

3. Studies of Cancer in Experimental Animals

The Working Group that evaluated smokeless tobacco previously noted that the majority of the early studies evaluated at that time (IARC, 1985) had various deficiencies, such as lack of quantitative and qualitative information on the nature of tobacco extracts and the degree of extraction, insufficient length of treatment, small group sizes and, in some cases, lack of appropriate controls. Since that time, new studies have been published and are included in this section. The cumulative published evidence for carcinogenicity of smokeless tobacco in experimental animals is summarized below and has also been reviewed recently (Hoffmann & Djordjevic, 1997; Grasso & Mann, 1998).

3.1 Tobacco

3.1.1 *Oral administration*

(a) *Mouse*

Groups [numbers unspecified] of male Swiss mice, 6–8 weeks of age, were administered a tobacco extract (ethanol extract from 50 g tobacco diluted in 10 mL distilled water) from a commercially available Indian chewing tobacco at a dilution of 1:25 or 1:50 [actual dose unspecified] by oral intubation for 15–20 months. A further group of mice was fed a diet that contained an extract of 10 g tobacco per 5 kg diet for up to 25 months. A group of 20 mice received distilled water only by intubation and served as controls. Administration of the 1:25 dilution was terminated at 18 weeks because of high mortality. Tumour incidences at 15–20 months were 0/4, 8/15 and 4/10 in the control, 1:50 dilution

and 1:25 dilution groups, respectively. At 21–25 months, 1/20 controls and 8/10 animals fed tobacco extract in the diet had developed tumours. The types of tumour observed were lung adenocarcinoma or hepatocellular carcinoma (Bhide *et al.*, 1984b). [The Working Group noted the incomplete reporting of the distribution of different types of neoplasm.]

(b) *Rat*

Weanling male Sprague Dawley rats were fed diets containing shark liver oil (sufficient in vitamin A, 60 rats) or without shark liver oil (vitamin A-deficient, 61 rats). Tobacco extract was prepared by extracting 100 g commercial tobacco with 1 L dichloromethane at room temperature for 72 h; the mixture was then filtered and dried under vacuum. Half of the rats in each group (29 vitamin A-sufficient, 31 vitamin A-deficient) received 3 mg tobacco extract dissolved in 0.05 mL dimethylsulfoxide (DMSO) by gavage five times per week for 21 months. The remaining (control) rats (31 vitamin A-sufficient, 30 vitamin A-deficient) received 0.05 mL DMSO by gavage five times per week for 21 months. All rats were necropsied, and all organs were examined for gross abnormalities; the liver, lung, stomach, brain and pituitary gland were examined histologically. No tumours were observed in control rats, irrespective of vitamin A status. Among vitamin A-sufficient rats given tobacco extract, 6/29 had single tumours: 3/29 had lung adenomas, 3/29 had forestomach papillomas, 0/29 had lung lymphomas or pituitary adenomas. Among vitamin A-deficient rats given tobacco extract, 29/31 had one or more tumours: 22/31 had lung lymphomas, 19/31 had pituitary adenomas, 28/31 had stomach papillomas and 0/31 had lung adenomas. The proportions of tumour-bearing rats were significantly greater in both tobacco extract-treated groups than in the corresponding control groups ($p < 0.001$, χ^2 test) (Bhide *et al.*, 1991). [The Working Group noted that primary lymphoma of the lung is extremely uncommon in rats. However, there was an increased incidence only of benign tumours in the vitamin-A sufficient rats.]

3.1.2 *Application to the oral mucosa or cheek pouch*

(a) *Mouse*

Groups of 9–16 male and female strain A (Strong) and Swiss mice, 2–3 months old, were administered different alkaloid-free extracts of an Indian chewing tobacco of the Vadakkan type (Meenampalayam variety). The extracts — a benzene extract and its neutral fraction, a water extract and four successive extracts (petroleum ether, benzene, chloroform and ethanol) — were applied by daily application to the oral mucosa for up to 18 months of age. No tumours were observed in mice exposed to the chewing tobacco extracts (Mody & Ranadive, 1959). [The Working Group noted the small number of animals used.]

(b) *Rat*

A group of 22 Wistar rats, 5 months of age, were painted on the oral mucosa with a 2% alkaloid-free extract of Vadakkan tobacco (Meenampalayam variety) in acetone twice

a week for life; 12 of these animals were also painted with a paste of lime (20% in distilled water) on the day after each treatment. Control groups of 10–14 rats received no treatment or were treated with lime only. No tumour was observed at the application site (Gothoskar *et al.*, 1975).

(c) *Hamster*

A group of 50 young Syrian golden hamsters [age unspecified] received an implantation of a 2-cm³ plug of chewing tobacco [unspecified] in the cheek pouch. The opening in the cheek pouch was ligated and the animals were followed for up to 30 months. Survival after 13 months was 21/50; eight were alive at 24 months, but none were alive at 30 months. No tumour was observed in any of the animals (Peacock & Brawley, 1959; Peacock *et al.*, 1960).

A group of 34 male and female Syrian golden hamsters, 1–2 months of age received an implant into the cheek pouch of pellets of Philippine leaf tobacco with 10% lime mixed with beeswax. Animals were allowed to live their lifespan (up to 22 months) and were killed when moribund. No tumour at the implantation site was reported (Dunham & Herrold, 1962).

Groups of 11–12 male Syrian golden hamsters, 9 weeks of age, received topical applications on the cheek-pouch mucosa of a DMSO extract of cured Banarsi chewing tobacco or DMSO alone thrice weekly for 21 weeks, at which time all animals were killed. No tumour was seen in treated or control hamsters, but 8/12 treated animals had leukoplakia (Suri *et al.*, 1971). [The Working Group noted the short duration of the experiment.]

A group of 12 male inbred Syrian golden hamsters, 2–3 months old, received topical applications to the cheek-pouch mucosa of DMSO extracts of an Indian chewing tobacco (Vadakkan) thrice weekly for life. A control group of seven animals received applications of DMSO alone. No local tumour but moderate hyperkeratosis was observed (Ranadive *et al.*, 1976). However, one animal developed a stomach tumour [pathology is not described] after exposure to DMSO tobacco extract. [The Working Group noted that a similar stomach tumour developed in another experiment when a mixture of tobacco and areca nut DMSO extract was applied.]

Groups of 30–41 Syrian golden hamsters [sex unspecified], weighing 40–50 g, received an application of 60 g tobacco ('Jada Jarda') alone, in combination with lime or in combination with lime plus vitamin A in the cheek pouch thrice weekly for 100–110 weeks, at which time 24–32 animals were still alive. Moderate to severe keratotic and dysplastic changes developed in the mucosa, but no neoplastic change was observed (Kandarkar *et al.*, 1981).

A group of 20 female Syrian golden hamsters, 6–7 weeks of age, received topical applications to the cheek-pouch mucosa of 1 mg lyophilized aqueous tobacco extract in 0.05 mL water twice daily for 6 months. Animals were observed for a further 6 months and were then killed. Squamous-cell papillomas and/or carcinomas occurred in 3/17 animals compared with none in 10 untreated and 10 vehicle (water) controls (Rao, 1984). [The findings were not statistically significant.]

Eighty male Syrian golden hamsters, 8 months of age, were divided into four treatment groups of 20 animals each: tobacco only, alcohol only, tobacco and alcohol and untreated controls. Smokeless tobacco (Skopal[®], US Tobacco Co., Nashville, TN; 200 mg) was placed in each cheek pouch of hamsters in the tobacco groups five times a week. In the alcohol groups, 2 mL 15% ethanol was placed in each cheek pouch five times weekly. Hamsters in the negative control group received mechanical stimulation of the right cheek pouch to simulate the placement of the tobacco. After 26 weeks, the hamsters were killed and pouches and abdominal organs were examined. Acanthosis of the pouch epithelium was noted more frequently in the groups treated with tobacco (14/20; $p < 0.005$) and tobacco plus alcohol (12/20; $p < 0.025$), but no tumours were observed in the cheek pouches. Adenomas of the adrenal gland were noted in 2/20 hamsters in the tobacco-treated group and in 1/20 hamsters in each of the other three groups. Squamous-cell papillomas of the forestomach occurred in 2/20 hamsters in the tobacco-treated group, 3/20 hamsters in the alcohol-treated group, 4/20 hamsters in the alcohol plus tobacco-treated group and 0/20 hamsters in the control group. Incidences of forestomach tumours in the treated groups were not significantly elevated above the zero incidence in controls (Summerlin *et al.*, 1992). [The Working Group noted the short duration of the study and the advanced age of the animals at the beginning of the treatment.]

3.1.3 *Skin application*

Mouse

Groups of 40 CAF₁ (Jackson) and 40 Swiss (Millerton) mice [sex and age unspecified] received topical applications of a 50% methanol extract of unburnt cigarette tobacco on the skin three times a week for 24 months. Groups of 30 CAF₁ and 30 Swiss mice that similarly received whole-tar extract for 21–24 months served as controls. Among the CAF₁ mice exposed to the tobacco extract, 11 developed papillomas; among the Swiss mice, three treated mice developed papillomas compared with 16 papillomas that developed in each of the control groups. One papilloma later developed into cancer in the extract-treated Swiss mice compared with three that transformed in control Swiss mice and eight in control CAF₁ mice (Wynder & Wright, 1957).

Groups of 8–17 male and female strain A (Strong) and Swiss mice, 2–3 months of age, received skin applications of five different extracts (petroleum ether, benzene, chloroform, chloroform ether and ethanol) of an Indian chewing tobacco (Vadakkan type, Meenam-palayam variety) up to 18 months of age; no tumour was observed at the site of application, and no excess incidence was reported at other sites (Mody & Ranadive, 1959). [The Working Group noted the small numbers of animals used.]

A group of 10 male and six female inbred strain C17 mice, 2–3 months of age, received thrice-weekly applications of a DMSO extract of an Indian chewing tobacco (Vadakkan type) on the skin of the interscapular region until 24 months of age. No skin tumour was observed (Ranadive *et al.*, 1976). [The Working Group noted the small number of animals used.]

3.1.4 *Other routes of administration*

(a) *Inhalation*

Mouse

Groups of 80 male strain A mice, 3 months of age, were exposed by inhalation to powdered tobacco leaf on alternate days for 30 months or served as untreated controls. The incidence of lung tumours (six alveogenic carcinomas, 35 squamous-cell carcinomas and three 'malignant adenomas'), leukaemia and hepatocellular carcinoma in animals surviving to 30 months was: 12/75 [$p < 0.001$; Fisher's exact test] and 1/80, 11/75 [$p < 0.01$; Fisher's exact test] and 2/80 and 3/75 and 0/80 in the treated and control groups, respectively (Hamazaki & Murao, 1969). [The Working Group noted that, while the incidence of lung tumours and leukaemia in treated animals was significantly increased, the incidence of lung and liver tumours in the untreated mice was unusually low.]

(b) *Subcutaneous administration*

Mouse

Two groups of 17 Paris albino XVII \times C57 black mice [age and sex unspecified] received multiple subcutaneous injections of 0.1 mL of a 2% solution of 'partially or completely alkaloid-free' extract of tobacco (Vadakkan, Meenampalayam variety) once a month for 41–95 weeks. One squamous-cell carcinoma [site not specified] developed in an animal that received the partially alkaloid-free extract (Ranadive *et al.*, 1963). [The Working Group noted that the results were inconclusive.]

(c) *Intravesicular implantation*

Mouse

Groups of 5–12 male and female inbred strain C17 and Swiss mice, 2–3 months of age, received a single intravesicular implantation of paraffin pellets that contained chewing tobacco (Jarda), a mixture of chewing tobacco and lime or an alkaloid-free chewing tobacco extract or paraffin pellets alone, and were observed until 10–30 months of age. Among the C17 mice that received the alkaloid-free tobacco implantation, 2/12 developed transitional-cell tumours of the bladder and one female developed a tumour described as a 'myosarcoma of the cervix with metastasis to the kidney'. No tumour was observed in the controls or in the other treated groups (Randeria, 1972). [The Working Group noted the small group size and the potential carcinogenic effect of intravesicular foreign bodies in mice.]

(d) *Vaginal application*

Mouse

A group of four female inbred C17 strain mice and four female Swiss mice, 2–3 months of age, received daily vaginal applications of a fine mixture of (Jarda) tobacco dust that

contained lime derived from sea shells for 10–30 months: no vaginal tumour was observed (Randeria, 1972). [The Working Group noted that no control group was used in this study.]

3.1.5 *Skin application with known carcinogens or modifiers*

Mouse

Groups of 11–36 Paris albino XVII × C57 black (hybrid) or inbred Swiss mice [sex and age unspecified] received twice-weekly skin applications of ‘total extract’ plus ‘partially alkaloid-free extract’ or ‘totally alkaloid-free extract’ of ‘Vaddakan’ tobacco of Meenampalayam variety or acetone (control) for 95 weeks followed by weekly applications of croton oil. No control group of Swiss mice was included. Between 61 and 95 weeks after the start of treatment, the incidence of papillomas and of squamous-cell carcinomas at the site of application was: 10/21 and 6/21, 9/25 and 2/25, 22/35 and 10/35 and 3/19 and 0/19 in the hybrid mice, respectively. [The increases in the incidence of papillomas and carcinomas were statistically significant, except in the ‘partially alkaloid-free extract’-treated group.] The incidence of papillomas in the Swiss mice was 2/9, 2/4 and 3/10, in the three tobacco-treated groups, respectively; no carcinoma was observed (Ranadive *et al.*, 1963).

The co-carcinogenic [promoting] effect of the ‘totally alkaloid free’ extract of ‘Vaddakan’ tobacco of Meenampalayam variety was tested in a group of 16 Swiss albino and 13 hairless Swiss (Baldy) mice [sex and age unspecified] that received a single topical application of benzo[*a*]pyrene [dose unspecified] followed by twice-weekly applications of the extract for 80 weeks. A group of seven Swiss albino and 10 Swiss (Baldy) mice received the benzo[*a*]pyrene treatment only and served as controls. Two carcinomas and four papillomas were observed in Swiss (Baldy) mice treated with the tobacco extract and benzo[*a*]pyrene; no tumour was observed in benzo[*a*]pyrene-treated controls (Ranadive *et al.*, 1963). [The Working Group noted the small number of animals and incomplete information concerning the initiating dose of benzo[*a*]pyrene.]

A total of six groups of 30 female ICR Swiss mice, 57 days of age, were untreated or received a single topical application of 125 µg 7,12-dimethylbenz[*a*]anthracene (DMBA) in 0.25 mL acetone. Twenty-one days later, mice received an application of 0.25 mL of either an acetone or ‘concentrated’ or ‘dilute’ barium hydroxide extract of unburnt commercial tobacco five times a week for 36 weeks. The amount of acetone extract was equivalent to 2.5 cigarettes per day. The barium hydroxide extract was prepared using two different extraction procedures (designated ‘concentrated’ and ‘dilute’) according to the yield: the ‘concentrated’ extract was equivalent to 0.5 cigarette per day and the ‘dilute’ extract was approximately 25% as strong as the ‘concentrated’ extract. Two control groups of 30 mice were untreated or received DMBA only. The incidence of tumours (all of which were small papillomas) was: acetone extract, 16 tumours in 7/30 mice (2.3 tumours per mouse); concentrated barium hydroxide extract, 18 tumours in 8/30 mice (2.2 tumours per mouse); and dilute barium hydroxide extract, six tumours in 2/30 mice (three tumours per mouse). No tumour was observed in either of the groups that received acetone or barium hydroxide

tobacco extract without DMBA pretreatment, in DMBA-treated or in untreated groups (Bock *et al.*, 1964).

Groups of 30 female ICR Swiss mice, 55–60 days of age, were untreated or received a single topical application of 125 µg DMBA in 0.25 mL acetone. Three weeks later, mice were either untreated or received applications of different aqueous extracts (crude, acidic, neutral and basic fractions) of an unprocessed, commercial, flue-cured tobacco five times per week for 26 weeks. A total of 12 papillomas developed in 6/30 mice treated with crude tobacco extract (equivalent to 0.5 g tobacco daily) after DMBA initiation. One mouse developed a papilloma after treatment with the acidic fraction and DMBA. No skin tumour was found in animals treated with neutral or basic fractions after DMBA initiation, DMBA alone or with the various fractions of tobacco alone. After treatment with half the concentration (0.25 g tobacco), one mouse treated with the crude extract developed a papilloma and one mouse treated with the neutral fraction developed three papillomas after DMBA initiation (Bock *et al.*, 1965).

Groups of 20 female Swiss ICR/Ha mice, 8 weeks of age, received a single application on the dorsal skin of 150 µg DMBA in 0.1 mL acetone followed 2–3 weeks later by thrice-weekly applications of solvent extracts (ether [25 mg], chloroform [1 mg], methanol [25 mg] or a reconstituted sample [25 mg]) of a flue-cured cigarette variety of tobacco leaf for 52 weeks. Groups of 20 mice that received DMBA alone or tobacco extracts alone served as controls. Two of 13 survivors in the DMBA/methanol extract group developed ‘cancers’. The numbers of mice with papillomas in the various groups were: 4/12 (ether extract), 1/10 (chloroform extract), 2/13 (methanol extract) and 5/14 (reconstituted extract). No tumour was observed in mice treated with DMBA or extracts alone (Van Duuren *et al.*, 1966).

3.2 Snuff tobacco

[The Working Group noted that specific brands of snuff used in most studies was not specified by the investigators.]

3.2.1 Oral administration

Hamster

A total of 13 male and female Syrian golden hamsters, 1.5 months of age, were fed three different test substances for 16 months: group 1 (two males and two females) was fed 0.75 g scented snuff [type unspecified] per week; group 2 (two males and two females) was fed 0.75 g scented snuff [type unspecified] and 0.75 g calcium hydroxide per week; and group 3 (five animals) [sex distribution not specified] received calcium hydroxide alone. One male hamster in group 2, estimated to have consumed 52 g snuff and 52 g calcium hydroxide during the 16-month period, developed a pancreatic carcinoid 4.5 months after the termination of treatment. Another hamster that was not fed snuff developed a carcinoid of the glandular stomach at the age of 26 months. The tumour

incidence in the remaining groups and at other sites was not reported; however, the authors stated that no carcinoids had been found in more than 700 hamsters necropsied previously in that laboratory (Dunham *et al.*, 1975). [The Working Group noted the relatively small group size used.]

Groups of 50 male BIO 15.16 and BIO 87.20 strain (carcinogen-susceptible) Syrian hamsters, 2–3 months of age, were fed one of the following five experimental diets for 2 years: diet containing 20% damp fresh US snuff; cellulose mixed with diet, such that the caloric content was reduced by 20% (negative control); control diet plus 50 treatments with 5 mg 20-methylcholanthrene per animal by stomach tube (positive control); cellulose diet plus 50 treatments with 0.5 mg 20-methylcholanthrene per animal by stomach tube; and snuff diet plus 50 treatments with 0.5 mg 20-methylcholanthrene per animal by stomach tube. The animals fed snuff diet alone showed a spectrum of tumours that was nearly identical to that of controls. No increased incidence of tumours was noted in animals administered snuff with 20-methylcholanthrene (Homburger *et al.*, 1976).

3.2.2 *Application to the oral mucosa or cheek pouch*

(a) *Rat*

A group of 21 male and 21 female Sprague-Dawley rats, 3 months of age, was administered snuff into a surgically created canal in the lower lip. Approximately 0.2 g of a standard Swedish snuff (Röda Lacket; pH 8.3), was injected into the canals morning and night on 5 days per week for up to 22 months. The calculated daily dose (Hirsch & Thilander, 1981) was 1 g/kg bw and the mean retention time after each administration was 6 h (range, 5–8 h). The rats were killed at 9, 12 and 18–22 months. A second group of five male and five female rats was treated similarly with the same snuff but at pH 9.3 [produced by the addition of 50% sodium carbonate (1% of the total weight)] and was killed at between 18 and 22 months. Of 42 animals administered the snuff, one developed a squamous-cell carcinoma of the oral mucosa at 8.5 months. No tumour was seen in rats exposed to the alkaline snuff or in 15 rats that had surgically created canals but were not given snuff. Benign tumours outside the oral cavity were observed at approximately equal frequency in control and treated groups in both experiments (Hirsch & Johansson, 1983).

Fifteen male and 15 female HMT rats, 6 months of age, received weekly applications of smokeless tobacco to the buccal mucosa for 1 year and were followed for an additional observation period of 6 months. A commercially available snuff tobacco (0.4 g per pack) was moistened with distilled water and applied with a cotton swab to both sides of the mandibular mucobuccal fold of each rat. Tobacco was gradually swallowed by the rats and disappeared after several hours. Fifteen male and 15 female control rats received sham treatments with cotton swabs that were wetted with distilled water. No oral carcinomas were observed in treated or control rats (Chen, 1989).

Beginning at 10 weeks of age, the lips and oral cavities of male Fischer 344 rats were swabbed with 0.5 mL water (controls; 21 rats, group 1), aqueous snuff extract (30 rats, group 2), aqueous snuff extract enriched with 10-fold the natural concentrations of the

tobacco-specific nitrosamines *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (30 rats, group 3) or an aqueous solution of NNN plus NNK in concentrations equal to those in the *N*-nitroso compound-enriched snuff extract group (30 rats, group 4). The snuff used was a moist snuff product commercially available in the USA. Swabbing was performed once or twice daily during weeks 1–23 and twice daily during weeks 24–131. The rats were observed until moribund or until survivors were killed at the end of the study, when complete necropsies were performed. Oral cavity tumours (papillomas of the cheek, hard palate or tongue) developed in 0/21, 0/30, 3/30 and 8/30 rats in groups 1–4, respectively. Lung tumours developed in 1/21 (adenoma), 0/30, 2/30 (adenomas) and 5/30 (one adenoma, four adenocarcinomas) rats in groups 1–4, respectively. The incidence of oral tumours in group 4 was significantly greater than that in group 1 ($p < 0.05$; t test and χ^2 test). Tumours were also seen at various other sites in all groups but were not related to treatment. Snuff extract alone induced no tumours of either the oral cavity or lung (Hecht *et al.*, 1986).

Surgery to create a test canal in the lower lip was performed on 95 male Fischer 344 rats at 10 weeks of age. Animals were tested for 2 weeks for wound healing which ensured that the epithelium of the lip canal was intact. Beginning at 13 weeks of age, rats received no further treatment (controls; 10 rats, group 1) or received moist snuff (a brand commercially available in the USA; 32 rats, group 2), water-extracted snuff (21 rats, group 3) or snuff enriched with its own aqueous extract (32 rats, group 4). Snuff preparations (approximately 50 mg per rat) were inserted into the surgically created test canals on 5 days per week and were generally retained in the test canal for 24 h. The experiment was terminated after 116 weeks. Tumours developed in the test canal or the oral cavity in 0/10 control rats (group 1), 3/32 snuff-treated rats (group 2; one papilloma and one squamous-cell carcinoma of the test canal, one papilloma of the hard palate), 2/21 rats treated with water-extracted snuff (group 3; one papilloma of the tongue, one papilloma of the hard palate) and 1/32 rats treated with enriched snuff (group 4; papilloma of the floor of the mouth). One olfactory tumour (esthesioepithelioma) also occurred in group 4. None of these results was statistically significant (Hecht *et al.*, 1986).

In an experiment of snuff-induced carcinogenesis, surgery to create a test canal in the lower lip was performed on male Sprague-Dawley rats at 8–9 weeks of age (see Table 80). Treatments were begun in groups of 30 rats 3–4 weeks after surgery and were continued for up to 108 weeks. One group received snuff (a brand available commercially in the USA), packed into the test canal with a spatula (at least 100 mg per application) twice daily on 5 days per week. (Snuff from the previous application was removed before the next treatment.) A second group of control rats received a cotton pellet dipped in saline twice daily on 5 days per week for 104 weeks. At the end of the study, complete necropsies were performed. Among the 29 rats of the snuff-treated group, five squamous-cell carcinomas (one lip, two hard palate, one nasal cavity, one forestomach), one squamous-cell carcinoma *in situ* (hard palate), three squamous-cell papillomas (one each of lip, hard palate and nasal cavity) and two undifferentiated lip sarcomas developed. No such tumours developed

Table 80. Tumour incidence in Sprague-Dawley rats following repeated treatment with snuff tobacco in the lip canal

Reference	Tumour site	Tumour type	Treated		Control	
			Benign	Malignant		
Johansson <i>et al.</i> (1989)	Lip		<i>n</i> = 29		<i>n</i> = 29	
		Sarcoma		2	0	
		Squamous-cell papilloma	1		0	
		Squamous-cell carcinoma		1	0	
		Hard palate	Squamous-cell papilloma	1		0
			Squamous-cell carcinoma		3 ^a	0
		Nasal cavity	Squamous-cell papilloma	1		0
			Squamous-cell carcinoma		1	0
		Forestomach	Squamous-cell carcinoma		1	0
Total tumours		3	8*	0		
Johansson <i>et al.</i> (1991)	Lip		<i>n</i> = 38		<i>n</i> = 30	
		Squamous-cell papilloma	2		0	
		Sarcoma		10	1	
		Hard palate		3	0	
		Total tumours	2	13*	1	

^a Including one 'in situ'

* $p < 0.01$

among the 29 control rats (all squamous-cell tumours, $p < 0.01$; malignant squamous-cell tumours, $p < 0.05$; Fisher's exact test) (Johansson *et al.*, 1989).

In an experiment designed to study the influence of 4-nitroquinoline *N*-oxide (4-NQO) and DMBA on snuff-induced carcinogenesis, male Sprague-Dawley rats underwent surgery to create a lip canal at 10 weeks of age (see Table 80). One group of 38 rats received 150–200 mg snuff (generic moist snuff type 1S3, University of Kentucky Research Center, USA) placed in the lip canal with a spatula twice daily on 5 days per week for 104 weeks. A second group of 30 control rats received cotton pellets dipped in saline once daily on 5 days per week for 100 weeks. Rats were killed when moribund, when they developed lip tumours or 104 weeks after the beginning of the study, and a complete necropsy was performed. Tumour incidences in different groups were compared by the Student *t* test and Fisher's exact test. Sarcomas of the lip occurred in 10/38 rats in the snuff-treated group ($p < 0.01$) and in 1/30 rats in the control group. Squamous-cell carcinomas and papillomas of the oral cavity (lip, palate and buccal mucosa) occurred in 3/38 rats in the snuff-treated group and in 0/30 rats in the control group. The incidence of epithelial tumours of the oral cavity of rats treated with snuff was not significantly different from that in controls. However, the combined incidence of malignant epithelial and mesenchymal tumours of lip and oral cavity was significantly greater in rats treated with snuff (three

squamous-cell carcinomas of the palate and 10 sarcomas of the lip; 13/38; $p < 0.01$) than in controls (one sarcoma of the lip; 1/30) (Johansson *et al.*, 1991).

(b) *Hamster*

Groups of 50 young Syrian golden hamsters [age and sex unspecified] received an instillation into the left cheek pouch of 10 mL of a thick paste of snuff. The opening of the pouch was ligated, and the animals were followed for up to 30 months. The contralateral pouches of 25 of these animals were filled with sand and gum and served as controls. After 13 months, 21/50 hamsters were still alive; 10 were alive at 24 months, but none were alive at 30 months. No tumour was observed in control or treated pouches (Peacock & Brawley, 1959; Peacock *et al.*, 1960).

A group of 35 male and female Syrian golden hamsters, 1–2 months of age, received a beeswax pellet that contained 20% snuff and 3% lime in the cheek pouch. A positive-control group of 71 hamsters was exposed to DMBA and 20-methylcholanthrene; a negative-control group of 36 animals was exposed to beeswax, which was used as a vehicle to prolong the retention time of the test substances. The animals were killed after 15–20 months or when moribund. Two of 35 animals exposed to snuff and lime and 2/36 exposed to beeswax only developed inflammatory lesions; among the positive controls, 23/56 developed malignant tumours (Dunham & Herrold, 1962).

Groups of four to seven male and female weanling Syrian golden hamsters [age unspecified] received twice-daily applications of 50 mg of a commercial US ‘Scotch’ (dry type) snuff, snuff and calcium hydroxide or calcium hydroxide alone into the cheek pouch on 5 days per week for up to 99 weeks. No local tumour was observed in any group (Dunham *et al.*, 1966).

A group of 84 male and female Syrian golden hamsters (BIO hamsters of the RB strain), aged 3–4 months, was exposed to 0.5 g snuff placed in a stainless-steel webbing cartridge attached to the lower incisors for 30 min per day on 5 days a week for 51 weeks. A group of 84 hamsters exposed to dry cotton served as negative controls and two groups of 84 animals exposed to benzo[*a*]pyrene and 24 animals exposed to DMBA served as positive controls. No tumour was found in the oral mucosa, except in the positive controls (Homburger, 1971). [The Working Group noted the short duration of this study.]

3.2.3 *Subcutaneous administration*

Rat

A group of 82 male and female albino (Händler) rats, 100 days of age, was given subcutaneous injections of 0.15 mL (50 mg) of an ethanol extract of Swedish snuff (Ettan) in tri-*n*-caprylin once a week for 84 weeks. A group of 81 male and female rats received the same schedule of injections of ethanol and tri-*n*-caprylin and served as controls. Malignant tumours developed in equal numbers in both test and control rats, and were ‘retothelsarcomas’ (one in each group), one uterine carcinoma (in a test animal) and one ovarian carcinoma (in a control animal) (Schmähl, 1965).

3.2.4 Administration with known carcinogens or modifiers

(a) Rat

Four groups of 10 female Sprague-Dawley rats, 3 months of age, with surgically created canals in the lower lip received the following treatments: group 1 was infected with herpes simplex type 1 virus (HSV-1) by scarification and topical application on the inside of the lower lip, followed, 10 days later, by administration of a standard Swedish (Röda Lacket) snuff into the canal morning and night on 5 days per week; group 2 was infected with HSV-1 and received no other treatment; group 3 was sham-infected with sterile saline followed by snuff treatment; and group 4 was given neither HSV-1 nor snuff and served as controls. The HSV-1 infection was repeated once after a 1-month interval, and snuff was administered 10 days later as before. Snuff treatment was continued for 18 months, after which time all animals were killed. Three animals each in groups 1 and 2 died from encephalitis shortly after the second infection with HSV-1. In the group exposed to HSV-1 and snuff, squamous-cell carcinomas of the oral cavity developed in 2/7 rats and a retroperitoneal sarcoma occurred in 1/7 rats. In the group exposed to snuff alone, 1/10 animals developed a squamous-cell carcinoma of the anus and 1/10 developed a retroperitoneal sarcoma. No such tumours occurred in the HSV-1-infected (0/7) or control (0/10) groups (Hirsch *et al.*, 1984a).

Surgery to create a test canal in the lower lip was performed on 150 male Sprague-Dawley rats at 8–9 weeks of age. Rats were randomized to five treatment groups initially of 30 rats each. Treatments were begun 3–4 weeks after surgery and continued for up to 108 weeks. Rats in group 1 received snuff (a brand available commercially in the USA) packed into the test canal with a spatula (at least 100 mg per application) twice daily on 5 days per week (snuff from the previous application was removed before the next treatment). Propylene glycol was applied three times a week for 4 weeks to the palate of each rat in group 2; no further treatment was given for the remainder of the study. 4-NQO dissolved in propylene glycol (approximately 0.13 mg/treatment) was applied three times per week for 4 weeks to the palate of each rat in group 3. Rats in group 4 received 4-NQO as for group 3 followed by snuff as for group 1. Group 5 (control) received a cotton pellet dipped in saline twice daily on 5 days per week for 104 weeks. At the end of the study, complete necropsies were performed; 28–29 rats in each group were evaluated. Squamous-cell papillomas and carcinomas of the lip, hard palate, tongue, nasal cavity, oesophagus and forestomach occurred only in groups 1, 3 and 4. Undifferentiated sarcomas of the lip occurred only in snuff-treated rats in groups 1 (two tumours) and 4 (three tumours). Among 29 rats in group 1 (snuff), five squamous-cell carcinomas (one lip, two hard palate, one nasal cavity, one forestomach), one squamous-cell carcinoma *in situ* (hard palate) and three squamous-cell papillomas (one each of the lip, hard palate and nasal cavity) developed. No such tumours developed among 29 rats in group 5 (all squamous-cell tumours, $p < 0.01$; malignant squamous-cell tumours, $p < 0.05$; Fisher's exact test) or among 28 rats in group 2 (propylene glycol control). At the sites specified above, a total of seven squamous-cell carcinomas and two squamous-cell papillomas occurred among 29 rats in group 3 (4-NQO) and eight squamous-cell carcinomas and

two squamous-cell papillomas among 28 rats in group 4 (4-NQO followed by snuff). Subsequent treatment with snuff did not enhance tumorigenesis by 4-NQO in the lip canal, oral cavity, nasal cavity, oesophagus or forestomach; the combined effects of 4-NQO and snuff were less than additive (Johansson *et al.*, 1989).

Male Sprague-Dawley rats underwent surgery to create a lip canal at 10 weeks of age. Group 1 (40 rats) was initiated with DMBA (0.1% in mineral oil) by placing cotton pellets containing approximately 70 mg of the solution in the lip canal three times per week for 4 weeks beginning at 12 weeks of age. Thereafter, the rats received a cotton pellet dipped in saline once daily on 5 days per week for 104 weeks. Group 2 was initiated with DMBA as for group 1 and subsequently received 150–200 mg snuff (generic moist snuff type 1S3, University of Kentucky Research Center, USA) placed in the lip canal with a spatula twice daily on 5 days per week (after removal of any material remaining from the previous application) for 104 weeks. Group 3 (38 rats) received snuff twice daily on 5 days per week for 104 weeks. Group 4 (40 rats) was initiated with 4-NQO dissolved (0.5%) in propylene glycol; approximately 70 mg of solution on a cotton pellet was placed in the lip canal three times per week for 4 weeks, after which rats were treated with a cotton pellet dipped in saline once daily on 5 days per week for 100 weeks. Group 5 (38 rats) was initiated with 4-NQO as for group 4 three times per week for 4 weeks, followed by snuff twice daily for 100 weeks. Group 6 (30 rats) received cotton pellets dipped in saline once daily on 5 days per week for 100 weeks. Rats were killed when moribund, when they developed lip tumours or 104 weeks after the beginning of the study, and a complete necropsy was performed. Tumour incidences in different groups were compared by the Student *t* test and Fisher's exact test. Sarcomas of the lip occurred in 0/40 rats in group 1, 9/40 rats in group 2, 10/38 rats in group 3, 1/40 rats in group 4, 25/38 rats in group 5 and 1/30 rats in group 6. The incidence of lip sarcomas in rats treated with snuff only (group 3) was significantly greater ($p < 0.01$) than that in controls (group 6), and was significantly increased by pretreatment with 4-NQO (group 5) but not DMBA (group 2). Squamous-cell carcinomas and papillomas of the oral cavity (lip, palate and buccal mucosa) occurred in 0/40 rats in group 1, 3/40 rats in group 2, 3/38 rats in group 3, 9/40 rats in group 4, 8/38 rats in group 5 and 0/30 rats in group 6. The incidence of epithelial tumours of the oral cavity in rats treated with snuff alone (group 3) was not significantly different from that in controls (group 6) and was not significantly modified by pretreatment with either DMBA (group 2) or 4-NQO (group 5). The combined incidence of malignant epithelial and mesenchymal tumours of lip and oral cavity was significantly greater in rats treated with snuff alone (group 3; three squamous-cell carcinomas of the palate and 10 sarcomas of the lip, 13/38; $p < 0.01$) than in controls (group 6; one sarcoma of the lip, 1/30) (Johansson *et al.*, 1991).

(b) *Hamster*

One hundred and twenty-five Syrian golden hamsters [age unspecified] were divided into seven groups of 15–20 animals, and the cheek pouches were inoculated with HSV-1, HSV-2 or culture medium. The mock and virus inoculations were performed once a

month for 6 consecutive months. In an effort to determine the effect of snuff on the mock- or virus-inoculated cheek pouches, a consistent amount (150 mg/pouch) of a commercially available snuff (USA) was placed into both the right and left pouches of half the animals twice daily on 5 days per week for 6 months. One group of animals was neither inoculated with HSV nor treated with snuff. At the end of the 6 months of simulated snuff dipping and 4 weeks after the final mock or virus inoculation, the hamsters were killed and the cheek pouches were removed for histopathological evaluation. Neither simulated snuff dipping nor HSV infection alone induced neoplastic changes in hamster cheek pouches (0/15 untreated controls, 0/15 mock inoculation, 0/15 mock inoculation plus simulated snuff dipping, 0/19 HSV-1 inoculation, 0/16 HSV-2 inoculation). HSV-1 or HSV-2 infection in combination with simulated snuff dipping resulted in epithelial dysplasia and invasive squamous-cell carcinoma in at least one cheek pouch in more than 50% of the animals (10/20 HSV-1 inoculation plus simulated snuff dipping; $p < 0.05$ (Fisher's exact test) versus untreated, mock inoculation or HSV-1-only groups; 11/20 HSV-2 inoculation plus simulated snuff dipping; $p < 0.05$ versus untreated, mock inoculation or HSV-2-treated groups) (Park *et al.*, 1986).

As part of an experiment to evaluate the effects of various modulating agents including various snuffs on hamster cheek pouch carcinogenesis initiated by DMBA, 110 randomly bred male Syrian golden hamsters, 4–6 weeks of age and weighing 90–100 g, were divided into six groups. DMBA in liquid paraffin solution at a concentration of 0.25% was applied to each cheek pouch of 55 hamsters at a dose of 0.125 mg in 50 μ L of oil twice a week for 1 month. Fifteen of these DMBA-treated hamsters served as positive controls. The snuffs studied included the Manglorian variety of ordinary (regular) snuff or scented snuff, both obtained from local markets. Snuff was suspended uniformly in liquid paraffin and applied to hamster cheek pouches at a dose of 20 mg per cheek pouch in a volume of 50 μ L twice a week. Each kind of snuff was administered to a group of 20 DMBA-initiated hamsters, beginning 2 weeks after the last DMBA treatment and continuing until 6 months after the first DMBA treatment, for a total of 4.5 months of snuff administration; the same treatments were applied to groups of 20 hamsters that had received no DMBA for an equivalent period. Fifteen untreated hamsters served as controls. All hamsters were killed 6 months after the first DMBA treatment, at approximately 7 months of age. No tumours were observed in either cheek pouches or forestomach in the 15 untreated control hamsters. Cheek pouch tumours occurred in 10/15 hamsters given DMBA only, in 3/20 hamsters given DMBA followed by regular (Manglorian) snuff and in 2/20 hamsters given DMBA followed by scented snuff. Cheek pouch tumours did not occur in hamsters given regular snuff or scented snuff alone. Forestomach tumours occurred in 15/15 hamsters given DMBA only, in 20/20 hamsters given DMBA followed by regular snuff and in 19/20 hamsters given DMBA followed by scented snuff. Forestomach tumours also occurred in 17/20 hamsters given regular snuff alone and in 15/20 hamsters given scented snuff alone. DMBA and both kinds of snuff induced forestomach tumours in hamsters. Neither kind of snuff induced tumours in hamster cheek pouches and both kinds of snuff inhibited carcinogenesis in cheek pouches by DMBA (Gijare *et al.*, 1990a).

3.3 *Bidi tobacco, mishri and naswar*

3.3.1 *Bidi tobacco*

Skin application

Mouse

In a study to determine the tumour initiation/promotion and complete carcinogenic potential of a processed blend of tobacco used for the manufacture of *bidis* [*bidi* tobacco is used for chewing by workers engaged in processing tobacco for the manufacture of *bidis*], groups of 15 female inbred hairless Swiss 'bare' mice (S/RVCri-ba strain), 6–7 weeks of age, were used. The supernatant of an aqueous extract of tobacco was lyophilized and dissolved in a minimal amount of DMSO; 1 μ L DMSO contained 2.5 mg aqueous extract of tobacco. An appropriate volume of DMSO was made up to 100 μ L with acetone to obtain the different doses. Doses of *bidi* tobacco extract corresponded to the original dry weight of tobacco. Eight groups of mice received topical applications on the back skin of 50 mg dried aqueous *bidi* tobacco extract in 100 μ L acetone twice a week for 40 weeks (complete carcinogenesis experiment), a single topical application of 5 mg *bidi* tobacco extract in 100 μ L acetone followed 1 week later by twice-weekly applications of 1.8 nmol 12-*O*-tetradecanoylphorbol-13-acetate (TPA) for 20 weeks (tumour initiation experiment) or a single topical application of 20 nmol DMBA followed by twice-weekly applications of acetone or 0.25, 2.5, 5 or 50 mg aqueous *bidi* tobacco extract or 1.8 nmol TPA (positive control) for 40 weeks (tumour promotion experiment). In order to determine the role of aqueous *bidi* tobacco extract in the progression of papillomas to carcinomas, skin papillomas were induced by initiation with 20 nmol DMBA and promotion with 1.8 nmol TPA for 20 weeks. TPA-dependent papillomas were allowed to regress during a 6-week treatment-free period and 50 mg aqueous *bidi* tobacco extract was applied twice weekly for 14 weeks. Aqueous extract of *bidi* tobacco did not exhibit skin tumour initiation, progression or complete carcinogenic activity. However, tumour promotion activity was observed with applications of 5 and 50 mg aqueous *bidi* tobacco extract after initiation with DMBA. The multiplicities of skin papillomas were significantly increased ($p < 0.01$) compared with DMBA-initiated controls. Tumour multiplicities were 9.69 ± 1.30 and 11.73 ± 1.38 tumours per mouse, respectively, versus 4.70 ± 1.01 tumours per mouse (control) (Bagwe *et al.*, 1994).

3.3.2 *Mishri*

(a) *Oral administration*

(i) *Mouse*

Four groups of Swiss mice, 8 weeks of age, were fed brown (26 males and 26 females) and black *mishri* (24 males and 26 females) in the diet at 10% for 20 months and were then maintained on standard diet. Animals were killed at 25 months of age or when moribund. Control animals (27 males and 31 females) received standard diet only. The incidence of

forestomach papillomas was 46% in male mice fed black *mishri*, 54% in male mice fed brown *mishri*, and 42% in female mice treated with *mishri* of either variety, which was significantly higher than that in control males (11%; $p < 0.001$) and females (3%; $p < 0.001$) (Kulkarni *et al.*, 1988).

(ii) *Rat*

Groups of 27 male and 24 female Sprague-Dawley rats, 8 weeks of age, were fed 10% brown *mishri* in the diet for 20 months. Animals were killed when moribund or at 25 months of age. Control animals (25 males and 30 females) received standard diet only. The incidence of forestomach papillomas was approximately 37% in both males (10/27) and females (9/24); no papillomas developed in control animals (0/25 males, $p < 0.001$; and 0/30 females, $p < 0.001$) (Kulkarni *et al.*, 1988).

Two groups of 30–31 male rats and two groups of 30 male Sprague-Dawley rats, 19–21 days of age, were maintained on vitamin A-sufficient and vitamin A-deficient diets, respectively. In one group of vitamin A-sufficient and one group of vitamin A-deficient rats, a daily dose of 3 mg *mishri* extract was administered by gavage five times a week over a period of 21 months. The two remaining groups (controls) received 0.05 mL DMSO for the same period. Autopsies were performed on all animals killed after 12 or 21 months. Liver, lung and stomach tissues were fixed and processed for microscopic examination. Rats given vitamin A-sufficient diet and *mishri* developed lung adenomas and stomach papillomas. Tumour incidence at 9–15 months and 16–21 months was 58% (7/12) and 5.5% (1/18), respectively. At these time periods, tumour incidences in vitamin A-deficient rats that received *mishri* extract were 88% (8/9) and 95% (20/21), respectively. Sixteen rats in the latter group developed malignant lung tumours. No tumours appeared in control rats given DMSO. Total tumour incidence in both vitamin A-sufficient and vitamin A-deficient rats given *mishri* extract was significantly higher ($p < 0.001$) than that in corresponding controls (Ammigan *et al.*, 1991).

(iii) *Hamster*

Two groups of 23 male and 26 female Syrian golden hamsters, 8 weeks of age, were fed a 10% black *mishri* diet and two groups of 28 males and 20 females were fed a brown *mishri* diet for 20 months. Twenty-three males and 23 females of the control groups were maintained on standard diet. Animals were killed at 25 months of age or when moribund. In male hamsters, the incidence forestomach papillomas was approximately 43% (12/28 brown *mishri* group and 10/23 black *mishri* group) and 25–27% in females (5/20 in the brown *mishri* group and 7/26 in the black *mishri* group), which was significantly higher than that in controls (9%, 2/23 males, $p < 0.01$; 4%, 1/23 females, $p < 0.02$). Forestomach carcinomas were observed in 2/23 male hamsters given the black *mishri* diet (Kulkarni *et al.*, 1988).

(b) *Skin application*

Mouse

Groups of 'nude' Swiss mice [number, sex distribution and age unspecified] received topical applications of 20 μ L of an acetone solution of the solid residue from a toluene extract of *mishri* [concentration not specified] on the midscapular region five times a week [duration of treatment not specified] or a single application of 200 nmol DMBA. The incidence of skin papillomas (~20%) was comparable in the two groups (Bhide *et al.*, 1987b).

Brown and black varieties of *mishri* were tested for their carcinogenic or promoting potential on skin in several groups of 8-week-old male Swiss mice (hairy) and male and female hairless Swiss bare mice. Three groups of 29–30 male Swiss mice received a single initiating dose of 200 nmol DMBA on the back skin and four groups of 16–21 male Swiss bare mice were initiated with doses of 50 or 200 nmol DMBA. Black or brown *mishri* extract was applied to the skin of two of the three groups of DMBA-initiated male Swiss mice and two groups of 30 uninitiated male Swiss mice (black *mishri* extract only) at doses of 2.5 mg on 5 days a week for 20 months. Two of the four groups of DMBA-initiated male Swiss bare mice and four groups of 17–24 uninitiated male or female Swiss bare mice were treated similarly with 2.5 mg or 1 mg black *mishri* extract. All mice were killed when moribund or at 24 months. Tumours more than 1 mm in diameter and lung and liver tissues were fixed and examined microscopically. Tumour incidence was analysed statistically using Yate's modification of the chi-square test. Skin tumours did not appear in either group of male Swiss mice treated with black *mishri* (2.5 mg dose) extract only or one control group of 30 male Swiss mice treated with acetone for 20 months. At the 1-mg dose of black *mishri* extract only, 6/21 male (33%) and 5/24 female (21%) Swiss bare mice developed skin papillomas and one male mouse developed a skin carcinoma. Six of 17 (35%) male and 5/23 (22%) female bare mice treated with 2.5 mg black *mishri* developed papillomas. No skin papillomas were observed in 21 male and 23 female controls treated with acetone only for 20 months. Four of 30 and 4/29 male Swiss mice treated with brown or black *mishri*, respectively, after DMBA initiation developed skin papillomas, but no carcinomas were observed. No tumours were observed in one group of 30 male Swiss mice treated with DMBA only. Eight of 20 and 7/16 male Swiss bare mice initiated with 200 or 50 nmol DMBA and promoted with 1 mg or 2.5 mg black *mishri* extract, respectively, developed skin papillomas. Carcinomas were observed in 2/20 and 4/16 animals, respectively. Skin papillomas were observed in two groups of male Swiss bare mice treated with both DMBA doses only (9/21 and 7/17, respectively). Two carcinomas were also observed in each group. Promotion with brown or black *mishri* extract significantly ($p < 0.05$) increased the total tumour incidence in Swiss mice but not in Swiss bare mice. However, application of *mishri* extracts alone to the skin induced papillomas in male and female Swiss bare mice (Kulkarni *et al.*, 1989).

3.3.3 Naswar

(a) *Application to the cheek pouch*

Hamster

In one experiment, a group of 28 female and 33 male Syrian hamsters [assumed to be 1–3 months of age] received applications of *naswar* (mixture of tobacco, lime, ash, plant oil and water) as a dry powder into the left cheek pouch for life; another group of 13 females and 24 males received *naswar* as a 50% suspension in refined sunflower oil in the cheek pouch (total dose per animal, 6.2–147.5 g; mean 53.8 ± 2.5 g). The animals were followed until death. No tumour was found at the site of *naswar* application. The average lifespan of animals that received *naswar* (50.8 weeks) was slightly shorter than that of untreated animals (57.3 weeks) or that of hamsters that received sunflower oil alone (57.6 weeks). Of 64 treated hamsters in both groups still alive at the time of appearance of the first tumour (17 and 37 weeks), 13 developed tumours: seven liver-cell tumours and one liver tumour of 'mixed structure', three tumours of the adrenal glands (described as a 'carcinoma of adrenal cortex' and as 'adenoma, chromaffinoma type' or 'carcinoma of adrenal cortex'), one forestomach papilloma, three uterine tumours (leiomyoma and/or fibromyoma and/or cysts), one skin melanoma, one benign skin tumour and one unspecified tumour of the large intestine. Among 110 untreated animals and 10 animals treated with sunflower oil, 53 survived to the appearance of the first tumour (59 weeks), and two developed tumours (one adrenal cortex neoplasm and one forestomach papilloma) (Kiseleva *et al.*, 1976).

In another experiment, *naswar* was introduced as a dry powder or as a 50% suspension in refined sunflower oil into the cheek pouch of 184 male and female hamsters, 1–3 months of age. *Naswar* was administered throughout life (total mean dose per animal, 53.8 ± 2.5 g). No tumour was found at the site of application. However, 26/138 hamsters that survived to the appearance of the first tumour (17 weeks after the experiment began) developed neoplasms at various sites: 13 tumours of the liver, six of the adrenal glands, five papillomas of the forestomach, four of the uterus and five other tumours. The mean survival time of the animals was 50.9 ± 1.9 weeks (Miličevskaja & Kiseleva, 1976). [The Working Group noted deficiencies in reporting the number of males and females and that the incidences of different tumour types were not indicated.]

(b) *Skin application*

Hamster

A group of 19 female and 31 male Syrian hamsters [assumed to be 1–3 months of age] received topical applications of a suspension of *naswar* (45% tobacco, 8% lime, 30% ash, 12% plant oil and 5% water) on the dorsal skin. The average lifespan was 44.4 weeks. Three of nine animals still alive at the time of appearance of the first tumours (53 weeks) developed neoplasms: one liver 'lymphangioendothelioma', one adrenal gland tumour and one forestomach papilloma. No local tumour occurred. In the untreated control group

(69 females and 41 males), 2/45 hamsters that survived to the appearance of the first tumour (59 weeks) developed tumours: one adrenal cortex neoplasm and one forestomach papilloma (Kiseleva *et al.*, 1976).

(c) *Administration with known carcinogens or modifiers*

Hamster

A group of 30 Syrian hamsters [age and sex unspecified] received a single application of 0.1 mg DMBA as a 0.1% solution in benzene in the cheek pouch. Another group of 30 hamsters received the same treatment, followed 7 weeks later by daily applications of *naswar* (composition as described above) as a dry powder in the cheek pouch; the total dose ranged from 11.2 to 102.5 g (mean, 38.9 ± 5.2 g). Three of 11 survivors at the time of appearance of the first tumour (23 weeks) that received DMBA alone developed tumours: one rhabdomyoblastoma of the cheek pouch and two papillomas of the forestomach. Six of 11 animals still alive at 50 weeks that received DMBA plus *naswar* had tumours: five papillomas of the forestomach and one cystic epithelioma of the skin of the jaw (Milievskaia & Kiseleva, 1976). [The Working Group noted the small number of animals that survived to the time of observation of the first tumour.]

[In consideration of the whole study by Kiseleva *et al.* (1976) and Milievskaia and Kiseleva (1976), the Working Group noted that the effective number, i.e. the number of animals that survived to the observation of the first tumour, was calculated separately for treated (number of survivors at 17 weeks with the dry powder) and control (59 weeks) animals. Therefore, the effective number of control animals should have been higher in the first experiment. High mortality of animals was noted, even in control groups, in the period preceding observation of the first tumour; average lifespan of untreated control animals was 57.3 weeks. The sex of animals in which liver tumours were found was not indicated.]