancer Lett., 26, 67–75.

Hoffmann, D., N.H. Harley, I. Fisenne, J.D. Adams and K.D. Brunnemann (1986) Carcinogenic agents in snuff, J. Natl. Cancer Inst., 76, 435–437.

Hoffmann, D., J.D. Adams, D. Lisk, I. Fisenne and K.D. Brunnemann (1987) Toxic and carcinogenic agents in dry and moist snuff, J. Natl. Cancer Inst., 79, 1281–1286.

International Agency for Research on Cancer (1985) Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco habits other than smoking, Vol. 37, IARC, Lyon, pp. 37–140.

ISO (1977) 2881.

Jansson, T., and L. Zech (1987) Effects of vanillin on sister-chromatid exchanges and chromosome aberrations in human lymphocytes, Mutation Res., 190, 221–224.

Jansson, T., M. Curvall, A. Hedin and C.R. Enzell (1986) In vitro studies of biological effects of cigarette smoke condensate. II. Induction of sister-chromatid exchanges in human lymphocytes by weakly acidic, semivolatile constituents, Mutation Res., 169, 129–139.

Jenssen, D. (1982) The induction of micronuclei, in: A.A. Sandberg (Ed.), Progress and Topics in Cytogenetics, Vol. 2, Sister Chromatid Exchange, Liss, New York, pp. 47–63.

Jenssen, D. (1984) A quantitative test for mutagenicity in V79 Chinese hamster cells, in: B.J. Kilbey, W. Nichols and C. Ramel (Eds.), Handbook of Mutagenicity Test Procedures, Elsevier, Amsterdam, pp. 269–290.

Jussawalla, D.J., and V.A. Deshpande (1971) Evaluation of cancer risk in tobacco chewers and smokers, Cancer, 28, 244–252.

Magnusson, J., and C. Ramel (1978) Mutagenic effects of vinyl chloride on *Drosophila melanogaster* with and without pretreatment with sodium phenobarbiturate, Mutation Res., 57, 307–312.

Maron, D.M., and B.N. Ames (1984) Revised methods for the Salmonella mutagenicity test, in: B.J. Kilbey, W. Nichols and C. Ramel (Eds.), Handbook of Mutagenicity Test Procedures, Elsevier, Amsterdam, pp. 93–141.

OECD (1981) Guidelines for Testing of Chemicals, with updates, Paris.

Palajda, M., and H.S. Rosenkranz (1985) Assembly and preliminary analysis of a genotoxicity data base for predicting carcinogens, Mutation Res., 153, 79–134.

Pet-Edwards, J., V. Chankong, H.S. Rosenkranz and Y.Y. Haimes (1985) Application of the carcinogenicity prediction by battery selection (CPBS) method to the Gene-Tox data base, Mutation Res., 153, 187–200.

Richold, M. (1987) Genetic toxicology, in: C.K. Atterwill and C.E. Steele (Eds.), In Vitro Methods in Toxicology, Cambridge University Press, Cambridge, pp. 299–318.

Stich, H.F. (1986) The use of micronuclei in tracing the genotoxic damage in the oral mucosa of tobacco users, Banbury Rep., 23, 99–111.

Smith, J.F. (1975) Snuff-dippers lesion, Arch. Otolaryngol., 101, 276–277.

Smith, J.F., H.A. Mincer, K.P. Hopkins and J. Bell (1970) Snuff-dipper's lesion. A cytological and pathological study in a large population, Arch. Otolaryngol., 92, 450–456.

Sobels, F.H. (1977) Some problems associated with testing for environmental mutagens and a perspective for studies in 'comparative mutagenesis', Mutation Res., 46, 245–260.

US Department of Health and Human Services (1986) The Health Consequences of Using Smokeless Tobacco: A Report of the Advisory Committee to the Surgeon General, DHHS (NIH) Publ. No. 86-2874, Public Health Service, Bethesda, MD.

Waterhouse, J., C. Muir, K. Shanmugaratnam and J. Powell (1982) Cancer incidence in five continents, IARC Sci. Publ., 42.

Whong, W-Z., R.G. Ames and T. Ong (1984) Mutagenicity of tobacco snuff: possible health implications for coal miners, J. Toxicol. Environ. Health, 14, 491–496.

Winn, D.M., W.J. Blot and J.R. Fraumeni Jr. (1981a) Snuff dipping and oral cancer, New Engl. J. Med., 305, 230–231.

Winn, D.M., W.J. Blot, C.M. Shy, D.W. Pickle, A. Toledo and J.R. Fraumeni (1981b) Snuff dipping and oral cancer

among women in the southern United States, New Engl. J. Med., 304, 745–749.

World Health Organization (1988) Smokeless Tobacco Control: Report of a WHO Study Group, Technical Report Series 773, WHO, Geneva.

Würgler, F.E., F.H. Sobels and E. Vogel (1984) Drosophila as an assay system for detecting genetic changes, in: B.J. Kilbey, W. Nichols and C. Ramel (Eds.), Handbook of Mutagenicity Test Procedures, Elsevier, Amsterdam, pp. 555–602.