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# Comparison of Fresh and Room-Aged Cigarette Sidestream Smoke in a Subchronic Inhalation Study on Rats

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Two experimental types of cigarette sidestream smoke (SS) were compared in a subchronic inhalation study on rats. Fresh SS (FSS) was generated continuously from the reference cigarette 2R1. Room-aged SS (RASS) was generated by aging FSS for 1.5 h in a room with noninert surfaces with materials typically found in residences or offices. Male Sprague–Dawley rats were head-only exposed to three dose levels of each SS type and to filtered, conditioned fresh air (sham-exposure) for 6 h/day, 7 days/week, for 90 days. Room-aging resulted in decreased concentrations of various SS components, *e.g.*, total particulate matter (TPM) and nicotine, while other components, such as carbon monoxide (CO), were not affected. The CO concentrations were 6, 13, and 28 ppm for both SS types. TPM concentrations were between 0.6 and 8.7 μg/liter and thus up to 100-fold above the maximum of average concentrations of respiratory suspended particles reported for environmental tobacco smoke. Slight reserve cell hyperplasia in the anterior part of the nose as well as hyperplastic and metaplastic epithelial changes in the larynx were the only observed dose-dependent findings. The metabolism of benzo(*a*)-pyrene—as a proxy for polycyclic aromatic hydrocarbon metabolism—was induced in the nasal respiratory epithelium and in the lungs while no effect was seen in the nasal olfactory epithelium. The lowest-observed effect level was 6 ppm CO or 0.6 μg TPM/liter. Most of the effects seen were less expressed in RASS- than in FSS-exposed rats when compared on the basis of the CO concentrations. When compared on the basis of TPM, these effects were equally pronounced for both SS types, suggesting a major role of particulate matter-associated compounds. All findings reverted to sham control levels following a 42-day postinhalation period. © 1998 Society of Toxicology.

Exposure to environmental tobacco smoke (ETS) has been reported to be associated with adverse health effects (e.g., US Environmental Protection Agency, 1992). Experimental toxicology can provide data on this association as discussed in a recent symposium overview by Witschi *et al.* (1995a), e.g., on possible effect thresholds via the determination of dose–response relationships. However, one of the most critical issues in investigating potentially toxic effects of environmental materials is the selection of an appropriate experimental surrogate for the test material.

ETS is a combustion product composed of sidestream smoke (SS) as well as exhaled mainstream smoke (MS) (First, 1985; Löfroth *et al.*, 1989; Benner *et al.*, 1989; Eatough *et al.*, 1989, 1990; Baker and Proctor, 1990; Guerin *et al.*, 1992). ETS is highly diluted with room air and undergoes chemical and physical changes in composition as a function of aging, e.g., by contact with various surfaces over time (Eatough *et al.*, 1990). Exhaled MS can contribute from 15 to 43% of the particulate matter in ETS, but only small amounts of the gas-phase constituents (Baker and Proctor, 1990).

Real environmental atmospheres are not reproducibly available as required for a laboratory experiment, most notably for a prolonged inhalation study. In previous rodent inhalation studies to assess the biological activity of ETS, diluted SS was used as a surrogate (e.g., von Meyerinck *et al.*, 1989; Coggins *et al.*, 1993a; Joad *et al.*, 1993; Teredesai and Prühs, 1994; Witschi *et al.*, 1995b). It was used fresh or moderately aged by contact with relatively inert surfaces in whole-body exposure chambers made of stainless steel and glass and for short duration (≤5 min; Ayres *et al.*, 1994; Teague *et al.*, 1994), due to the high number of air-changes per hour in these exposure systems. These aging conditions are less representative of human residences or office environments, where there are materials with large surface areas and adsorption potential, such as curtains or carpets. In addition, mean air changes of approximately 0.5 per hour are characteristic (Seppänen, 1995) for residences, which would correspond to a mean ETS age of 2 h.

In order to address the relevance of SS aging in experimental studies, the objective of the present study was to compare respiratory tract responses in the rat to fresh SS (FSS) and room-aged SS (RASS). RASS was generated by aging FSS for 1.5 h (mean age) under experimental conditions which are more realistic for the human environment than those previously used, or which even exaggerate realistic conditions for the purpose of the experiment. To enable a direct comparison,

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dosing of FSS and RASS was based on carbon monoxide (CO), an SS component which has not been found to change during aging (Eatough *et al.*, 1990; Voncken *et al.*, 1994). In addition, in experimental studies, the CO concentration is proportional to and thus representative of the number of cigarettes smoked, a measure frequently applied to determine human ETS exposure in experimental studies (Muramatsu *et al.*, 1983; Scherer *et al.*, 1990) and epidemiological studies (pack-years: e.g., Fontham *et al.*, 1994). Rats were head-only exposed 6 h/day, 7 days/week for 90 days. The concentration levels used in the present study are comparable to those used in previous SS inhalation studies ("extreme" to "exaggerated"; Coggins *et al.*, 1993a) to determine lowest- or no-observed effect levels. The major end points in this study were respiratory tract histopathology and the benzo(*a*)pyrene (B(*a*)P) metabolism in nasal epithelia and the lungs.

## MATERIALS AND METHODS

***Experimental design.*** Rats were head-only exposed to FSS and to RASS as well as to filtered, conditioned fresh air (sham-exposed group) 6 h per day, 7 days per week, for 90 days. FSS and RASS were administered at three dose levels. The CO concentrations for the respective levels were equal for both SS types and were up to 29 ppm The TPM concentrations were up to 9 μg/liter. There was a 42-day postinhalation period for rats of the control and high concentration groups to investigate the delayed occurrence, persistence, and/or reversibility of findings. The histopathology of the respiratory tract and the B(*a*)P metabolism—as a proxy for polycyclic aromatic hydrocarbon metabolism—in nose and lungs were the major end points studied. The main dose parameter to compare the biological activity of FSS and RASS was the CO concentration The results are also discussed on the basis of the respective TPM concentrations.

The study was performed in conformity with Good Laboratory Practice (OECD, 1981a; German Law on Chemicals, 1990) and the American Association for Laboratory Animal Science (AALAS) Policy on the Humane Care and Use of Laboratory Animals (1991).

***Experimental animals.*** Male outbred Sprague–Dawley rats (Crl:CDBR), bred under specified pathogen-free conditions, were obtained from Charles River (Raleigh, NC). This strain was chosen because of the large amount of published data available, in particular because of its frequent use in cigarette smoke inhalation studies (e.g., Joad *et al.*, 1993; Coggins *et al.*, 1993a,b; Teredesai and Prühs, 1994). A previous study did not show differences between male and female rats in susceptibility to SS-induced effects (Coggins *et al.*, 1992). Thus, only one sex was used to allow sufficiently large group sizes and group numbers. Male rats were preferred based on our greater experience with them.

The respiratory tracts of randomly selected rats were examined histopathologically on arrival; no unusual findings were observed. Serological screening performed on arrival, after 90 days of inhalation, and at the end of the postinhalation period did not detect antibodies to rat-related viruses, such as lymphocytic choriomeningitis virus, mouse adenovirus, murine poliovirus, parainfluenza virus type 1, parvovirus H-1, rat parvovirus, pneumonia virus of mice, rat coronavirus/sialodacryoadenitis virus, and reovirus type 3, as well as to the bacterium *Mycoplasma pulmonis.*

The rats were identified individually using subcutaneous transponders (IMI-1000, Plexx, Elst, Netherlands; data acquisition by DAS-4001, Uno, Zevenaar, Netherlands). Following a 16-day preexposure acclimatization period they were randomly allocated to the six SS exposure groups and the sham exposure group (48 rats per group). At the end of the inhalation period, 20 and 10 rats per group were used for the histopathological examination and the analysis of the B(*a*)P metabolism, respectively. At the end of the postinhalation period, 12 and 6 rats per sham and high-dose groups were used for the aforementioned two end points. The age of the rats at the start of the inhalation period was 47 days. The body weight at that time was 196 g (SD: 13 g).

The rats were kept in an animal laboratory unit with controlled hygienic conditions. The laboratory air (filtered, fresh air) was conditioned. Positive pressure was maintained inside the laboratory unit. Room temperature and relative humidity were maintained at 22°C (SD: 1°C) and 69% (SD: 10%), respectively. The light/dark cycle was 12 h / 12 h. The rats were housed in transparent polycarbonate cages, two rats per cage. The bedding material was autoclaved softwood granulate (SK-20/50, Braun & Co., Battenberg, Germany). A sterilized, fortified pellet diet (MRH FF, Eggersmann, Rinteln, Germany) from cage lid racks and heat-treated tap water from water bottles with autoclaved sipper tubes were supplied *ad libitum* in each cage. Food and drinking water were not available to the rats during the daily exposure periods. Good hygienic conditions within the animal housing and exposure room were maintained as evidenced, among other criteria, by negative results for the bacteriological examinations of the rat diet, drinking water, and the laboratory air and selected surfaces.

***Generation of FSS and RASS.*** The University of Kentucky reference cigarette 2R1 was used to generate the test atmospheres (MS yields per cigarette: 44.6 mg TPM, 2.45 mg nicotine, and 25.1 mg CO; Tobacco and Health Research Institute, 1990). They were conditioned and smoked in basic accordance with the International Organization for Standardization (ISO) standards 3402 (1978) and 3308 (1986), respectively, as generally applied to MS generation The cigarettes were conditioned at 22°C, 60% relative humidity, for at least 8 days. Two automatic 30-port smoking machines were used for smoke generation (Reininghaus and Hackenberg, 1977). Mean puff volumes of 35 ml were generated taking 1 puff/min with a 2-s puff duration using a four-piston pump (Battelle, Geneva, Switzerland) resulting in approximately 12 puffs/cigarette at a mean butt length of 23 mm. The MS was exhausted. SS was collected using a circular hood made of glass and stainless steel on top of the smoking machines. The three FSS concentrations were obtained by dilution with filtered, conditioned fresh air. The maximum age of the FSS was approximately 10 s. Using a second smoking machine, RASS was generated by continuously passing diluted FSS at a rate of 20 $m^3$/h through a 30-$m^3$ experimental aging room with non-inert surfaces, resulting in RASS of a mean age of 1.5 h. In the aging room were materials usually found in residences and/or offices, such as wallpaper painted with a latex-based white paint (29 $m^2$), window glass (2 $m^2$), and a wool carpet (11 $m^2$). For experimental purposes, the surface areas of some of the materials were exaggerated relative to the size of the room, i.e., a 26-$m^2$ wool curtain and a bookshelf with a surface area of 7 $m^2$ untreated pine wood with approximately 50 books or magazines with a surface area of 3 $m^2$. The materials in the aging room were unexposed at the start of the inhalation and not replaced throughout the 90 days. A ceiling fan was operated to facilitate uniform distribution of the RASS. The room was illuminated by fluorescent "daylight" lamps (Lumilux L58W/11, Osram, Munich, Germany). Two painted heat exchangers (approximately 60 $m^2$ surface area) were used to keep the room temperature constant (mean: 22.6°C, SD: 1.9°C). FSS and RASS were conveyed via glass tubing to the exposure chambers. RASS generation was started approximately 3 h before the start of the daily exposure to achieve a steady-state test atmosphere for inhalation. During overnight, nonsmoking periods, the room was flushed with filtered, conditioned fresh air at 20 $m^3$/h.

***Analytical characterization of FSS and RASS.*** At designated time intervals, specified analytes were determined to characterize the test atmospheres, to evaluate the reproducibility of the test atmosphere generation, and to exclude cross-contamination in the sham-exposed group. Samples were collected directly at the exposure chambers. CO was continuously monitored using nondispersive infrared photometry (Ultramat 5E, Siemens, Brussels, Belgium) of the gas phase of the test atmospheres. TPM was gravimetrically (A200S, Sartorius, Göttingen, Germany) determined at least once per day after trapping particulate matter on a Cambridge type glass fiber filter (Gelman, Ann Arbor, MI). The other analytes were determined at least at weekly intervals.

TABLE 1
Chemical Characterization of Test Atmospheres: Sham-Exposed Control and High-Concentration FSS and RASS Groups

| Parameter | N | Sham | FSS | RASS |
|---|---|---|---|---|
| TPM (μg/liter) | 91 | 0 | 8.7 ± 0.7 | 2.6 ± 0.3 |
| Carbon monoxide (ppm) | 89 | <1.5 | 27.8 ± 1.2 | 28.7 ± 1.7 |
| Carbon dioxide (ppm) | 7–13 | 302 ± 75 | 452 ± 63 | 502 ± 48 |
| Nicotine (μg/liter) | 51 | <0.05 | 2.21 ± 0.43 | 0.52 ± 0.10 |
| Nitric oxide (ppm) | 12 | 0.01 | 0.68 ± 0.09 | 0.69 ± 0.14 |
| Nitrogen oxides (ppm) | 12 | 0.02 ± 0.02 | 0.68 ± 0.08 | 0.69 ± 0.13 |
| Formaldehyde (ppm) | 12–13 | <0.02 | 0.54 ± 0.05 | 0.16 ± 0.01 |
| Acetaldehyde (ppm) | 12–13 | <0.04 | 0.68 ± 0.06 | 0.74 ± 0.05 |
| Acrolein (ppm) | 11–13 | <0.02 | 0.10 ± 0.01 | 0.10 ± 0.01 |
| Ammonia (μg/liter) | 12 | — | 3.71 ± 0.65 | 1.39 ± 0.37 |

*Note.* In the sham-exposed control group, the raw data showed a median TPM concentration of 0.29 μg/liter (25 and 75% quartiles: 0.19 and 0.43 μg/liter) which was subtracted from the raw TPM means for all groups. The nitric oxide concentration in the sham-exposed group is given as the median (25 and 75% quartiles: 0.00 and 0.02 ppm).

Carbon dioxide was analyzed using nondispersive infrared photometry (Ultramat 5E) of the gas phases. For nicotine determinations, samples were drawn on sulfuric acid-impregnated diatomite tubes (Extrelut, Merck, Darmstadt, Germany). Extraction was performed with *n*-butylacetate containing 5% (v/v) triethylamine. Nicotine was analyzed by capillary gas chromatography (HP5890, Hewlett Packard, Waldbronn, Germany) with a DB-5 column (15m × 0.25 mm, J and W, Carlo Erba, Hofheim, Germany) using a nitrogen-phosphorus detector. Nitrogen oxides were determined by chemoluminescence in the gas phase of the test atmospheres after catalytic reduction and reaction with ozone ($NO/NO_x$-analyzer CLD 700AL, Tecan, Hombrechtikon, Switzerland). The aldehydes were determined by reverse-phase HPLC (Hypersil ODS, 5 μm, 250 × 4 mm, Hewlett Packard) and UV detection (HP1050 Multiple Wavelength Detector, Hewlett Packard) of the 2,4-dinitrophenylhydrazine (DNPH) derivatives after trapping in acid DNPH/acetonitrile solution. Ammonia was determined by liquid chromatography (Lichrosorb RP-18, 10 μm, 250 × 4.5 mm, Merck) and fluorescence detection (650-10S, Perkin Elmer, Überlingen, Germany) of fluorescamine derivatives after trapping on sulfuric acid-impregnated diatomite. For the determination of the particle size distribution, the particles were precipitated on a filter strip in a spinning spiral duct (Stöber and Flachsbart, 1969) followed by a fluorometric determination of the particulate matter eluted from sequentially cut filter pieces. The particle size distribution was calculated using linear regression analysis after probit transformation (Finney, 1971).

The temperature in the exposure chambers was monitored continuously using a digital thermometer (Tastotherm D700, IMPAC Elektronic, Frankfurt, Germany). The relative humidity was determined psychrometrically (Therm 2246, Ahlborn, Nürnberg, Germany) in the atmosphere of the sham-exposed group, which also served as a proxy for the filtered, conditioned fresh air used to generate and dilute the SS.

***Animal exposure system.*** The rats were head-only exposed to the test atmospheres for 6 h/day, 7 days/week for 90 days. The head-only exposure mode was used to ensure reproducible inhalation of the test atmospheres and to minimize uptake by noninhalation routes, e.g., by dermal absorption or ingestion following preening of the fur (Mauderly *et al.*, 1989). The exposure chambers consisting of glass, stainless steel, and brass (INBIFO, octagonic cross-section, 303 $cm^2$; height, 72 cm) were equipped with custom-made glass tubes for animal exposure that were conical at the front end to fit the rat skull and sealed with rubber stoppers at the rear end. The rats were restrained in the front part of the tube with their heads protruding into the stream of the test atmosphere, which passed through the exposure chamber at a rate of approximately 100 liters/min corresponding to approximately 2 liters/(min × rat). The tubes tilted slightly caudally in order to minimize contact of the rat with its urine. The glass tube size was varied according to the body weight of the rats. The position of the rats in the exposure chambers was systematically changed on a daily basis.

Sham-exposed rats were exposed to filtered, conditioned fresh air under the same conditions as the test atmosphere-exposed rats.

During the postinhalation period, 18 rats of the high dose and the sham-exposed groups were kept in polycarbonate cages, 2 per cage. Diet and drinking water were available to the rats *ad libitum*.

***In-life observations.*** The rats were observed for mortality, signs of overt toxicity, or injuries when they were transferred from their cages to the exposure chambers and when being transferred back to their cages. More detailed checks on general condition and behavior of the rats were performed on three rats per group and day shortly after the end of the daily exposure.

***Body weight determinations.*** The body weight of individual rats was determined one day after arrival of the rats, at the start of the inhalation period, and once per week during the inhalation and postinhalation periods.

***Biomonitoring.*** In order to provide an estimate of the amount of test atmosphere taken up by the rats, respiratory frequencies and volumes were determined on at least six rats per group during exposure by whole-body plethysmography in the exposure tubes (Coggins *et al.*, 1981). The differential pressure signals (Validyne MP45, HSE, March-Hugstetten, Germany) were digitized and analyzed using the Ratvent program developed by S. A. Buch, Stowmarket, Suffolk, United Kingdom.

To monitor exposure to the test atmospheres, steady-state proportions of blood carboxy-hemoglobin (HbCO) were determined once in three rats per group according to Klimisch *et al.* (1974). Blood samples were taken after at least 5 h of exposure from rats withdrawn for a short period of time from the exposure tubes by puncturing the retro-orbital sinus with glass micropipettes.

To provide a rough estimate of the amount of nicotine taken up by the rats, urine was collected from five rats per group during the 6-h exposure period using specially modified exposure tubes and during the following 18 h using custom-made metabolism cages. The two samples per rat were combined to determine nicotine, cotinine, and trans-3′-hydroxycotinine by gas chromatography (Voncken *et al.*, 1989).

***Necropsy and gross pathology.*** The rats were not fasted before necropsy On the day following the last exposure, 20 rats per group were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body w) and subsequently sacrificed by exsanguination by transsecting the abdominal aorta. The carcasses were weighed and subjected to a complete gross examination under supervision of a veterinary pathologist with special attention paid to the respiratory tract. The same procedure was followed for 12 rats per group of the high-dose and sham-exposed groups at the end of the postinhalation period.

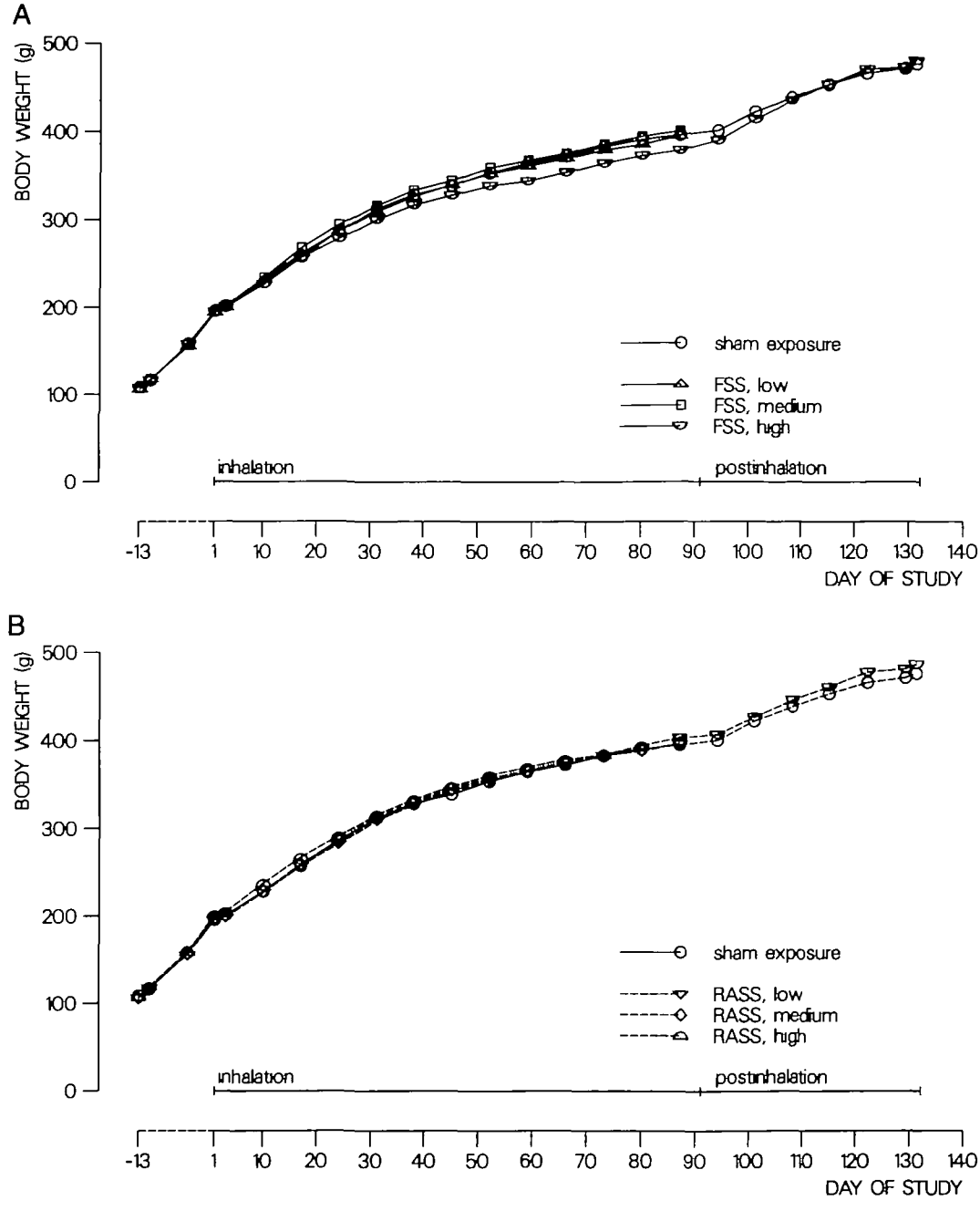


FIG. 1. Body weight development during the inhalation (all groups, 48 rats/group) and postinhalation periods (sham-exposed and high-dose groups, 18 rats/group). (A) Sham-exposed control and FSS groups; (B) sham-exposed control and RASS groups. Relative standard deviations for the individual groups were <14% for each time point.

***Organ weights.*** Absolute weights of lungs with larynx and trachea, liver, adrenal glands, testes, and kidneys were determined. The organ weights relative to body weight were calculated using the weights of the exsanguinated carcasses. The time between exsanguination and organ weight determination was kept to a minimum and did not exceed 15 min.

***Histopathology.*** Lungs were excised with larynx and trachea and fixed by intratracheal instillation with EAFS (40% ethanol, 5% acetic acid, 10% formaldehyde, 45% isotonic saline, v/v, Harrison, 1984) at 20 cm water pressure to achieve physiological distention of the lung. The skin, eyes, lower jaw, and brain were removed from the head and the nose was gently flushed with 10% neutral buffered formaldehyde solution via the nasopharyngeal duct. The head was fixed in 10% neutral buffered formaldehyde solution for 1 day and, subsequently, in 4% solution for 3 to 4 days.

Prior to trimming, the head was decalcified with 5% nitric acid in an ultrasonic bath. The nose was trimmed and transverse sections were cut according to Young (1981) to obtain two tissue slices at the following levels: (1) immediately posterior to the upper incisor teeth, (2) at the incisive papilla. The laryngeal transverse sections were cut at the base of the epiglottis and at

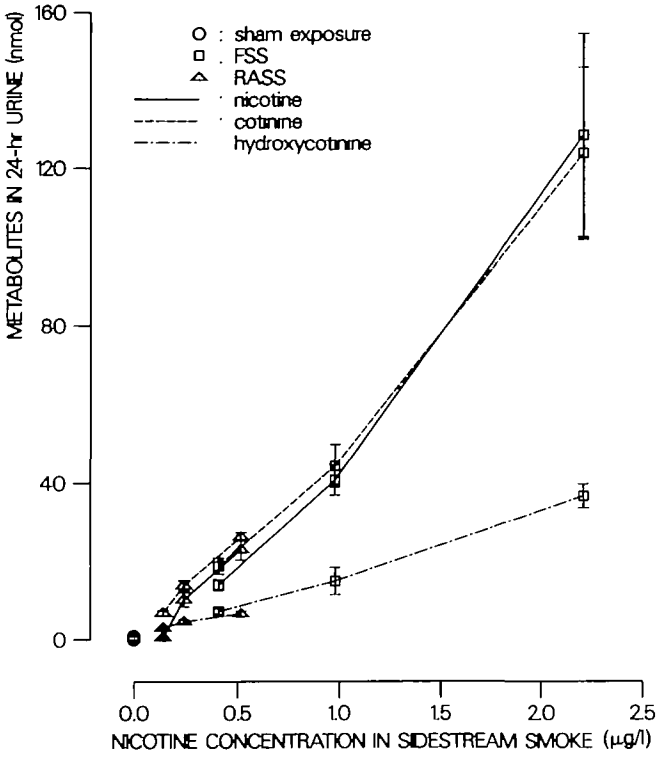


FIG. 2. Amounts of nicotine and its main metabolites recovered from urine samples collecting during the 6-h exposure period and the 18 h between exposures as a function of the SS nicotine concentrations (means ± SE).

the arytenoid projections according to Lewis (1981). The trachea was trimmed and longitudinally cut at the tracheal bifurcation A frontal section of the left lung including the left main bronchus and its major branches was cut according to Lamb and Reid (1969). The tissues were processed, embedded in Paraplast, cut at 5 to 6 µm thickness, and stained with hematoxylin and eosin (HE). In addition, the sections of the trachea and lung were stained with Alcian blue/periodic acid Schiff's reagent to demonstrate goblet cells.

All slides were read by a veterinary pathologist with experience in cigarette smoke-related changes in the respiratory tract of rodents without knowledge of the treatment groups. All histopathological findings were scored according to a defined severity scale from 0 to 5 (marked effects). Mean severity scores were calculated based on all rats in a group.

***Morphometrical analysis of larynx.*** The thickness of the laryngeal epithelium was determined without knowledge of the treatment groups on a standardized HE-stained section at the arytenoid projections, which included the ventral depression, floor of the larynx, and vocal cords. At each of these sites, the epithelial thickness was measured at 10 specified locations directly on the microscopic image using a Leica Microvid system (Bensheim, Germany).

***Analysis of the B(a)P metabolism.*** The microsomal B(*a*)P metabolism was assessed by fluorescence detection of 5 B(*a*)P metabolites after reverse-phase HPLC separation. The activity was normalized using the microsomal protein content. Reference materials for the B(*a*)P metabolites (3-hydroxy-, 9-hydroxy, 4,5-diol-, 7,8-diol-, and 9,10-diol-B(*a*)P) were obtained from the U.S. National Cancer Institute (Chemical Carcinogen Repository Midwest Research Institute, Kansas City, MO).

Rats were euthanized as described above. The right lung and the nasal respiratory and olfactory epithelia (NRE, NOE) were removed and stored at −70°C. Lung and nasal microsomes were isolated by differential ultracentrifugation according to Grover *et al.* (1974) with minor modifications.

Microsomal suspensions (lungs, 100 to 600 µg protein; NRE, 100 to 200 µg protein, NOE, approx. 150 µg protein) were incubated for 60 min at 37°C in Tris/HCl buffer (50 mM, pH 7.6) in the presence of B(*a*)P (80 µM), $NADP^+$ (370 µM), glucose-6-phosphate (2.5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), $MgCl_2$ (5 mM), and EDTA (240 µM) (three replicates per rat and tissue). The incubation was stopped by adding methanol containing benz(*a*)anthracene as an internal standard. After centrifugation, the supernatant was directly injected into the HPLC. Mono and dihydroxy B(*a*)P metabolites were separated using a Hewlett Packard HPLC 1090 with a Nucleosil 100-5 C-18 precolumn (5 µm, 4 cm × 4 mm, Knauer KG, Oberursel, Germany) linked to a Novapak RP-18 column (5 µm, 15 cm × 3 9 mm, Millipore/Waters, Eschborn, Germany). The solvents for step gradient elution were solvent A (10 mM $KH_2PO_4$, pH 4.8) and solvent B (acetonitril). Peak detection and quantitation were performed using a Hewlett Packard 1046 A fluorescence detector equipped with a 5-µl flow cell. Excitation/emission wavelengths were as follows (in the order of elution from the column): 287/421 nm for 9- and 3-OH-B(*a*)P; 248/400 nm for 4,5- and 7,8-diol-B(*a*)P; 287/404 nm for 9,10-diol-B(*a*)P; and 287/421 nm for benz(*a*)anthracene and B(*a*)P.

The protein concentrations of the microsomal suspensions were determined according to Lowry *et al.* (1951), as modified by Peterson (1983), using an automated micromethod with bovine serum albumin as a standard. Duplicate determinations were performed.

***Statistical analyses.*** For the comparisons of the FSS- and RASS-exposed groups with the sham-exposed group, respectively, the following statistical tests were performed: for the overall comparison, the one-way analysis of variance for continuous data and the generalized Cochran–Mantel–Haenszel test (Koch and Edwards, 1988) for ordinal data were used with the CO concentration as the stratifying variable. If the overall comparison showed a significant difference, then for a pairwise comparison the Duncan test (Duncan, 1955) was applied for continuous data and the generalized Cochran–Mantel–Haenszel test for ordinal data. For the comparison of FSS- with RASS-exposed groups, the two-way analysis of variance for continuous data and the Cochran–Mantel–Haenszel test for ordinal data were used.

All tests were conducted at the nominal level of significance of $\alpha = 0.05$ (two-tailed). Due to the large number of parameters analyzed, no correction for multiple testing was applied, which would have made the tests very insensitive. Statistical significances, therefore, have to be considered as explorative indicators rather than confirmatory evidence. No correction for multiple testing was applied.

## RESULTS

### *Test Atmospheres*

The test atmospheres were generated reproducibly throughout the 90-day inhalation period. As targeted, the CO concentrations of FSS and RASS were equal for each of the three dose levels. The CO concentrations (mean ± SD) for the low-, medium-, and high-dose levels of FSS were 5.5 ± 0.4, 12.6 ± 0.6, and 27.8 ± 1.2 ppm, respectively. The respective values for RASS were 5.5 ± 0.4, 12.2 ± 0.6, and 28.7 ± 1.7 ppm. The TPM concentrations for the low-, medium-, and high-dose levels of FSS were 1.5 ± 0.6, 3.6 ± 1.2, and 8.7 ± 0.7 µg/liter, respectively. The respective values for RASS were 0.6 ± 0.3, 1.2 ± 0.7, and 2.6 ± 0.3 µg/liter. The time course of the daily mean TPM concentrations in the high-dose groups was reported separately (Voncken *et al.*, 1994). The analytical characterization of the high-dose test atmospheres as well as that of the sham-exposed group is presented in Table 1. The individual smoke components in the medium- and low-dose FSS and

TABLE 2
Histopathological Findings after 90 Days of Inhalation

| Parameter | Sham | FSS | | | RASS | | |
|---|---|---|---|---|---|---|---|
| | | Low | Medium | High | Low | Medium | High |
| Nasal cavity, level 1 | | | | | | | |
| Reserve cell hyperplasia of | 0 | 0 | 0 | 0.6 ± 0.2* | 0 | 0 | 0.1 ± 0.1 |
| respiratory epithelium† | 0/20 | 0/20 | 0/20 | 8/20 | 0/20 | 0/20 | 1/19 |
| Larynx | | | | | | | |
| Base of epiglottis | | | | | | | |
| Squamous metaplasia of | 0 | 0.2 ± 0.1 | 0.6 ± 0.1* | 1.0 ± 0.2* | 0 | 0.2 ± 0.1* | 0.7 ± 0.1* |
| pseudostratified epithelium† | 0/17 | 3/19 | 10/18 | 15/19 | 0/19 | 4/19 | 12/20 |
| Hyperplasia of squamous | 0 | 0.2 ± 0.1 | 0.8 ± 0.2* | 1.4 ± 0.3* | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.7 ± 0.2* |
| epithelium† | 0/17 | 3/19 | 11/18 | 14/19 | 2/19 | 3/19 | 12/20 |
| Arytenoid projections | | | | | | | |
| Ventral depression | | | | | | | |
| Hyperplasia of | 0 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0 | 0.1 ± 0.1 | 0.4 ± 0.2* |
| cuboidal epithelium | 0/18 | 3/16 | 2/18 | 1/20 | 0/18 | 2/16 | 4/17 |
| Squamous metaplasia of | 0 | 0 | 0 | 0.1 ± 0.1 | 0 | 0.1 ± 0.1 | 0 |
| cuboidal epithelium | 0/18 | 0/16 | 0/18 | 1/20 | 0/18 | 1/16 | 0/17 |
| Vocal cords, lower medial region | | | | | | | |
| Hyperplasia of | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.7 ± 0.2* | 0.9 ± 0.2* | 0.3 ± 0.1 | 0.9 ± 0.2* | 1.0 ± 0.2* |
| squamous epithelium | 2/18 | 3/16 | 9/18 | 11/20 | 4/18 | 9/16 | 10/17 |
| Trachea | | | | | | | |
| Goblet cell hyperplasia of | 0.1 ± 0.1 | 0.2 ± 0.2 | 0.2 ± 0.1 | 0 | 0.1 ± 0.1 | 0.3 ± 0.2 | 0.1 ± 0.1 |
| respiratory epithelium | 1/17 | 2/14 | 3/17 | 0/18 | 1/19 | 3/15 | 1/20 |
| Lungs | | | | | | | |
| Goblet cell hyperplasia of | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.7 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.5 ± 0.2 |
| respiratory epithelium | 4/20 | 3/20 | 2/20 | 9/20 | 2/20 | 3/20 | 6/20 |

*Note.* Histopathological findings are given as mean score ± SE and incidence.
* Indicates statistically significant difference to sham-exposed control group.
† Indicates statistically significant differences between the two SS types.

RASS groups were found to be diluted at relatively constant proportions from the high- to the medium- (2.2 ± 0.2) and from the high- to the low- (4.6 ± 0.9) dose levels, respectively. The TPM concentrations for RASS decreased by 60 to 70% compared to FSS. The aging-related decreases found for nicotine, formaldehyde, and ammonia were numerically similar to that found for TPM, but may not necessarily result from the same mechanism since, e.g., nicotine was found only in the gas phase and was not associated with particulate matter. On average, the mass median aerodynamic diameter was slightly higher following room-aging (changed from 0.36 to 0.42 μm) with no effect on the geometric standard deviation (1.8 to 2.0) of the particle size distribution. No oxidation of nitric oxide occurred during the aging process. Acetaldehyde and acrolein were not affected by SS room-aging.

The relative humidity in the sham-exposed group was 54 ± 4% (mean ± SD); this is considered to be representative for the other exposure groups and complies with the exposure conditions specified by the OECD (1981b). The temperature within the exposure chambers was 26 ± 1°C for all groups and thus also met OECD (1981b) specifications.

*In-Life Observations*

There was no smoke-associated mortality, nor were there effects on general condition and behavior of the rats.

*Body Weight Development*

The body weights of the rats increased during the inhalation and postinhalation periods (Fig. 1). The mean body weight of the high-dose FSS exposure group was statistically significantly lower than that of the sham-exposed group between inhalation days 59 and 73. At the end of the inhalation period, a numerical body weight gain reduction of 4% for this exposure group compared to the sham-exposed group was calculated (no statistically significant difference). No body weight effect was seen following RASS inhalation. During the postinhalation period, the body weights of the rats in all groups increased to the same level, indicating a reversal of the reduction in body weight gain associated with the tube restraint during the inhalation period.

*Biomonitoring*

No relevant effects on the respiratory minute volume of the rats were observed (data not shown). The steady-state blood

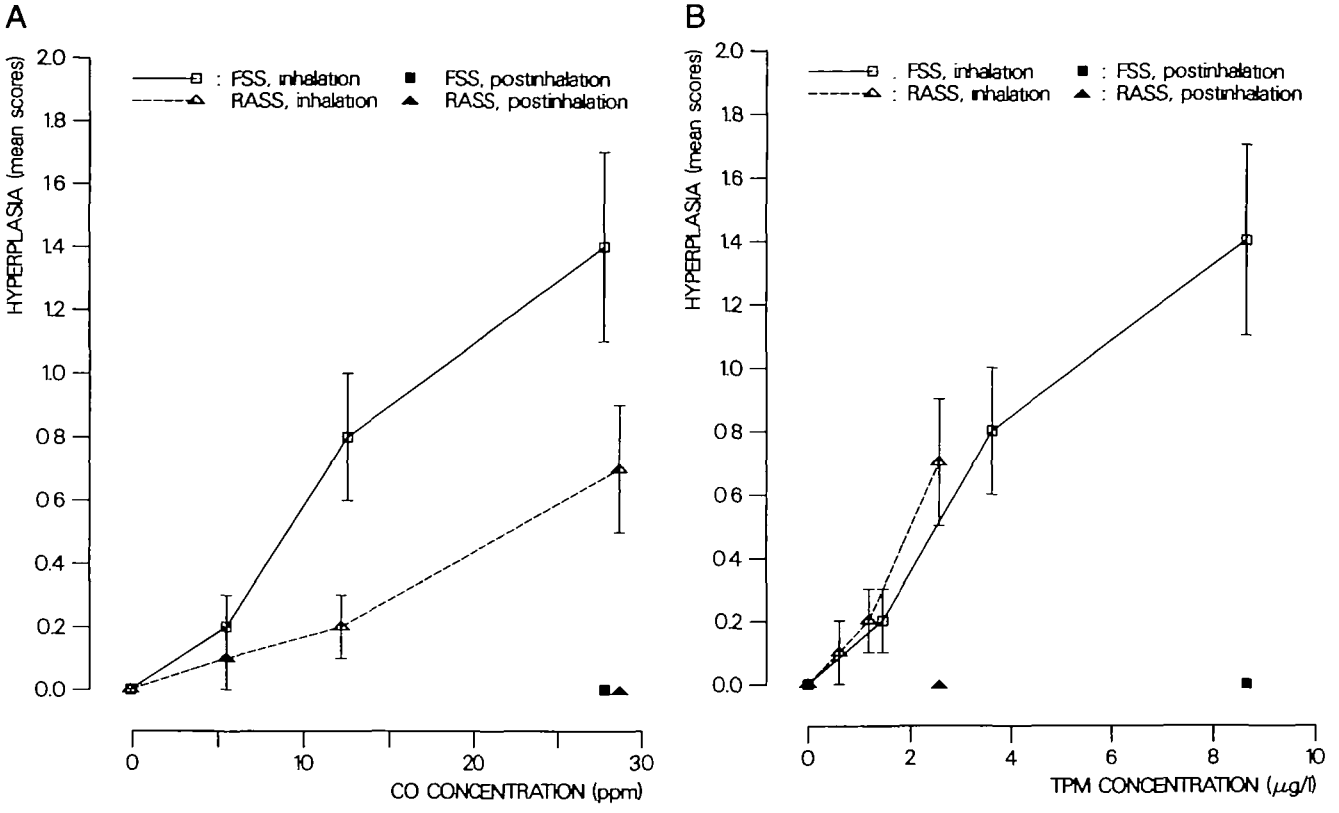


FIG. 3. Histopathological findings in the larynx. Effects are shown based on both dose parameters, i.e., CO and TPM concentrations, in FSS and RASS. (A, B) Hyperplasia of the squamous epithelium at the base of epiglottis. (A) Effects based on the CO concentrations as dose parameter; (B) effects based on the TPM concentrations as dose parameter. Results are given as mean scores ± SE.

HbCO proportions (1, 2, and 4% above sham control values (0.26%) for the low-, medium-, and high-dose levels, respectively) were in agreement with those expected from the CO concentrations in both SS types.

The amounts of nicotine, cotinine, and trans-3′-hydroxycotinine found in the urine samples collected during and for 18 h after exposure showed an almost linear increase with increasing concentrations of nicotine in FSS and RASS (Fig. 2). The absolute amounts found in the urine samples did not account for the total uptake of nicotine, since only nicotine and two of its metabolites were determined. There is no difference in the relative proportion of nicotine and the two metabolites between the two SS types.

HbCO and nicotine metabolite data in the sham-exposed group confirmed nonexposure to SS.

*Gross Pathology*

There were no SS-related gross pathological findings. Slight yellow–brown discoloration of the fur was observed which was roughly dose-dependent; the cause of this discoloration is unclear.

*Organ Weights*

The absolute weights of the lungs with larynx and trachea, kidneys, and liver were statistically significantly lower (maximum effect: 11%) in the high-dose FSS-exposed group compared to the sham-exposed group. No effects in organ weights were seen in the RASS-exposed rats. For organ weights relative to body weight, no differences between FSS- or RASS-exposed and sham-exposed groups were seen. At the end of the postinhalation period, the organ weights of the FSS-exposed rats returned to those of the sham-exposed rats.

*Histopathology*

At the end of the 90-day inhalation period, only slight histopathological changes in the upper respiratory tract in the FSS- and RASS-exposed groups were consistently observed in almost all rats (Table 2).

In the nose at the most anterior level (level 1), slight patchy reserve cell hyperplasia was observed in rats of the high-dose groups only, the mean score for this finding being statistically significantly higher for FSS compared to RASS. This difference is related to the relatively high incidence of this finding in the high-dose FSS group compared to only one rat in the

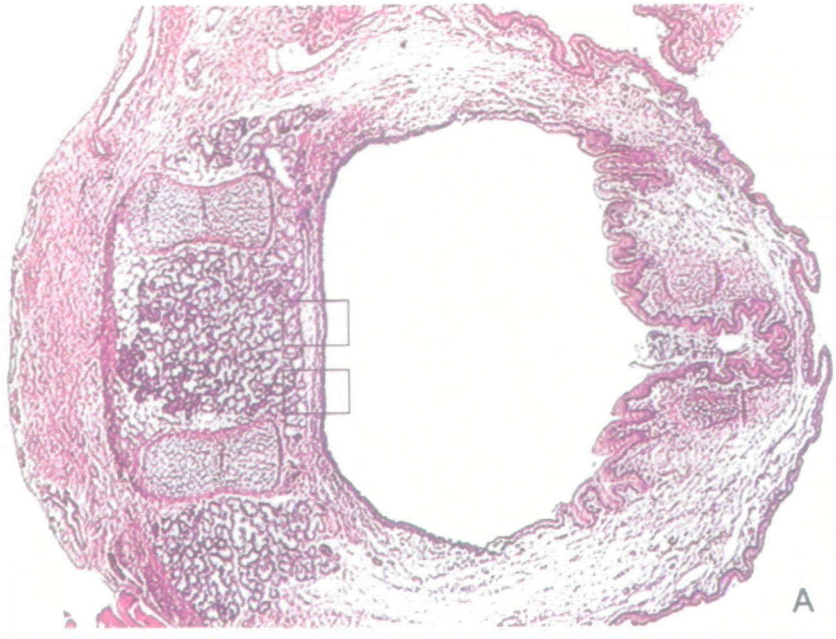


FIG. 4. Transverse sections at the larynx, base of epiglottis. (A) Overview (sham-exposed rat); (B, C) sham-exposed rat showing normal squamous epithelium at the ventromedial site and pseudostratified epithelium at ventrolateral sites; (D, E) high-dose FSS-exposed rat; (F, G) high-dose RASS-exposed rat, both showing hyperplasia of the squamous epithelium (ventromedial site) and squamous metaplasia of the pseudostratified epithelium (ventrolateral site).

high-dose RASS group, the severity of this finding being similarly low for both SS types. At the second nose level, no changes were seen.

The larynx was found to be the most sensitive organ for histopathological changes following FSS or RASS exposure (Table 2; Fig. 3). At the base of the epiglottis, a dose-dependent diffuse squamous metaplasia of the pseudostratified epithelium and hyperplasia of the squamous epithelium (Fig. 4) were found. The mean scores were statistically significantly higher for the FSS- compared to the RASS-exposed groups based on the CO concentration. As was the case for nasal epithelial hyperplasia, the incidence was higher in the FSS groups compared to the RASS groups, while the severity of these findings showed no remarkable difference. At the ventral depression (arytenoid projections), very slight hyperplasia and squamous metaplasia were observed in few rats. The statistically significant difference between the high-dose RASS and the sham-exposed group concerning the hyperplasia at this site is considered to be incidental since a very similar mean score and incidence were obtained for the low-dose FSS group with no indication for dose dependency. There was no difference between the two SS types at the ventral depression. At the lower medial region of the vocal cords, a dose-dependent increase in hyperplasia of the squamous epithelium was observed. This finding was also seen in two rats of the sham-exposed control and is considered to be incidental. No difference between the two SS types was seen for this effect when compared on the basis of CO concentration. This is the only morphologic effect for which, on the basis of the TPM concentration, RASS was slightly more active than FSS. There was no finding in the alveolar region of the lungs.

At the tracheal bifurcation, minimal goblet cell hyperplasia was seen in few rats of all exposure groups with no indication for a SS-related effect (Table 2). Slight goblet cell hyperplasia was also seen in the bronchial respiratory epithelium with a slightly higher incidence in both high-dose groups (no statistical significance). No difference between the two SS types was observed.

Morphometric determination of the laryngeal epithelial thickness at the arytenoid projections showed numerical increases at all sites measured in the SS-exposed groups compared to that of the sham-exposed group in a roughly dose-dependent manner (Table 3), although the increases were statistically significant in only a few cases. The only statistically significant difference between the two SS types was seen at the vocal cords, where RASS was more active than FSS when compared on the basis of the CO concentration.

At the end of the 42-day postinhalation period, no relevant SS-related histopathological changes were observed. The epithelial changes observed at the end of the inhalation period reverted completely.

### *B(a)P Metabolism*

In the NRE, the formation of the bay region metabolites, i.e., 9-OH-, 7,8-diol-, and 9,10-diol-B(*a*)P, was induced in all SS-exposed groups, the highest factor of induction being seen for 9,10-diol-B(*a*)P (Table 4). The induction was roughly dose-dependent and statistically significant.

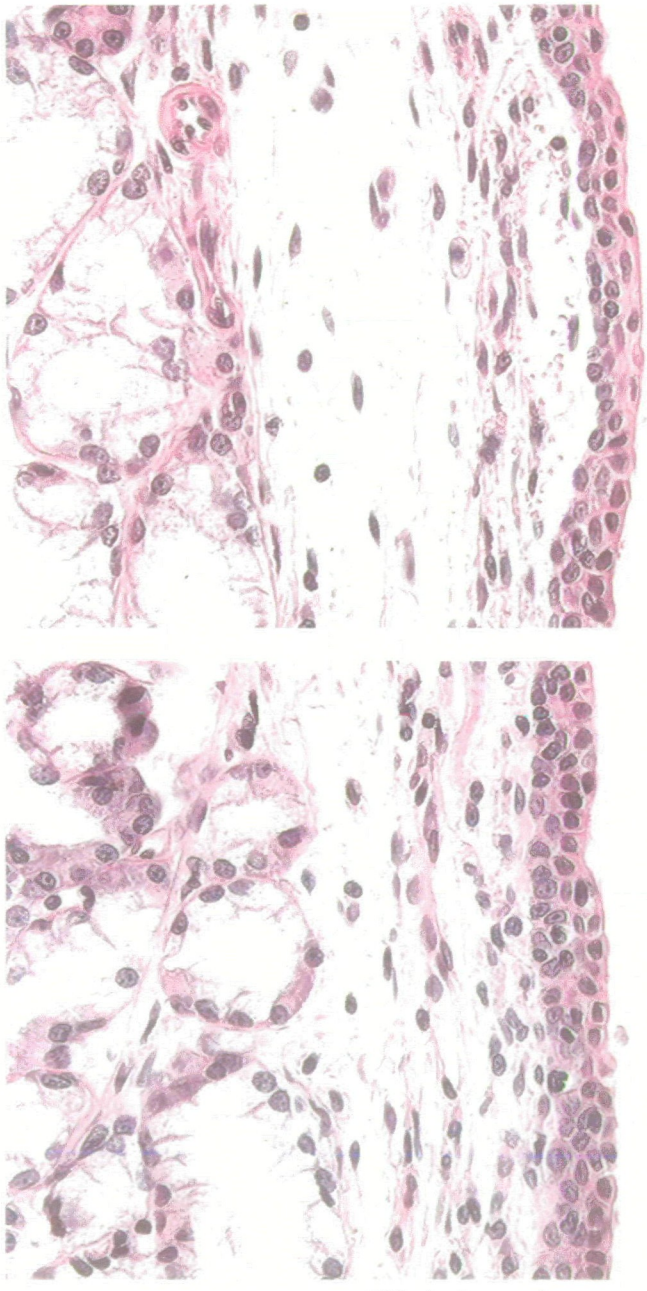


FIG. 4—*Continued*

In the NOE, the baseline values of the sham-exposed group determined after 90 days were 1151 ± 79, 44 ± 4, 773 ± 38, 72 ± 5, and 42 ± 3 nmol/(g protein × h) for 3-OH-, 9-OH-, 4,5-diol-, 7,8-diol-, and 9,10-diol-B(*a*)P, respectively, and thus about two-fold higher than in NRE. No relevant SS-related effects were seen in this tissue.

In the lungs, the formation of all metabolites except 4,5-diol-B(*a*)P was dose-dependently induced, the highest factor of induction being seen for 9,10-diol-B(*a*)P (Table 5). The induction was up to a factor of 8 higher than in the NRE. For all induced metabolites, the induction was higher in the FSS- than in the RASS-exposed groups on the basis of the CO concentration. The induction was similar for both SS types when compared on the basis of the TPM concentration.

At the end of the postinhalation period, no differences between sham and SS-exposed groups were found.

## DISCUSSION

The SS concentrations used in the present study ranged from 6 to 29 ppm CO and from 0.6 to 8.7 μg TPM/liter. These TPM concentrations were up to two orders of magnitude higher than

the maximum of average concentrations of RSP reported for ETS, particularly in residences and offices (Oldaker *et al.*, 1990; Guerin *et al.*, 1992; U.S. Environmental Protection Agency, 1992; Jenkins *et al.*, 1996). The lowest concentrations used here may overlap with those encountered in extreme human exposure situations. The concentration range between 0.1 and 10 $\mu$g TPM/liter has been used widely by the scientific community for subchronic or chronic toxicological testing of experimental ETS surrogates in rodents (e.g., von Meyerinck *et al.*, 1989; Coggins *et al.*, 1993a; Joad *et al.*, 1993; Teredesai and Prühs, 1994; Witschi *et al.*, 1995b), and enables the determination of lowest-observed effect levels. In only a few cases was this concentration range exceeded, for example in the chronic study reported by Witschi *et al.* (1997), in which this concentration range was exceeded by one order of magnitude. However, this concentration was highly toxic, as evidenced by the body weight loss in the exposed mice. The concentration range between 0.1 and 10 $\mu$g TPM/I was used to investigate a variety of respiratory tract end points, such as morphological (von Meyerinck *et al.*, 1989) or biochemical (Ji *et al.*, 1994) changes, genotoxicity (Lee *et al.*, 1992), or increased DNA synthesis (Rajini and Witschi, 1994). Therefore, this concentration range was deemed useful for a comparative inhalation study on the effects of room-aging in the rat respiratory tract.

No information on the effects of SS on the nasal xenobiotics metabolism has been reported to date. Therefore, to assess the effect of SS inhalation on the xenobiotics metabolism in the respiratory tract, the formation of B(*a*)P metabolites in the nasal olfactory and respiratory epithelia and lungs was also investigated in the present study.

SS was room-aged under steady-state dynamic conditions. The room-aging-related changes in the chemical composition of the SS confirmed previous experience about the instability of SS due to the physicochemical and chemical nature of its components (e.g., reviews by Baker and Proctor, 1990, and Eatough *et al.*, 1990). Details on the contribution of various materials to the overall aging effect seen in this study are given by Voncken *et al.* (1994).

The decrease in TPM concentrations can be attributed primarily to particle deposition on all surfaces in the aging room as indicated, e.g., by the yellowish staining of the wallpaper. During the aging process, the mass median aerodynamic diameter of the aerosol slightly increased, the geometric standard deviation remaining unchanged. This small shift is not expected to influence the particle deposition probability in the respiratory tract of the rats (Raabe *et al.*, 1997). Reports about aging-related changes in SS particle size distribution have been inconsistent describing both initial size decrease (Ingebrethsen and Sears, 1986) and increase (Benner *et al.*, 1989) upon aging in relatively inert chambers. In contrast to MS, nicotine in SS is a gas-phase component (reviewed by Eatough *et al.*, 1990). It readily adsorbs to surfaces and reevaporates upon cleansing the ambient atmosphere (Piadé *et al.*, 1996). Formaldehyde and ammonia are chemically reactive compounds. They reacted differently with various surface materials in a room (Voncken *et al.*, 1994). The gas-phase components acetaldehyde and acrolein were less reactive than formaldehyde and did not change in spite of their aldehyde functional groups. Due to the low concentration of nitrogen oxides, NO remained stable under the conditions of this study with no oxidation to $NO_2$ being detectable. Also, CO was not affected by aging, and thus proved to be useful as a marker in experimental studies on SS and the leading dosing parameter in the present study. In field studies, however, CO is not useful as a marker for ETS since the majority of the indoor CO stems from sources other than ETS (Eatough *et al.*, 1990).

The chemical and physical characteristics of RASS remained constant over the 90-day period of inhalation, resulting in stable and reproducible test atmospheres throughout the study. Thus, no saturation of reactive surfaces or shifting equilibria with built-up deposits were observed.

The lack of a detectable body weight effect by SS exposure in the present study is in agreement with the results seen in previous studies at similar SS concentrations (von Meyerinck *et al.*, 1989; Coggins *et al.*, 1993a; Teredesai and Prühs, 1994).

The histopathological findings seen in previous SS inhalation studies of similar design were also seen in the present study. Slight hyperplasia of the respiratory epithelium was observed in the anterior part of the nose with no findings at the next posterior level, which includes the olfactory epithelium. This is consistent in type, location, severity, and sensitivity with the findings described by von Meyerinck *et al.* (1989), Coggins *et al.* (1993a), and Teredesai and Prühs (1994). The metaplasia described by von Meyerinck *et al.* (1989) was seen neither by Coggins *et al.* (1993a) and Teredesai and Prühs (1994) nor in the present study. As in the previous studies, no statistically significant histopathological findings were seen in the lower respiratory tract, although there was an indication in the present study of an increased incidence of bronchial goblet cells in both high-dose groups.

The most sensitive site for histopathological changes in the present study was the larynx, showing slight hyperplasia and metaplasia of a number of epithelia at different locations within the larynx, in particular the base of epiglottis. Similar changes were seen in a previous subchronic SS inhalation study performed in the same laboratory (Teredesai and Prühs, 1994). However, no morphological changes in laryngeal epithelia were observed in the studies by von Meyerinck *et al.* (1989) and Coggins *et al.* (1993a). The few differences in the experimental designs among these studies are not considered to account for this discrepancy in larynx findings. Rather, differences in sectioning levels might affect the optimal detection of these changes. The laryngeal hyperplasia at the arytenoid projections was confirmed by morphometric analyses of the epithelium at this site, the increase in the epithelial thickness being up to approximately 30% in the present and previous (Teredesai and Prühs, 1994) studies. Squamous metaplasia at

TABLE 3
Laryngeal Epithelial Thickness at Three Sites of the Arytenoid Projections after 90 Days of Inhalation

| Organ/site | Sham | FSS | | | RASS | | |
|---|---|---|---|---|---|---|---|
| | | Low | Medium | High | Low | Medium | High |
| Larynx | | | | | | | |
| Arytenoid projections | | | | | | | |
| Ventral depression | 7.9 ± 0.2 | 8.3 ± 0.3 | 8.7 ± 0.3 | 10.0 ± 1.0 | 8.4 ± 0.2 | 8.9 ± 0.4 | 8 5 ± 0.2 |
| | | 6% | 10% | 27%* | 7% | 13% | 8% |
| Floor of the larynx | 10.5 ± 0.4 | 11.2 ± 0.4 | 11.3 ± 0.4 | 12.2 ± 0.4 | 10.7 ± 0.4 | 12.1 ± 1.0 | 12.0 ± 0.9 |
| | | 7% | 7% | 16%* | 2% | 15% | 14% |
| Vocal cords† | 22 2 ± 1.2 | 22.6 ± 1.2 | 24.5 ± 1.4 | 26.1 ± 1.3 | 24.2 ± 1.1 | 27 5 ± 1 3 | 27.5 ± 1.1 |
| | | 2% | 10% | 17% | 9% | 24%* | 24%* |

*Note.* Epithelial thickness ($\mu$m) is given as mean ± SE and percentage increase relative to the sham-exposed control group.
* Indicates statistically significant difference to sham-exposed control group.
† Indicates statistically significant differences between the two SS types.

the base of the epiglottis was similarly observed following subchronic glycerol inhalation (Renne *et al.*, 1992) and has been discussed as a commonly observed adaptive response to repeated inhalation of aerosols (Gopinath *et al.*, 1987; Burger *et al.*, 1989).

The lowest-observed effect level for histopathological changes was 12 ppm CO, equivalent to 3.6 and 1.2 $\mu$g TPM/liter for FSS and RASS, respectively. The no-observed-effect levels were 6 ppm CO, equivalent to 1.5 and 0.6 $\mu$g TPM/liter for FSS and RASS, respectively.

As discussed before, the relevant basis of comparison between the two SS types is the number of cigarettes smoked per unit of air volume, a dose parameter which is represented in the present study by the CO concentration in the test atmospheres. On this basis of comparison, the biological activity of RASS is approximately two- to three-fold lower than that of FSS for the histopathological findings in the anterior nose and in the larynx at the base of the epiglottis. FSS and RASS are equally active for changes in the larynx at the arytenoid projections when compared on a CO concentration basis. Previous SS inhalation studies with experimental animals have usually been based on the TPM concentration as dose parameter. FSS and RASS were equally active based on TPM concentrations with one exception, i.e., histopathological findings at the arytenoid projections which were more pronounced for RASS than for FSS.

The described differences in the biological activity of FSS and RASS may also give some clues as to the mechanism and the SS components which may be involved in inducing such effects: Most of the histopathological changes observed seem to correlate with the TPM concentration. In the larynx, at the base of the epiglottis, this may be interpreted as a consequence of particle impaction on the sites where the inhaled air stream bends. Except for the base of the epiglottis, this correlation with the TPM concentration was not expected. For example, among the gas-phase components of SS analyzed, the aldehydes were described to induce epithelial changes in the nose, in particular acrolein as the most active of the three aldehydes analyzed at their respective dose levels (Feron *et al.*, 1978; Appelman *et al.*, 1986; Woutersen *et al.*, 1987). Apparently, the concentration of the gas-phase aldehydes was not high enough to substantially impact the SS-related morphological effects at this site. There is only one site where histopathological findings were not seen to depend on the particle concentration, i.e., at the arytenoid projections, namely the vocal cords. No explanation for this is available to date. The data may suggest a dependence on the SS gas phase, but a qualitative change of the particulate matter by room-aging cannot be excluded either. In order to clarify the role of particulate and gas phase, a subchronic study comparing the separate phases would be useful.

During the postinhalation period, all histopathological changes reverted to the sham control level, confirming their adaptive nature (cf., Burger *et al.*, 1989).

The B(*a*)P metabolism was investigated in the present study by determining the amounts of five individual metabolites formed. This is different from the method employed in previous SS-related studies in which the "aryl hydrocarbon hydroxylase" activity was determined by analyzing the total amounts of B(*a*)P metabolites formed. The pulmonary aryl hydrocarbon hydroxylase in rats was induced following subchronic inhalation (Gairola, 1987) or intraperitoneal administration of SS condensate or condensate fractions (Pasquini *et al.*, 1987). No direct measurement of the SS concentration used was made by Gairola (1987), but based on the HbCO proportions reported, it can be assumed that it was approximately fivefold higher than those in the high-dose groups of the present study. To date, no studies have been reported that investigate dose responses for the B(*a*)P metabolism at SS concentrations that are closer to the realistic human environment. In addition, the effect of room-aging has not been investigated previously.

In the present study, the formation of four of the five B(*a*)P

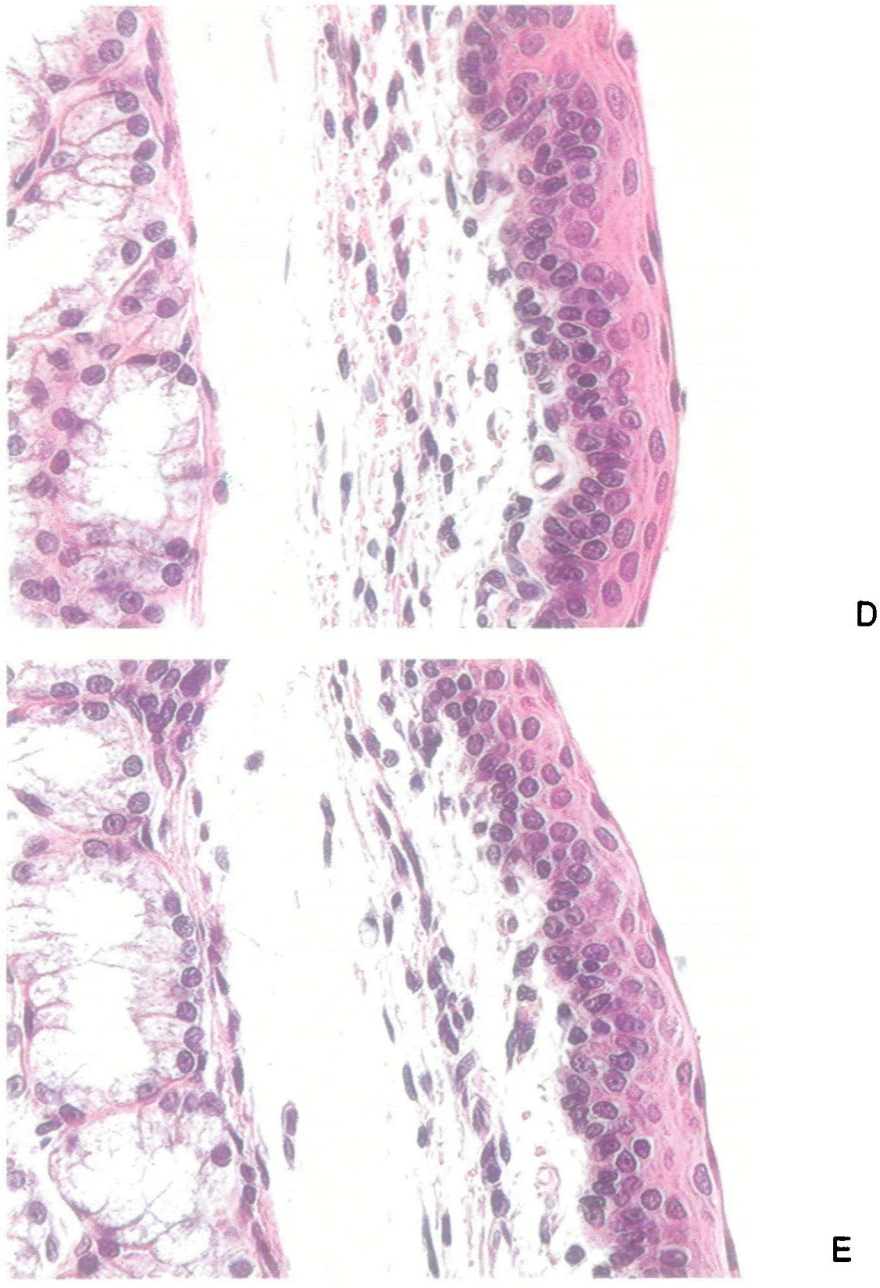


FIG. 4—*Continued*

metabolites analyzed was dose-dependently induced in the lungs, with different maximum induction factors. Only the formation of 4,5-diol-B(*a*)P, the most abundant metabolite, was not inducible by SS inhalation. This difference is most probably attributable to the involvement of different cytochrome P450 isoenzymes in the formation of the five metabolites analyzed. For example, subchronic inhalation of SS in rats at a concentration of 1 μg TPM/liter resulted in an increased expression of the cytochrome P450 isoenzyme 1A1 in nonciliated bronchiolar epithelial (Clara) as well as alveolar type II cells (Ji *et al.*, 1994), which was accompanied by an induction of 1A1-associated pulmonary metabolic activities (Gebremichael *et al.*, 1995). However, the cytochrome P450 2B1-associated activity was not inducible in this study. Similarly, chronic inhalation of SS in A/J mice at a concentration of 4 μg TPM/liter resulted in an induction of cytochrome P450 1A1 in pulmonary endothelial cells with no effect on isoenzymes 2B1, 2E1, and 2F2 (Pinkerton *et al.*, 1996). Thus, immunohistochemical and metabolic data fit together since cytochrome P450 1A1 is considered to play a major role in the metabolic activation of B(*a*)P (Dogra *et al.*, 1990; Voigt *et al.*, 1993).

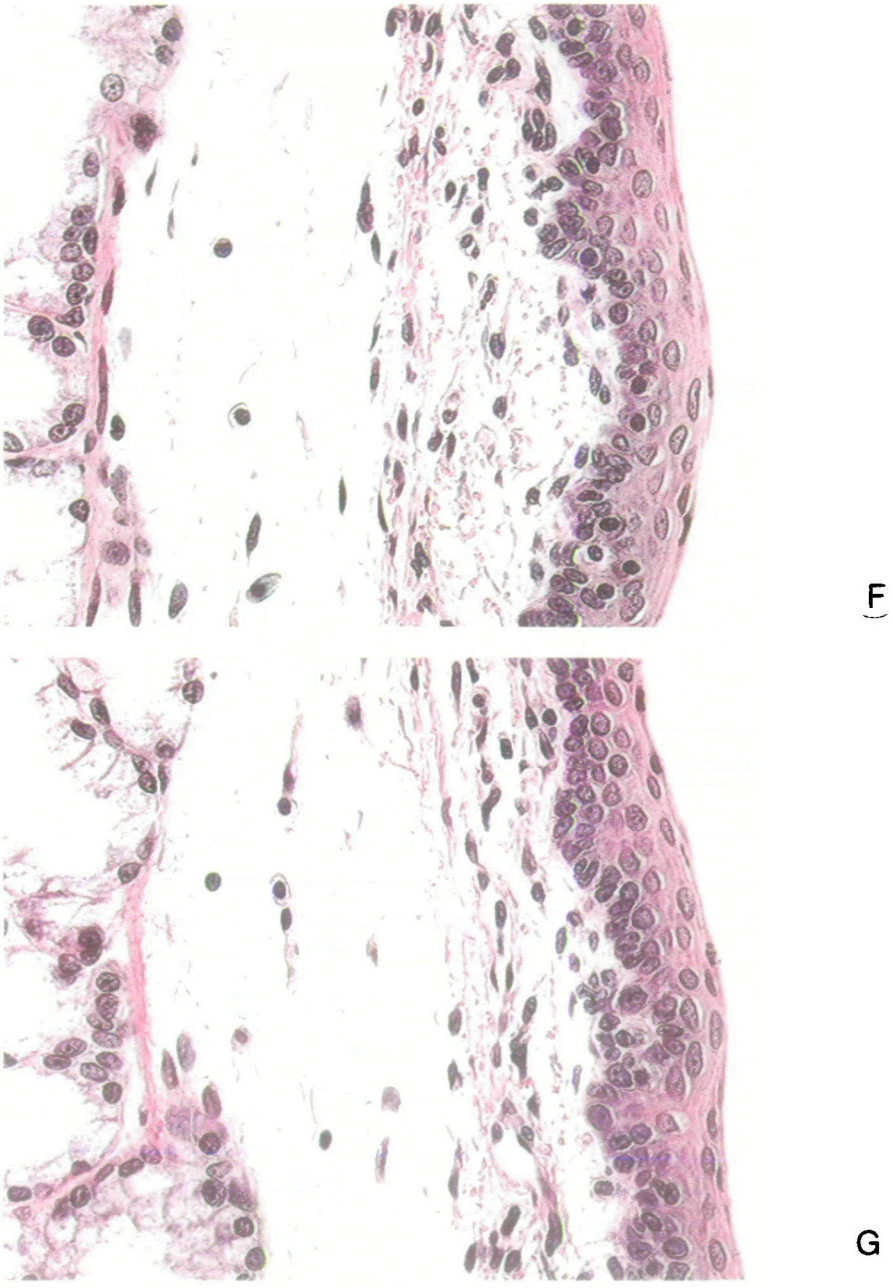


FIG. 4—*Continued*

The induction factors reported by Gairola (1987) and Pasquini *et al.* (1987) were similar irrespective of the route of administration, i.e., SS inhalation or intraperitoneal SS condensate injection, respectively. In addition, no adaptive or progressive changes were seen for the induction of the pulmonary cytochrome P450-dependent metabolic activities with prolonged SS inhalation (Gebremichael *et al.*, 1995; Pinkerton *et al.*, 1996). Thus, the induction of the pulmonary B(*a*)P metabolism is considered a stable biomarker for the pulmonary concentration of inducers following both short-term and prolonged SS exposure at relatively low experimental SS concentrations.

For the nasal epithelia, the results of the present study show a higher baseline activity for B(*a*)P metabolism in the NOE than in the NRE. This is in agreement with the site-specific distribution of the B(*a*)P metabolism described by Bond and Dahl (1989). Following FSS or RASS inhalation, there was a distinct but slight induction of the B(*a*)P metabolism in the NRE, while no effect was seen in the NOE. Since this distribution of SS-related changes parallels those observed his-

TABLE 4
B(*a*)P Metabolism in the Nasal Respiratory Epithelium

| | | FSS | | | RASS | | |
|---|---|---|---|---|---|---|---|
| Metabolite | Sham | Low | Medium | High | Low | Medium | High |
| 9-OH-B(*a*)P† | 16 ± 1 | 24 ± 2 | 24 ± 1 | 35 ± 2 | 22 ± 1 | 22 ± 1 | 23 ± 2 |
| | | 1.5* | 1.5* | 2.1* | 1.4* | 1.3* | 1.4* |
| 7,8-Diol-B(*a*)P† | 37 ± 2 | 54 ± 4 | 60 ± 3 | 91 ± 6 | 50 ± 3 | 48 ± 2 | 53 ± 5 |
| | | 1.5* | 1.6* | 2.5* | 1.3* | 1.3* | 1.4* |
| 9,10-Diol-B(*a*)P† | 22 ± 1 | 38 ± 2 | 51 ± 3 | 79 ± 6 | 33 ± 2 | 34 ± 2 | 40 ± 5 |
| | | 1.7* | 2.3* | 3.5* | 1.5* | 1.5* | 1.8* |
| 3-OH-B(*a*)P | 602 ± 37 | 726 ± 60 | 571 ± 31 | 650 ± 22 | 686 ± 35 | 643 ± 34 | 613 ± 35 |
| | | 1.2 | 1.0 | 1.1 | 1.1 | 1.1 | 1.0 |
| 4,5-Diol-B(*a*)P | 343 ± 24 | 398 ± 36 | 313 ± 19 | 350 ± 21 | 380 ± 29 | 339 ± 27 | 328 ± 19 |
| | | 1.2 | 0.9 | 1.0 | 1.1 | 1.0* | 1.0 |

*Note.* Metabolic activities (nmol/(g protein × h)) are given as mean ± SE and factor of induction relative to the sham-exposed control group.
* Indicates statistically significant difference to sham-exposed control group.
† Indicates statistically significant differences between the two SS types.

topathologically, it might be speculated that the changes in B(*a*)P metabolism reflect the changed distribution of cell types following SS inhalation, or that there is cell-specific induction. Model cytochrome P450 inducers, such as phenobarbital, 3-methylcholanthrene, and B(*a*)P itself, which most probably do not affect the morphology of the nasal epithelia, failed to induce the nasal B(*a*)P metabolism (Bond, 1986; Voigt *et al.*, 1993). Only the administration of the most potent inducers, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Bond, 1986) and Aroclor 1254 (Voigt *et al.*, 1993), resulted in an induction of the B(*a*)P metabolism in the nasal epithelia. The Aroclor 1254-mediated induction of the B(*a*)P metabolism was more pronounced in the NRE than in the NOE and did not coincide with the distribution of the cytochrome P450 1A1 induction, suggesting the involvement of multiple enzymes in the nasal metabolism of B(*a*)P.

Based on the CO concentrations, the induction of the B(*a*)P metabolism was more pronounced following inhalation of FSS compared to RASS. Based on the TPM concentrations, there was no difference in response. This could be expected since the components of cigarette smoke that induce the B(*a*)P metabolism, e.g., polycyclic aromatic hydrocarbons, are found mainly in the particulate matter fraction of the smoke (Pasquini *et al.*, 1987), and there seems to be no qualitative change in the SS particulate material by room-aging with regard to cytochrome P450 induction. Filtered SS did not induce cytochrome P450 1A1-associated metabolic activities in rat lungs (Gebremichael *et al.*, 1995).

The dose-dependent induction of the respiratory tract B(*a*)P metabolism may be useful as a biomarker of exposure to inducing agents, particularly at relatively low doses, although

TABLE 5
B(*a*)P Metabolism in the Lungs

| | | FSS | | | RASS | | |
|---|---|---|---|---|---|---|---|
| Metabolite | Sham | Low | Medium | High | Low | Medium | High |
| 9-OH-B(*a*)P† | 13 ± 4 | 143 ± 30 | 177 ± 14 | 235 ± 22 | 55 ± 8 | 71 ± 13 | 135 ± 15 |
| | | 10.6* | 13.2* | 17.5* | 4.1* | 5.3* | 10.1* |
| 7,8-Diol-B(*a*)P† | 22 ± 4 | 258 ± 55 | 339 ± 21 | 476 ± 46 | 107 ± 14 | 144 ± 24 | 316 ± 28 |
| | | 11.6* | 15.2* | 21.4* | 4.8* | 6.5* | 14.2* |
| 9,10-Diol-B(*a*)P† | 15 ± 4 | 179 ± 42 | 246 ± 18 | 362 ± 39 | 75 ± 11 | 105 ± 21 | 256 ± 25 |
| | | 11.9* | 16.3* | 24.0* | 4.9* | 7.0* | 16.9* |
| 3-OH-B(*a*)P† | 109 ± 13 | 379 ± 57 | 436 ± 28 | 576 ± 53 | 214 ± 18 | 237 ± 39 | 442 ± 40 |
| | | 3.5* | 4.0* | 5.3* | 2.0* | 2.2* | 4.1* |
| 4,5-diol-B(*a*)P | 1126 ± 311 | 808 ± 126 | 1089 ± 134 | 1417 ± 193 | 1100 ± 257 | 935 ± 283 | 1381 ± 243 |
| | | 0.7 | 1.0 | 1.3 | 1.0 | 0.8 | 1.2 |

*Note.* Metabolic activities (nmol/(g protein × h)) are given as mean ± SE and factor of induction relative to the sham-exposed control group.
* Indicates statistically significant difference to sham-exposed control group.
† Indicates statistically significant differences between the two SS types

independent from the route of exposure. Conclusions as to its toxicological relevance are limited by the experimental model used and the complexity of the toxification pathway leading to the ultimate animal carcinogen. For example, the microsomal fraction used in this study to assess the B(*a*)P metabolism does not account for most phase II detoxification pathways which might be induced in parallel to cytochrome P450. A better approach to assess the relevance of the described induction would be obtained by determining DNA adducts in target organs, although their specific determination would require higher B(*a*)P doses than those taken up by the rats in this study.

The lowest TPM concentration effective in inducing the pulmonary B(*a*)P metabolism, i.e., 0.6 μg/liter, is consistent with the lowest reported concentration effective in inducing the cytochrome P450 1A1, i.e., 1 μg TPM/liter (Ji *et al.*, 1994; Gebremichael *et al.*, 1995).

No differences between sham- and SS-exposed groups were seen at the end of the postinhalation period. The lack of a persistent induction after cessation of SS exposure strongly suggests an effective clearance of the lungs from materials inducing the B(*a*)P metabolism.

In the present study, the amount and/or surface area of materials present in the SS-aging room were exaggerated compared to those typically found in residences or office environments in order to investigate in principle the effects associated with room-aging. Correspondingly, the mean age of ETS in human indoor environments other than residences, e.g., offices or public buildings, is probably shorter than the mean age used to generate RASS in the present study (Seppänen, 1995). However, the results of this study show that room-aging in general reduces the biological activity of FSS. This may impact the risk evaluation based on experimental studies using more or less fresh SS. In this context, RASS is considered a more realistic experimental surrogate for ETS than FSS.

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