Submission by Philip Morris U.S.A.

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- Appendix 1 -

References

March 20, 1998

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- Appendix 2 -

Long-Term Inhalation Study on Room-Aged Sidestream Smoke and Diesel Engine Exhaust

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Introduction

Philip Morris is funding a long-term inhalation study on room-aged sidestream smoke (RASS) and diesel engine exhaust (DEE), currently underway at INBIFO (Institut für biologische Forschung, Köln, Germany), with the objective of comparing classic and mechanistic endpoints considered to be relevant in experimental carcinogenesis.

Appended to this chapter are the following manuscripts prepared and submitted or accepted for publication based on several completed pilot studies:

- Haussmann, H.-J., Anskeit, E., Becker, D., Kuhl, P., Stinn, W., Teredesai, A., Voncken, P., Walk, R.-A., Comparison of fresh and room-aged cigarette sidestream smoke in a subchronic inhalation study on rats, *Toxicol. Sci.* 41: 100-116 (1998). **[Tab A]**

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- Voncken, P., Stinn, W., Haussmann, H.-J., Anskeit, E., Influence of aging and surface contact on the composition of cigarette sidestream smoke -- Models for environmental tobacco smoke, In: Dungworth, D.L., Mauderly, J.L., Oberdörster, G. (Eds.): *Toxic and carcinogenic effects of solid particles in the respiratory tract*, Washington: ILSI Press, ILSI Monographs, pp. 637-641 (1994). **[Tab C]**

Concept of the Long-Term Inhalation Study

The study will correlate mechanistic data gained from intermediate biomarker assays with tumor development, as well as compare these data for the two test materials. The study is designed to investigate the test materials at toxicologically relevant concentrations that bear a more realistic relationship to human exposure.

This study will also contribute to the discussion on the biological plausibility of the tumorigenic risk purportedly attributable to ETS and DEE exposure. Mechanistic investigations on the interaction between the test model and the test materials will be an integral part of the study in order to provide a comprehensive interpretation of effects. The integration of mechanistic endpoints in long-term bioassays and the use of these data in risk assessment has been recommended by the NTP Board of Scientific Counselors (1992) and the International Agency for Research on Cancer (IARC) Working Group (1992), as well as in the Guidelines for Carcinogen Risk Assessment recently proposed by the U.S. Environmental Protection Agency (EPA) (1996).

The study has been designed to generally comply with regulatory requirements and recommendations (OECD guideline 451, 1986; NTP, 1991). There are two major exceptions from these guidelines in the present study design. The use of only two instead of three dose levels is motivated by the comparative nature of the study design; it is not the intention to perform a classical carcinogenesis study with the determination of a no-effect level. Also, doses have not been selected

based primarily on the concept of maximum tolerated dose (MTD), but rather are linked to certain multiples of realistic human environmental concentrations.

In the following, critical parameters of the study concept will be discussed.

Test Atmosphere Definitions

Environmental tobacco smoke (ETS) is a complex mixture mainly composed of aged sidestream smoke (SS) as well as of small amounts of exhaled mainstream smoke (MS) (First, 1985; Baker and Proctor, 1990). Since "real" ETS cannot be generated for long-term laboratory research, RASS will be used in the long-term study as a surrogate for ETS. It will be obtained by diluting and aging SS generated from the standard reference cigarette 1R4F in a controlled, noninert environment. International standards applying to the generation of MS will be adapted to produce SS. Generally, the same chemical compounds are found in SS, RASS, and ETS, but there are quantitative differences in the concentrations and phase distribution (Guerin et al., 1992; Voncken et al., 1994).

DEE is a complex combustion aerosol like SS. The composition of DEE depends on the fuel and lubricants used as well as on the engine and its mode of use. In the long-term study, standard fuel and lubricant will be used, and international standards for diesel engine operation and the dilution of DEE will be applied (EPA, 1990).

Both ETS and DEE are reported in the environment at similar concentrations. The most significant difference in the chemical composition of the two combustion aerosols is the insoluble carbon core of DEE particles, whereas ETS particles are almost completely soluble (Zaebst et al., 1991).

Test Atmosphere Generation

RASS will be generated from the SS of the standard reference cigarette 1R4F, a lower yield filter cigarette supplied by the University of Kentucky (Tobacco and Health Research Institute, 1990). The 1R4F is in line with current consumer preference.

Dilution and aging of SS involve physicochemical changes (e.g., particle/gas phase distribution and particle size distribution), particle losses due to deposition and adsorption on surfaces, and chemical reactions (Benner et al., 1989; Eatough et al., 1989 and 1990; Baker and Proctor, 1990). In comparison to other SS constituents, nicotine has been shown to interact most readily with surfaces (Neurath et al., 1991; Voncken et al., 1994). To quantify ETS concentrations in field studies, particle and nicotine concentrations have most frequently been determined. Due to different kinetics, the ratio of particle/nicotine mass concentrations changes with time and has thus been considered as a determinant to characterize ETS and fresh SS (Eatough et al., 1990; Nelson et al., 1992; Guerin et al., 1992; Sterling et al., 1996). Average concentration ratios of 4 up to 100 were

determined in field studies and 2 to 4 in fresh SS.[1] This ratio may be distorted at lower ETS concentrations by unaccountable portions of nonsmoke-related particles (dust).

In order to approach "real" ETS conditions as far as possible, RASS with a mean age of at least 30 min will be used in the long-term study. A mean air residence time of 30 min (corresponding to 2 air changes per hour) can be found in less ventilated rooms.

The RASS concentrations (3 and 10 μg/l) required for the long-term study may be obtained either by aging SS at the high TPM concentration and subsequent dilution to the lower concentrations or by aging SS separately at each of the RASS concentrations required. As the intention of the long-term study is to investigate a single test atmosphere at two different concentrations, the former approach is preferred.

In our previous investigations, the chemical composition of RASS was found to change with the amount and type of surface materials in the aging room (e.g., with paper or wool) (Voncken et al., 1994). In general, particles and particle-associated components decreased, while most vapor phase components remained unchanged. In order to provide reasonable aging of the SS and to optimize the reproducibility of the setup, a noninert empty room with defined characteristics will be used in the long-term study.

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1. The ratio indicative of fresh SS was also found in previous subchronic and chronic inhalation studies on rats (Adlkofer et al., 1988; von Meyerinck et al., 1989; Coggins et al., 1992; Coggins et al., 1993; Rajini and Witschi, 1994; Witschi et al., 1995).

In most field studies as well as in all published experimental inhalation studies, the particle concentration has been used as the key analyte to determine the concentration of ETS and ETS surrogates. In order to be able to compare our analytical and biological results with published data. TPM concentration will be used as the key analyte in the long-term study. In addition, the particle concentration has been the key analyte in all DEE inhalation studies as well as the basis for DEE risk assessment.

DEE will be generated using standard fuel and lubricant and a passenger car engine frequently in use in Europe. The engine will be driven using a standardized protocol to simulate city driving behavior.

Fresh DEE will be used as the test atmosphere to keep comparability with previously published DEE long-term inhalation studies (Karagianes et al., 1979; Iwai et al., 1986; Ishinishi et al., 1986; Vallyathan et al., 1986; Mauderly et al., 1987; Brightwell et al., 1989; Heinrich et al., 1986. 1992, and 1995; Nikula et al., 1995). Fresh DEE may be less environmentally relevant but its use in long-term inhalation studies is consistent with published literature.

A comparison of DEE composition in published inhalation studies revealed some differences. The most important difference may be the proportion of elemental carbon in the particulate phase. In the most recent published long-term DEE inhalation studies, elemental carbon was reported to be 60 % (Heinrich et al., 1995) and 92 % (Nikula et al., 1995) of TPM. It is well known that the proportion of elemental carbon in DEE particulates fluctuates (Hering et al., 1990;

Watts, 1995). Major factors contributing to this fluctuation include, apart from emission control devices, the engine type, duty cycle, fuel, and lubricant consumption. Elemental carbon generally accounts for about 40 to 60 % of DEE particulate matter mass (Klingenberg et al., 1991; Zaebst et al., 1991).

As for SS, DEE concentrations in experimental studies are commonly based on particulate matter mass as collected on glass fiber filters. Sometimes, soot seems to be synonymously used.

Dose Levels

According to the principle of maximum tolerated dose (MTD), the highest test substance dose in a carcinogenicity study should be "sufficiently high to elicit signs of minimal toxicity without substantially altering the normal life span due to effects other than tumors. Signs of toxicity are those that may be indicated by alterations in certain serum enzyme levels or slight depression of body weight gain (less than 10 %)" (OECD Guideline 451, 1986). Setting the upper limit of doses to the MTD level should prevent toxicity from substantially interfering with tumorigenicity. On the other hand, dosing as high as this limit should prevent false negative carcinogenicity studies.

Historically, the MTD has been frequently determined in pilot subchronic/90-day studies, and histopathological lesions other than those that may be related to carcinogenesis were

also considered (Morrow et al., 1996). However, in only approximately 20 % of the NTP inhalation studies, reduced body weight was used as the rationale for selecting the highest dose. In one study, a multiple of the human therapeutic dose was applied. In the long-term studies completed by the NTP, only 60 % had reduced body weight at the highest dose, and the magnitude of these body weight effects did not correlate with the carcinogenic response of the test substances. This is in line with recent trends within the NTP in the interpretation of the MTD concept, i.e., from the "maximum tolerated dose" to the "minimally toxic dose," which would also consider slight body weight effects in the long-term study as minimally toxic. A special workshop dealt with establishing a rationale for aerosol exposure concentrations in long-term inhalation studies (Lewis et al., 1989). Emphasis was given to substances which are relatively insoluble and of low systemic and respiratory tract toxicity. This workshop concluded that the highest dose should only minimally affect pulmonary clearance. This is in line with the concept of a functionally defined MTD (MFTD) for highest exposure concentrations in long-term bioassays (Muhle et al., 1990a), which should permit the extrapolation of observed biological effects to realistic concentrations to which humans are exposed. The MFTD concept considers biokinetic and mechanistic aspects (NTP Board of Scientific Counselors, 1992). However, more specific recommendations with regard to the type or degree of respiratory tract responses that constitute evidence of an appropriate minimally toxic response or an MFTD were not made (Lewis et al., 1989; Morrow et al., 1996). Especially, the relevance of particle overload in the lungs and the resulting inflammatory response and cell proliferation with regard to risk assessment remains open (Oberdörster, 1995).

Altered serum enzyme levels or hematological changes as signs of toxicity were not found in a subchronic SS inhalation study at a TPM concentration of 4 μg/l (Adlkofer et al., 1988). At concentrations of up to 10 μg/l, no clinical signs of toxicity were found using "aged and diluted SS" (Coggins et al., 1993).

The rationale for the selection of the SS concentration of 4 μg TPM/l in one fully published long-term SS inhalation study was not made quite clear (Witschi et al., 1995). At this concentration, there was no significant effect on body weight. In a previous (pilot?) study performed by the same laboratory, cell proliferation was observed in bronchi and bronchioli at 1 μg TPM/l (Rajini and Witschi, 1994). The same effects as well as cell proliferation in the nasal epithelia were seen during the initial part of the chronic study (4 μg TPM/1). Obviously, the dose for the chronic study was selected based on the response in the pilot study of biomarkers considered to be related to carcinogenesis. Taking this approach for the dose selection, i.e., subchronic responses in biomarkers related to carcinogenesis, a RASS dose of 10 μg TPM/l for the present study would seem to be justified based on the biomarker responses noted above. In a second study, Witschi et al. (1997) used a TPM concentration of roughly 90 μg/l.

In the long-term study on female rats performed by Heinrich et al. (1995), the body weight of the high dose DEE group started to deviate significantly from the control on study day 200 (7 μg TPM/1, 18 hours/day, 5 days/week, weekly particle dose: 630 hours x μg/l). The final body weight difference was 17 %. Nikula et al. (1995) found a body weight gain reduction of approximately 10 % for both sexes following about 180 days of inhalation (6.5 μg TPM/1, 16

hours/day, 5 days/week weekly particle dose: 520 hours x μg/l). This body weight gain reduction did not further increase for the male rats but increased to approximately 20 % for the female rats following approximately 500 days of inhalation. Thus, these long-term DEE inhalation studies which were both positive for lung tumors do not meet the historical MTD definition (10 % body weight gain reduction within 90 days), but are in line with the minimally toxic dose concept (body weight differences at chronic time points).

The survival of DEE-exposed rats in the study by Heinrich et al. (1995) was not affected, as was the case for female rats in the study by Nikula et al. (1995). However, the male rats in the latter study showed reduced survival in the high dose group.

Slight changes in serum clinical chemical and hematological data were reported following DEE inhalation (Ishinishi et al., 1986; Lewis et al., 1986). The relevance of these changes is unclear. These parameters have not been regularly investigated in long-term DEE inhalation studies.

DEE inhalation at the high doses mentioned above reproducibly resulted in an impairment of the macrophage-associated particle clearance from the lungs, which is connected to a particle overload phenomenon (Proceedings of Symposium on Particle Lung Interactions: Overload Related Phenomena, 1990; HEI, 1995). According to a current hypothesis, the overloaded macrophages attract PMNL to the alveolar lumen as an inflammatory response. This persisting inflammation may ultimately result in hyperplastic and metaplastic changes leading to lung fibrosis

and carcinogenesis. The definition of an MTD or MFTD as well as the relevance of tumors arising under particle or other overload conditions for human risk assessment is under discussion. Excessive particle overload as found in high dose long-term DEE inhalation studies is certainly no condition which can be readily extrapolated to the human situation. On the other hand, prolonged particle clearance as well as associated inflammation, cell proliferation, and fibrotic changes can also be found in humans, such as coal workers, occupationally exposed to particles Oberdörster, 1995).

Deposition of soot particles in the lungs, carbon-loaded alveolar macrophages, and influx of PMNL and lymphocytes were also observed in our subchronic DEE pilot inhalation study. The magnitude of these biomarker responses suggests that the dosing regimen selected would be sufficient to elicit lung tumors in a long-term study.

The tumorigenic response to DEE in long-term inhalation studies on rats varies from 4 (spontaneous tumor prevalence) to 54 % lung tumor prevalence at approximately 7 μg particles/l (Karagianes et al., 1979; Brightwell et al., 1986; Mauderly et al., 1986; Heinrich et al., 1992). This large variance has been mainly attributed to differences in the exposure regimen (Heinrich et al. 1986), but major differences in test atmosphere generation are also probable. For weekly particle doses, a threshold of approximately 120 hours x μg/l has been suggested for lung tumorigenicity (Nikula et al., 1995). The lung soot burden is generally considered to be the major determining factor for particle-associated tumor responses. No relevant difference in the lung particle burden or associated effects were found when changing the daily exposure pattern or rate of delivery (Henderson et al., 1992). Thus, the relatively short daily exposure duration applicable to head-only

exposure can be compensated by increasing the DEE particle concentrations over those usually used in whole-body exposure studies for longer daily durations.

Taking together the foregoing discussion on the applicability of the MTD concept to the present test atmospheres, the dose levels chosen seem to be compatible with the minimally toxic dose concept. Another aspect for setting dose levels is the intention to correlate the responses of selected biomarkers to the lung tumor response. This requires the use of doses which have been shown to be positive in previously published long-term inhalation studies. This rationale fully applies to DEE. It cannot be applied to RASS, since only few data are available on the long-term responsiveness of mice (Witschi et al., 1995; Witschi and Pinkerton, 1996; Witschi et al., 1997) but none on rats.

Maximum mean ETS concentrations in terms of smoke-related, respirable suspended particles (RSP) are reported to be approximately 0.1 μg/l in residences, offices, transportation vehicles, or other places where smoking occurs (EPA, 1992; Guerin et al., 1992). 600-1000 $\mu g/m^3$ seems to be the upper limit for the most extreme ETS concentrations reported for all types of occupied spaces. In the present study, the highest RASS particle concentration of 10 μg/l will exceed extreme human exposure concentrations by a factor of 10 and typical concentrations by a factor higher than 100. For the low dose group of this study, a TPM concentration of 3 μg/l will be used. As for the planned high RASS concentration, the high DEE concentration in the present study will exceed extreme and normal mean environmental DEE concentrations by factors of 10 and 100,

respectively (Woskie et al., 1988; Lehmann, 1991; Pott, 1991; Elbers and Muratyan, 1991; Kühn and Bireft, 1992; projected ambient concentrations by McClellan, 1986; Brightwell et al., 1989).

Due to the comparative nature of the present study design as well as the relationship of the doses to reported environmental concentrations, the use of the same particle concentrations for both test atmospheres is considered straightforward.

Animal Model

Rats, mice, and hamsters have been preferred in carcinogenicity studies "because of their relatively short life span, . . . their widespread use in pharmacological and toxicological studies, their susceptibility to tumor induction, and the availability of sufficiently characterized strains" (OECD Guideline 451, 1986). The same recommendation was made by Lewis et al. (1989), particularly for aerosol inhalation studies.

For head-only exposure, rats are technically more suitable than mice and hamsters. This fact and our long experience with the rat in inhalation studies recommend its use in this long-term inhalation study. The mouse could be considered as a second species for possible future work.

In order to facilitate the detection of a low prevalence of induced tumors in the test groups, the spontaneous tumor prevalence in target organs should be as low as possible. The spontaneous prevalence of nasal cavity tumors seems to be negligible (i.e., <1 %) in the three rat

strains considered. However, approximately 4-fold spontaneous lung tumor prevalences were reported for the Fischer 344 rat (Goodman et al., 1978; Solleveld et al., 1984; Haseman et al., 1985) compared to the Sprague Dawley (MacKenzie and Garner, 1973; Prejean et al., 1973) and Wistar rat (Vandenberghe, 1990; Kroes et al., 1988; Deerberg et al., 1980, 1982; Rehm et al., 1984; Ueberberg and Luetzen, 1979; Takizawa and Miyamoto, 1976; Boorman and Hollander, 1973; Bomhard et al., 1986). Fischer 344 rats have been used in carcinogenicity studies performed by the NTP, a preference which was based on the availability of historical data rather than scientific reasons (Gregory, 1992).

Test animals should be exposed to the test material for a major portion of their life span (OECD Guideline 451, 1986). Survival in all groups should be not less than 50 % at 24 months for rats in order for a negative test result to be accepted. In past years, the longevity of laboratory rat strains has decreased substantially, possibly due to breeding targets for rapid growth (White, 1992). The Sprague Dawley rat, which has been used in our previous MS and SS inhalation studies, is no longer considered to fulfill the above requirement (British Society of Toxicological Pathologists, 1992; White, 1992; Mariani et al., 1992). In addition, the increasingly high body weight and associated large size of adult Sprague Dawley rats do not recommend their use in a long-term head-only inhalation study.

Based on the reported low spontaneous lung tumor prevalence, the sufficient longevity and the body weight development suitable for head-only exposure, the Wistar rat seems to be particularly appropriate for the proposed long-term inhalation study. The suitability of the

Wistar rat was corroborated in a recent historical control study. In addition, besides the Fischer 344 rat, the female Wistar rat was used in previous DEE inhalation studies (Heinrich et al., 1986, 1995).

Exposure Regimen

The head-only or nose-only exposure modes are most appropriate for rat inhalation studies with aerosols (Pauluhn, 1984; Phalen et al., 1984; Hahn, 1993). These modes diminish test substance deposition on the fur of the animals and subsequent dermal or oral uptake by grooming, which was observed following whole-body exposure (Wolff et al., 1982; Iwasaki et al., 1988; Mauderly et al. 1989; Chen et al., 1995). In a mainstream cigarette smoke inhalation study, the two exposure modes were compared: plasma and urinary nicotine concentrations were 5- to 6-fold higher in whole-body compared to nose-only exposed rats when normalized to the nicotine concentrations in the test atmospheres (Mauderly et al., 1989). In whole-body exposure, the filtering of the test atmosphere by the fur may impair reproducible uptake by inhalation. In addition, the test atmosphere is in contact with the animal excretion products. In previous studies we found that nicotine and reactive test atmosphere components such as formaldehyde are efficiently trapped by urine and feces.

Thus, to enhance the controllability of the test atmosphere administration to the rat as well as the route of the test substance uptake by the rats, the head-only exposure mode will be used in the present study.

In inhalation studies, "long-term exposures are usually patterned on projected industrial experience, giving the animals a daily exposure of 6 hours . . . for 5 days a week (intermittent exposure)" (OECD Guideline 451, 1986). In all published DEE inhalation studies with rats, whole-body exposure was used. The daily exposure duration lasted up to 19 hours/day (Heinrich et al., 1986). Head-only exposure should not last longer than 7 hours/day due to the restraint of the rats and other technical reasons. Recently published inhalation studies on man-made vitreous fibers used head-only exposure for 6 hours/day, 5 days/week, 24 months (Smith et al., 1987; Hesterberg et al., 1993). In order to maximize our weekly doses, the present study will be conducted for 6 hours/day and 7 days/week.

Depending on the longevity of the rat strain used, it is recommended to terminate carcinogenicity studies after 24 or 30 months of exposure (OECD Guideline 451, 1986). Studies with DEE-exposed rats demonstrated that nearly 80 % of the exposure-related tumors were observed later than 24 months of inhalation (Mauderly et al., 1987). However, for the expression of tumors in this late stage of the rats' lifespan, a continuation of the inhalation period over 24 months does not seem to be necessary, since the particle load will not be substantially cleared under these conditions due to a complete loss of clearance. No information is available for SS-induced pulmonary tumorigenicity in rats, but Witschi et al. (1997) followed a similar exposure regimen for their A/J mice, i.e., inhalation followed by a postinhalation observation period. For the present study, an inhalation period of 24 months followed by a postinhalation period of 6 months is planned.

TABLE 3. Histopathological Findings Following 12 Months of RASS Inhalation

Parameter	Exposure Groups			
	WB0	WB6	WB12	HO12
nasal cavity, level 1				
respiratory epithelium				
reserve cell hyperplasia	0 0/8	0.8 ± 0.2 * 6/8	1.9 ± 0.1 * 8/8	1.6 ± 0.3 7/8
squamous metaplasia	0 0/8	0.1 ± 0.1 1/8	1.0 ± 0.3 * 5/8	0.3 ± 0.2 2/8
goblet cell hyperplasia	0 0/8	0 0/8	0.4 ± 0.4 1/8	0.1 ± 0.1 1/8
nasal cavity, level 2				
respiratory epithelium				
reserve cell hyperplasia	0 0/8	0.1 ± 0.1 1/8	0.1 ± 0.1 1/8	0 0/8
olfactory epithelium				
reserve cell hyperplasia	0 0/8	0 0/8	0.1 ± 0.1 1/8	0 0/8
atrophy	0 0/8	0 0/8	0.3 ± 0.2 2/8	0.3 ± 0.2 2/8
eosinophilic globules	0.1 ± 0.1 1/8	1.1 ± 0.5 3/8	1.0 ± 0.5 4/8	1.3 ± 0.5 4/8
nasal cavity, level 3				
olfactory epithelium				
eosinophilic globules	0 0/8	0.6 ± 0.4 2/8	0.9 ± 0.5 3/8	1.5 ± 0.6 4/8
larynx				
base of epiglottis				
squamous metaplasia of pseudostratified epithelium	0 0/1	2.8 ± 0.3 4/4	4.0 ± 0.0 3/3	2.6 ± 0.2 5/5
hyperplasia of squamous epithelium	0 0/1	3.3 ± 0.3 4/4	4.0 ± 0.0 3/3	2.8 ± 0.2 5/5

or fibrotic processes. Especially for particle inhalation studies, deposition and/or clearance studies are recommended (Lewis et al., 1989).

In the present study, in addition to classical pathology, several endpoints are planned to be investigated which are thought to be mechanistically related to chronic disease and chemical carcinogenesis in the respiratory tract. The reasons for choosing these endpoints is the current mechanistic understanding of the processes under investigation as well as in-house scientific expertise. The extent of these investigations is limited by the available number of rats in a long-term head-only inhalation study.

Biomonitoring for both RASS and DEE will include the determination of the carboxyhemoglobin proportion in the blood and of the steady-state content of aminobiphenyl adducts to hemoglobin. For RASS, nicotine metabolites in urine will serve as a specific monitor. If possible, TSNA-derived hemoglobin adducts will also be investigated. In addition, the green autofluorescence of alveolar macrophages is considered for use as an estimate for the steady-state lung particle dose. The feasibility of this parameter has not yet been fully validated. .

The determination of the lung burden of inhaled particles or the pulmonary clearance efficacy are mandatory in chronic inhalation studies using particle-containing aerosols (Lewis et al., 1989). For DEE lung burden, nonlinear time-response relationships but linear dose-response relationships were found (McClellan, 1986; Heinrich et al., 1992). The nonlinearity has been

attributed to particle overload which impairs pulmonary clearance. For SS-exposed rats, particle overload has not been published.

The oxidative modification of deoxyguanosine by forming 8-OHdG is a rather frequent event due to the permanent cellular oxidative stress (Fraga et al., 1990). Therefore, efficient repair mechanisms are set in operation which, in conjunction with the impaired catabolism of the modified base, result in the urinary excretion of 8-OH-dG. Guanidine oxidation reportedly leads to base mispairing, resulting in G:C to T:A transversions (Cheng et al., 1992). In some experimental systems, a correlation between the presence of 8-OH-dG in DNA and tumor development was observed (Floyd, 1990). This endpoint has never been investigated in long-term DEE inhalation studies to date. In the present study, both the excretion of 8-OH-dG in urine during inhalation as well as the respiratory tract tissue level of this base modification will be investigated.

Bulky DNA adducts were observed in a subchronic SS inhalation study at 10 μg TPM/l in lungs, heart, and larynx tissue using the ^{32}P-postlabeling technique (Lee et al., 1993; Brown et al., 1995). This effect was not observed at lower concentrations. This type of adduct could not be found in ETS-exposed nonsmokers (Scherer et al., 1993) but has been described for smokers (Phillips et al., 1990). DNA adducts were also detected in rat lungs following subchronic DEE inhalation (Bond et al., 1990). Although the ^{32}P-postlabeling technique seems to be the most sensitive method to detect DNA adducts, it lacks specificity. In the present study, DNA adducts will be evaluated. For this purpose, we prefer to use specific mass spectrometric methods which still

have to be established in our laboratory. If the latter is not possible, samples will be extramurally analyzed by the ^{32}P-postlabeling technique.

Classical histopathology remains a basic endpoint for use in the present study. Apart from the microscopic confirmation and classification of tumors, persistent hyperplastic, metaplastic, and dysplastic tissue changes are considered as essential indicators of preneoplastic and neoplastic lesion induction. In subchronic inhalation studies on SS, hyperplasia and metaplasia of nasal and laryngeal epithelia were found in the rat (von Meyerinck et al., 1989; Coggins et al., 1993; Teredesai and Prühs, 1994). Due to the general reversibility of these findings after cessation of the SS inhalation, these changes have been considered an adaptive response to the irritating activity of the test atmosphere (Burger et al., 1989).

Mutations are a prerequisite for initiating carcinogenesis, and most probably also play a role in epigenetically induced carcinogenesis such as by particle overload (Driscoll et al., 1996). Apart from the latter study, there is limited expertise in the detection of early mutations in rat long-term studies. The single-strand DNA conformation assay can be used to detect unknown mutations. Recently, we have been able to increase the sensitivity of this assay by several orders of magnitude. The attempt to further increase the assay sensitivity was limited by the fidelity of the DNA polymerase used in the polymerase chain reaction to amplify DNA probes. The present assay sensitivity is not considered to be sufficient enough to detect early mutations to fulfill the function of an intermediate biomarker. However, the assay will contribute to tumor differentiation in the final part of this study. Emphasis will be given to mutations in the *p*53 tumor suppressor gene. Mutations

of this gene can be found in about half of all human cancer cases. The location and characteristics of these mutations may reveal clues about their etiology. The predominant base changes in *p53* in human lung cancers (G:C to T:A transversions) were suggested to be indicative of causal lesions on the nontranscribed DNA strand by polycyclic aromatic hydrocarbons (Harris. 1993). Possible correlations of *p53* mutations with cigarette smoking have recently been discussed (Suzuki et al., 1992; Spruck et al., 1993; Habuchi et al., 1993; Brennan et al., 1995). The mutational activation of the protooncogene *ras*, another endpoint frequently associated with lung tumor development (Carbone and Minna, 1992), was not considered for the present study since the rat seems to be less sensitive to *ras* mutations by agents positive in the mouse and hamster such as N-nitrosamines (Belinsky et al., 1990).

Activation of cell proliferation, in conjunction with changes at the DNA level, is considered to be essential for initiation and tumor development. Cell division is necessary for conversion of adducts or DNA strand breaks to mutations or gaps, and also allows for mitotic recombination. However, the direct correlation between increased cell proliferation and development of neoplasia in target organs or morphological sites has been questioned (Yoshida et al., 1993). Cell proliferation, which does not give rise to formation of neoplasia, may simply be induced by the cytotoxicity of the test material in the absence of initiation. On the other hand, persistent cell proliferation may increase the probability of converting spontaneous DNA lesions to neoplastic changes. In subchronic inhalation studies on aged and diluted SS, an increased incorporation of BrdU into the rat nasal respiratory epithelium was found at a concentration of 10 μg TPM/l (Brown et al., 1995). Following a shorter period (5 days) of inhalation, the effect was

already seen at 1 μg/l. A similar pattern of response was found for the A/J mouse at concentrations of 1 and 4 μg TPM/l (Rajini and Witschi, 1994; Witschi et al., 1995), whereas the C57Bl/6 mouse did not respond. Following DEE inhalation, a transient initial increase in rat lungs was described by Wright (1986). Following chronic DEE inhalation, increased cell proliferation measured by ^{3}H-thymidine incorporation was detected in the bronchi and bronchioli as well as at particular sites in rat lungs (McClellan et al., 1986). Cell proliferation will be investigated in the present study using BrdU incorporation.

Cytokeratins have been used for the differential characterization of preneoplastic and neoplastic changes in epithelial tissues (Broers et al., 1988; Moll et al., 1988; Lindberg and Rheinwald, 1989; Schaafsma et al., 1990; Smedts et al., 1990). In contrast to the well-defined human cytokeratin expression patterns, those of the rat have been less extensively characterized. Alterations in cytokeratin expression were found to precede the histological expression of squamous metaplasia in several epithelial tissues in vitamin A-deficient rats (Gijbels et al., 1992). Recently, rat lung tumor types could be differentiated using monoclonal antibodies to human cytokeratins (Kal et al., 1993).

Inflammatory and fibrotic processes have often been associated with particle overload and carcinogenesis (Heinrich et al., 1986; Henderson et al., 1988; Muhle et al., 1990b; Morrow, 1992; Oberdörster, 1995). The PMNL proportion of lavagable bronchoalveolar cells as a sign of persistent acute inflammation was found to increase time- and dose-dependently following chronic exposure to DEE or toner.

Lactate dehydrogenase (LDH) activity is a cellular enzyme which is commonly determined extracellularly, e.g., in the supernatant of lung lavage, as a measure for cell lysis or cytotoxicity. Increased LDH activity was found in the lavage of DEE-exposed rats (Henderson et al., 1988) and may either be ascribed to alveolar macrophage lysis or to damage to the alveolar epithelium subsequent to particle overload. The determination of lung lavage LDH activity will enhance the interpretation of data in the present study.

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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.

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 *mg*/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 Prüfs, 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, formula 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, 2.43 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, Brusseis, 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	3.21 ± 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 wt) 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.

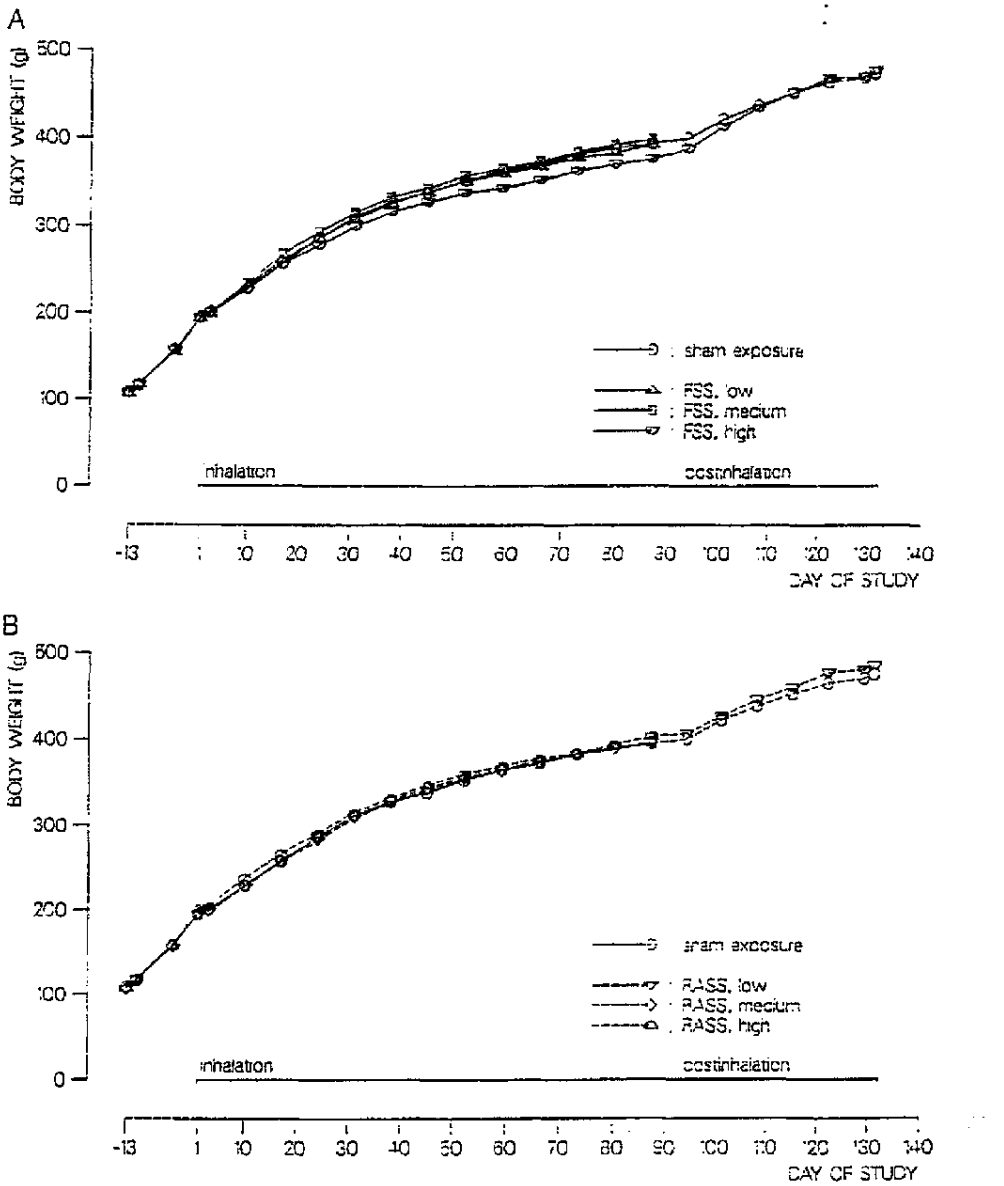

FIG. 1. Body weight development during the inhalation (all groups, 48 rats/group) and postinhalation periods (sham-exposed and high-dose groups, 18 rats/group). (A) Sham-exposed control and FSS groups; (B) sham-exposed control and RASS groups. Relative standard deviations for the individual groups were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

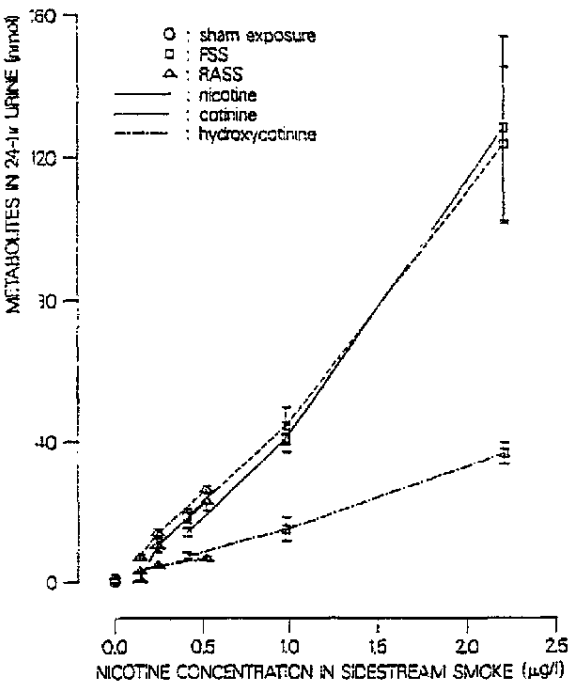

FIG. 2. Amounts of nicotine and its main metabolites recovered from urine samples collecting during the 6-h exposure period and the 18 h between exposures as a function of the SS nicotine concentrations (means ± SE).

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 ± 0.2*	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

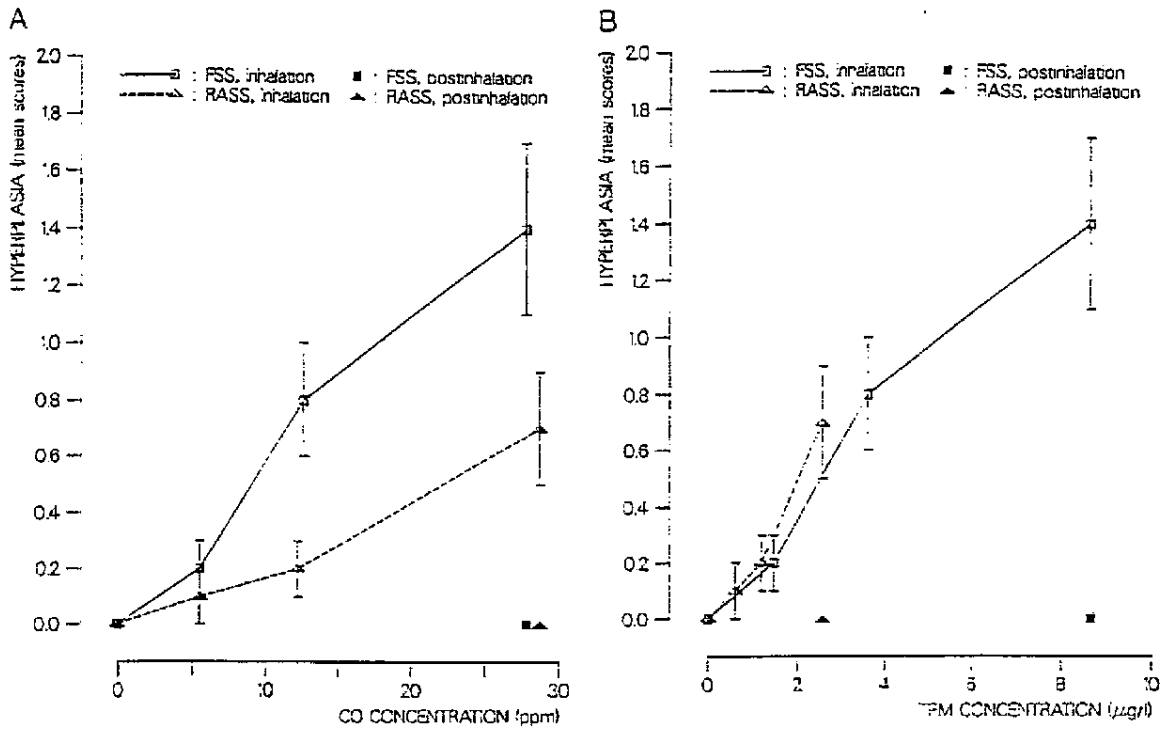

FIG. 3. Histopathological findings in the larynx. Effects are shown based on both dose parameters, i.e., CO and TPM concentrations, in FSS and RASS. (A, B) Hyperplasia of the squamous epithelium at the base of epiglottis. (A) Effects based on the CO concentrations as dose parameter; (B) effects based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

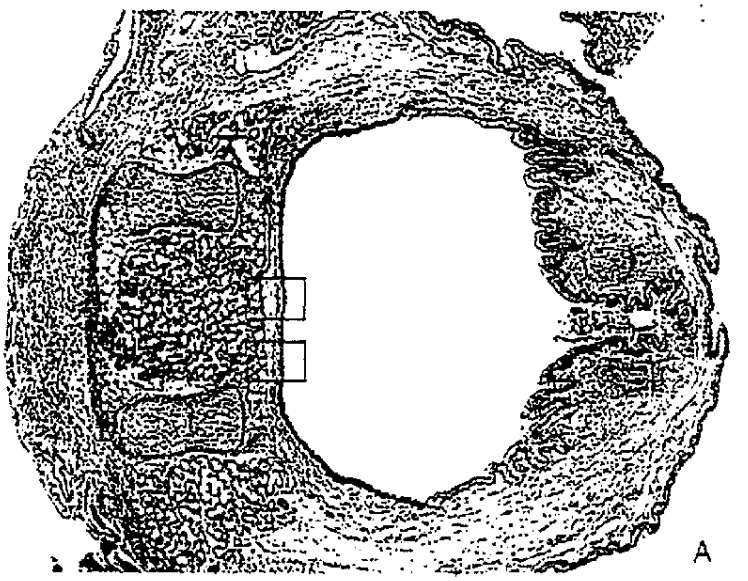

FIG. 4. Transverse sections at the larynx, base of epiglottis. (A) Overview (sham-exposed rat); (B, C) sham-exposed rat showing normal squamous epithelium at the ventromedial site and pseudostratified epithelium at ventrolateral sites; (D, E) high-dose FSS-exposed rat; (F, G) high-dose RASS-exposed rat,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

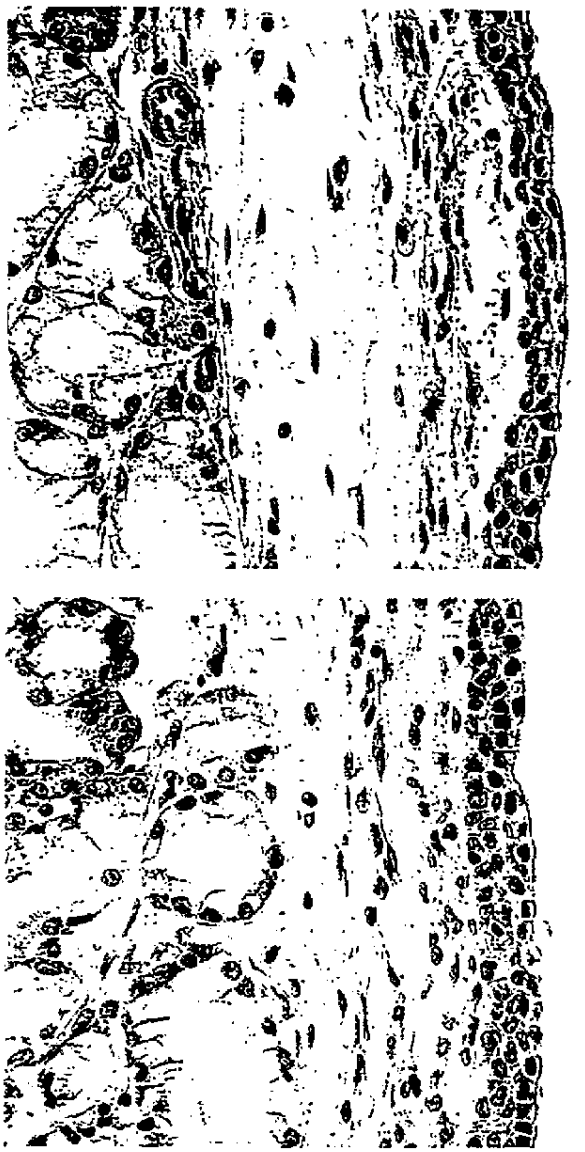

FIG. 4—*Continued*

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 3.7 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 μg 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 μg TPM/l 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 6%	8.7 ± 0.3 10%	10.0 ± 1.0 27%*	8.4 ± 0.2 7%	8.9 ± 0.4 13%	8.5 ± 0.2 8%
Floor of the larynx	10.5 ± 0.4	11.2 ± 0.4 7%	11.3 ± 0.4 7%	12.2 ± 0.4 16%*	10.7 ± 0.4 2%	12.1 ± 1.0 15%	12.0 ± 0.9 14%
Vocal cords†	22.2 ± 1.2	22.6 ± 1.2 2%	24.5 ± 1.4 10%	26.1 ± 1.3 17%	24.2 ± 1.1 9%	27.5 ± 1.3 24%*	27.5 ± 1.1 24%*

Note. Epithelial thickness (μm) 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 μg TPM/liter for FSS and RASS, respectively. The no-observed-effect levels were 6 ppm CO, equivalent to 1.5 and 0.6 μg 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

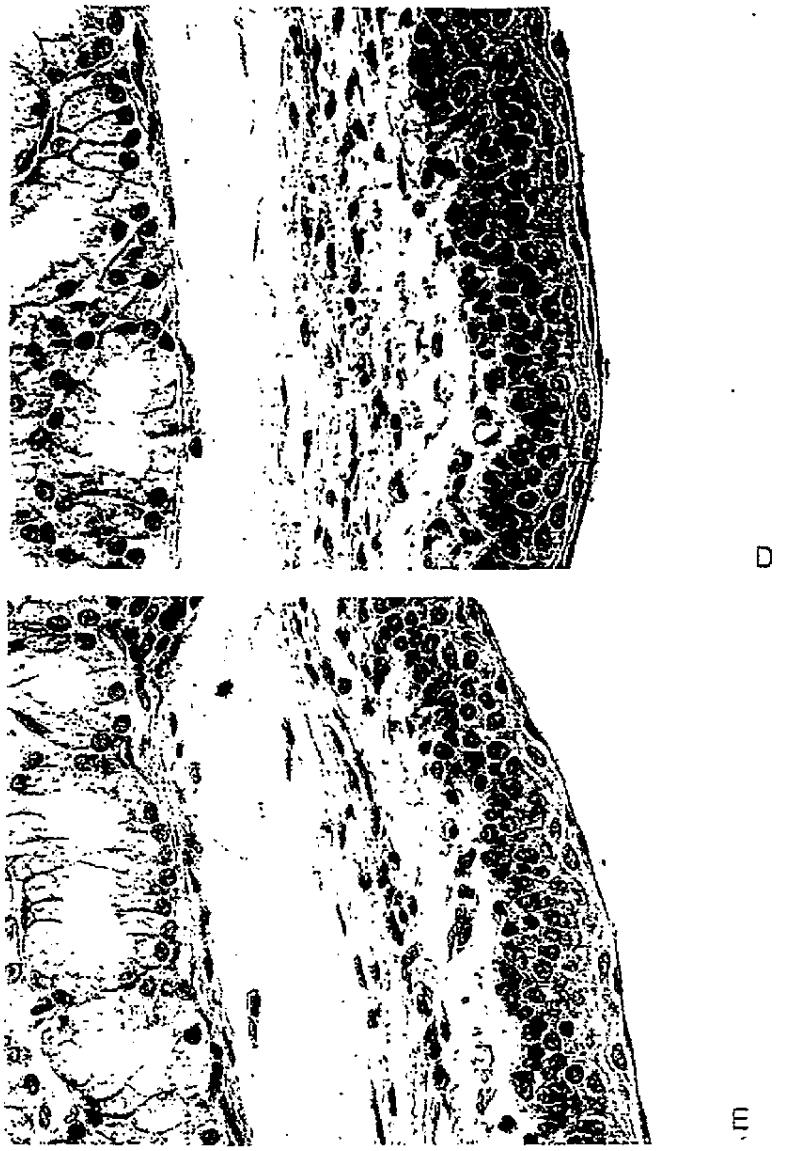

FIG. 4—*Continued*

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).

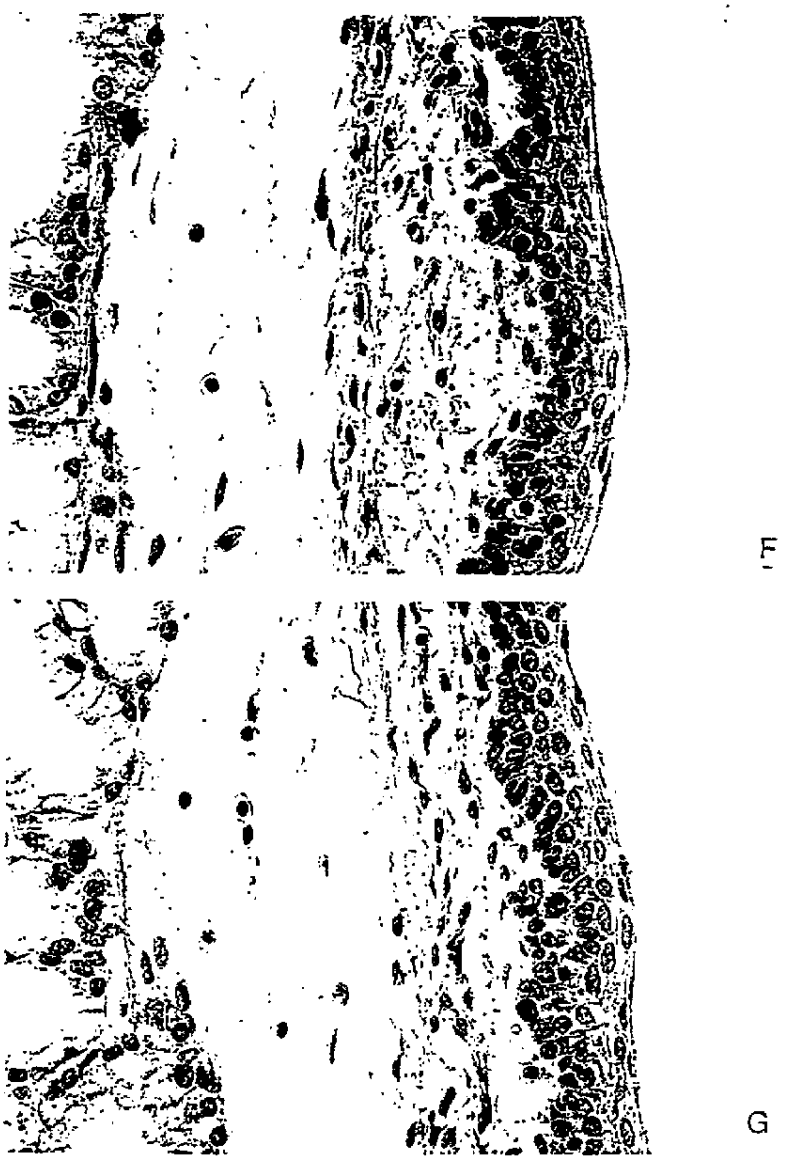

FIG. 4—*Continued*

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

Metabolite	Sham	FSS			RASS		
		Low	Medium	High	Low	Medium	High
9-OH-B(*a*)P†	16 ± 1	24 ± 2 1.5*	24 ± 1 1.5*	35 ± 2 2.1*	22 ± 1 1.4*	22 ± 1 1.3*	23 ± 2 1.4*
7,8-Diol-B(*a*)P†	37 ± 2	54 ± 4 1.5*	60 ± 3 1.6*	91 ± 6 2.5*	50 ± 3 1.3*	48 ± 2 1.3*	53 ± 5 1.4*
9,10-Diol-B(*a*)P†	22 ± 1	38 ± 2 1.7*	51 ± 3 2.3*	79 ± 6 3.5*	33 ± 2 1.5*	34 ± 2 1.5*	40 ± 5 1.8*
3-OH-B(*a*)P	602 ± 37	726 ± 60 1.2	671 ± 31 1.0	650 ± 22 1.1	686 ± 35 1.1	643 ± 34 1.1	613 ± 35 1.0
4,5-Diol-B(*a*)P	343 ± 24	398 ± 36 1.2	313 ± 19 0.9	350 ± 21 1.0	380 ± 29 1.1	339 ± 27 1.0*	328 ± 19 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

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

Room-Aged Cigarette Sidestream Smoke in Rats

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room-aged sidestream smoke inhalation

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The present study extends the current scope of rat inhalation studies on surrogates of environmental tobacco smoke. The 12-month inhalation period enabled an investigation of the potential progression or occurrence of new morphologic effects from subchronic to chronic inhalation. In addition, pulmonary inflammation and oxidative DNA damage were investigated. Female Wistar rats were whole-body exposed to room-aged cigarette sidestream smoke (RASS) generated from the reference cigarette 1R4F at 6 and 12 µg total particulate matter/l for 12 h/day, 5 days/week, and 12 months. To enable an evaluation of the exposure mode, another group of rats was exposed head-only to 12 µg total particulate matter/l for 7 h/day. Whole-body exposure conditions per se *resulted in changes of the RASS composition. An analysis of urinary nicotine metabolites showed that with whole-body exposure, RASS components, such as nicotine, were additionally taken up by routes other than inhalation. Independent from the exposure mode, blood carboxyhemoglobin and the hemoglobin adduct of 4-aminobiphenyl were used as biomarkers for the RASS concentration and dose, respectively. Histopathological changes were minimal to moderate reserve cell hyperplasia and slight squamous metaplasia of the respiratory epithelium as well as minimal reserve cell hyperplasia and atrophy of the olfactory epithelium in the anterior nasal cavity; slight eosinophilic globules in sustentacular cells of the olfactory epithelium in the anterior and posterior nasal cavity; pronounced squamous metaplasia and hyperplasia in the larynx at the base of epiglottis; and slight reserve cell hyperplasia in the bronchial respiratory epithelium. Most of the changes were adaptive and similar in type and degree to those seen in previous subchronic RASS inhalation studies. A flow cytometric analysis of bronchoalveolar lavage cells,* i.e., *alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes, did not show signs of pulmonary inflammation after 6 or 12 months of inhalation. As a measure for oxidative DNA modifications, 8-hydroxy-deoxyguanosine was determined in the lungs and nasal epithelia. No change was seen for this parameter at either time point in the lungs. There*

was a slight but not consistent increase in the nasal respiratory and olfactory epithelia as well as in urinary 8-hydroxy-deoxyguanosine excretion. In summary, there was little indication for progression or occurrence of new effects from 3 or 6 months to 12 months of RASS inhalation. There were also no signs of inflammation or oxidative DNA modification in the lungs. Chronic head-only exposure to RASS was shown to be technically feasible and is generally considered preferable for smoke inhalation studies over whole-body exposure to avoid artificial changes in smoke composition and the non-inhalative uptake of smoke constituents.

Environmental tobacco smoke (ETS) was classified as a class A (known human) carcinogen by the US Environmental Protection Agency (US EPA, 1992). The agency based this decision on the analogy to mainstream cigarette smoke (MS) as well as on the results of epidemiological studies. It acknowledged that lifetime animal inhalation studies were lacking, which implies that further experimental toxicology is necessary to evaluate the claimed biological plausibility of US EPA's classification.

Since this classification, three long-term inhalation studies on A/J mice were reported using different sidestream smoke (SS) surrogates for ETS (Witschi et al., 1995a, 1997a,b). The first study, six months of exposure to relatively fresh SS at a concentration of 4 μg total particulate matter (TPM)/l, did not show a difference in the rate of lung tumors compared to controls. In the second and third study, five months of exposure to a mixture of SS and MS at concentrations up to 87 μg TPM/l plus a 4-month postinhalation period, increased rates of pulmonary adenomas in the exposed mice were reported. However, apart from the highly toxic dose levels used, there were several inconsistencies in the latter studies, such as the large variation in the spontaneous lung tumor rate and the lack of smoke exposure-related non-neoplastic lesions in the lungs, which leave some questions open about the relevance of this experimental design. For rats, no long-term SS inhalation study has been reported to date although the rat is the most frequently used species in subchronic inhalation studies (Witschi et al., 1995b).

The present chronic study extends the current scope of rat inhalation studies on ETS surrogates. With an inhalation period of 12 months, an investigation of the potential progression of respiratory tract histopathological changes or occurrence of new changes from subchronic to chronic inhalation is made possible. In addition, the investigation of mechanistic end points has been included in this study in line with EPA's proposed guidelines for risk

assessment (US EPA, 1996) to obtain information on potential long-term/tumorigenic effects of SS using this experimental design. Pulmonary inflammation was assessed by investigating the composition of bronchoalveolar lavage cells, while oxidative DNA damage was assessed by determining tissue and urinary 8-hydroxy-deoxyguanosine (8-OHdG) levels. Other end points, such as cytokeratin expression, an epithelial cell differentiation marker, were also investigated and will be reported elsewhere (*cf.*, Schlage et al., 1997). The results of this study should help in the design and interpretation of possible long-term bioassays with regard to exposure levels, exposure mode, and/or mechanisms involved in chronic toxicity and possibly carcinogenicity, and thus contribute to a quantitative risk assessment of ETS (*cf.*, Witschi et al., 1995b).

Since ETS can not be reproducibly generated as required for chronic laboratory experiments, ETS surrogates have been developed for laboratory studies, e.g., aged and diluted SS (Coggins et al. 1993; Ji et al., 1994) and, more recently, room-aged SS (RASS) (Voncken et al., 1994; Haussmann et al., 1998). Although the carbon monoxide (CO) concentration - as a proxy for the number of cigarettes smoked per air volume - may be the most relevant basis to evaluate experimental SS-related effects, the TPM concentration was used in the present study to enable a comparison with environmental or other experimental SS studies. Concentrations in the range used in the present study (6 and 12 µg TPM/l) have shown to be effective in subchronic studies on rodents in producing a spectrum of histopathological (von Meyerinck et al. 1989), biochemical (Ji et al., 1994), genotoxic (Lee et al., 1993), and cell proliferative changes (Witschi et al., 1995a) and were, therefore, considered suitable for a chronic RASS inhalation study. The RASS concentrations employed in this study were approximately 100-fold higher than the maximum of the average concentrations of respiratory suspended particles (RSP) reported for ETS (Guerin et al., 1992; US EPA, 1992; Jenkins et al., 1996).

The aforementioned long-term SS inhalation studies (Witschi et al., 1995a, 1997a,b) as well as some subchronic SS inhalation studies (e.g., von Meyerinck et al., 1989) were conducted using the whole-body exposure mode. This is most probably related to the convenience for both experimental animals and staff. However, when using an aerosol such as cigarette smoke, this exposure mode might be subject to confounding influences, such as oral uptake during grooming. Differences between the whole-body and head-only exposure modes were reported for the uptake of smoke constituents (Mauderly et al., 1989; Chen et al., 1995). Similarly, pronounced differences in the uptake and toxicity of test atmospheres other than cigarette smoke were observed between whole-body and head-only exposure modes (Langard and Nordhagen, 1980; Wolff et al., 1982; Iwasaki et al., 1988). In the present study, therefore, the head-only exposure mode was compared to the whole-body mode with regard to various biomarkers of exposure and effect. The test substance uptake was maximized by extending the daily exposure duration as long as feasible for each exposure mode.

MATERIALS AND METHODS

Experimental Design

Female rats were whole-body exposed to RASS at concentrations of 6 and 12 µg TPM/l (WB6 and WB12) or to filtered, conditioned fresh air (WB0, sham-exposed group) for 12 h per day, 5 days per week, for 12 months (Table 1). For the comparison of exposure modes, another group of rats was head-only exposed for 5 days per week for 12 months to the high RASS concentration (HO12), but only for 7 h per day, which is considered the maximum daily exposure duration for this exposure mode. Due to laboratory capacity restraints, the inclusion of a head-only sham-exposed control group was not possible. However, this is not considered to seriously impact the comparison of the two exposure modes for most of the end points investigated. The two exposure modes were compared based on the TPM concentration as well as on the daily TPM dose for those end points presumably unaffected by tube restraint. Interim investigations of pulmonary inflammation and oxidative DNA damage, end points not previously included in subchronic SS inhalation studies, were performed after 6 months of inhalation for WB0 and WB12 only.

The study was performed in conformity with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (1991).

Experimental Animals

Female outbred Wistar rats (Crl: (WI)WU BR), bred under specified pathogen-free conditions, were obtained from Charles River (Sulzfeld, Germany). Wistar rats were used in the present study because of their potential suitability for long-term inhalation bioassays in terms of longevity, moderate body weight development, low rate of spontaneous tumors, particularly in the lungs, and sensitivity to rat respiratory tract carcinogens (Woutersen et al.,

1986; Kroes et al, 1988, Gupta et al., 1990; Vandenberghe, 1990; Bomhard and Rinke, 1994; Heinrich et al., 1995; Sanders and Lundgren, 1995). Female rats were used, because they showed a higher sensitivity in several long-term aerosol inhalation studies (Brightwell et al., 1989; Nikula et al., 1995), while in subchronic SS inhalation studies no difference was reported for the histopathological changes between male and female rats (Coggins et al., 1992). Wistar rats have not been used for the evaluation of SS-induced histopathological changes in the respiratory tract to date.

The respiratory tracts of 5 randomly selected rats were histopathologically examined on arrival; no abnormal findings were observed. Serological screening of 10 to 15 rats performed on arrival and after 6 and 12 months of inhalation did not detect antibodies to rat-related viruses, such as hantavirus, lymphocytic choriomeningitis virus, murine adenovirus, parvovirus H-1, pneumonia virus of mice, rat coronavirus/sialodacryoadenitis virus, rat virus, reovirus 3, rodent orphan parvovirus, Sendai virus, and Theiler's murine encephalomyelitis virus, to the bacteria *Clostridium piliforme*, *Mycoplasma pulmonis*, and cilia-associated respiratory bacillus, nor to the protozoon *Encephalitozoon cuniculi*.

The rats were individually identified using subcutaneous transponders (IMI-1000, Plexx, Elst, Netherlands; data acquisition by DAS-4001, Uno, Zevenaar, Netherlands). Following a 7-day acclimatization period before exposure, they were randomly allocated to the two RASS groups and the sham exposure group for whole body exposure (WB0 and WB12: 96 rats/group; WB6: 48 rats) as well as to the high RASS concentration group for head-only exposure (HO12: 48 rats). The age of the rats at the start of the inhalation period was between 55 and 70 days. The mean body weight at that time was 110 g (SD: 10 g).

The rats were barrier maintained 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 61 % (SD: 9 %), respectively. The light/dark cycle was 14 h/10 h. The whole-body exposed rats were exposed and housed in wire mesh cages (2 rats/cage), whereas the head-only exposed rats were housed between exposures in transparent polycarbonate cages (2 rats/cage) on sterilized softwood granulate (Braun & Co., Battenberg, Germany). A sterilized, fortified pellet diet (MRH FF, Eggersmann, Rinteln, Germany) from cage lid racks and sterilized water from bottles with sterilized sipper tubes were supplied ad libitum in each cage. Food was not available to the rats during the daily exposure periods. During exposure, drinking water was not available to the head-only exposed rats. Chemical analyses of food, water, and bedding material confirmed compliance with the requirements set forth by the National Toxicology Program (1991). Good hygienic conditions within the animal housing and exposure rooms were maintained as evidenced by the results of the bacteriological examinations of the laboratory surfaces and air as well as of the rat diet and drinking water.

RASS Generation

The University of Kentucky reference cigarette 1R4F (MS yields per cigarette: 10.8 mg TPM, 0.80 mg nicotine, and 11.6 mg CO; Tobacco and Health Research Institute, 1990) was used for SS generation as previously described for another reference cigarette (Haussmann et al., 1998). The cigarettes were smoked in basic accordance with the International Organization for Standardization as generally applied to MS generation. Room-aging was performed by continuously passing diluted SS at a rate of 56 m^3/h through a 28-m^3 experimental aging room with non-inert surfaces, resulting in RASS of a mean age of 0.5 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), vinyl floor tiles (11 m^2), and a polycarbonate

window (2 m^2) (*cf.*, Voncken et al., 1994). The materials in the aging room were unexposed at the start of the inhalation. The painted wallpaper was replaced every 13 weeks. 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 heat exchangers (approximately 60 m^2 surface area) were used to keep the room temperature constant. RASS was conveyed via glass tubing to the exposure chambers. RASS generation was started approximately 2.5 h before the start of the daily exposure to achieve a steady-state test atmosphere for inhalation. During overnight, non-smoking periods, the room was flushed with filtered, conditioned fresh air at 56 m^3/h.

The test atmosphere for WB6 was obtained by diluting the RASS from the aging room with filtered, conditioned fresh air. Whole-body exposure normally results in slight losses of TPM, mainly due to particle deposition in the chamber and on fur. In order to obtain the same TPM concentration in WB12 and HO12, the RASS from the aging room was diluted by 20 % with filtered, conditioned fresh air before entering the head-only exposure chamber.

Sham-exposed rats (WB0) were exposed to filtered, conditioned fresh air under the same conditions as the whole-body RASS-exposed rats.

Analytical Characterization of the Test Atmospheres

At designated time intervals, a broad range of analytes was determined to characterize the test atmospheres, to evaluate the reproducibility of the test atmosphere generation, and to detect possible cross contamination in the sham-exposed group. Samples were collected within the exposure chambers at sites representative for the breathing zone of the rats. CO was continuously monitored. TPM was determined at least once per day. The other analytes were determined at less frequent intervals from weekly to twice a year.

The analytical methods used to determine TPM, CO, nicotine, nitrogen oxides, aldehydes, ammonia, and the particle size distribution as well as those for temperature and relative humidity in the exposure chambers were performed as previously described (Haussmann et al., 1998). The other analytes were determined as follows: 3-Ethenyl-pyridine and nicotine were determined in parallel using 2-ethenyl-pyridine as internal standard. Solanesol was determined in particulate matter after trapping on Fluoropore membrane filters (pore size: 1 µm; Millipore, Eschborn, Germany) by reversed phase HPLC (Lichrospher RP-select B, 5 µm, 125 x 3 mm, Merck, Darmstadt, Germany) and UV detection (HP 1090, Hewlett Packard, Waldbronn, Germany). Isoprene, toluene, 1,3-butadiene, and benzene were trapped in methanol at -78 °C and determined by gas chromatography/mass spectrometry (GC/MS; Hewlett Packard 5890A/5970B) with a DB-5.625 column (30 m x 0.25 mm, J and W, Fisons, Wiesbaden, Germany). Phenols were determined in the particulate phase after trapping on Cambridge type glass fiber filters (Gelman, Ann Arbor, MI, USA), extraction, and silylation using GC/MS with a DB-5.625 column. Polycyclic aromatic hydrocarbons were extracted from a TPM-loaded glass fiber filter with methanol/water followed by back extraction with hexane, clean-up by solid phase extraction (Bakerbond amino, Baker, Gross-Gerau, Germany), and analyzed by GC/MS with a DB-17 column (30 m x 0.25 mm, J and W). N-nitrosamines were trapped in citrate/phosphate buffer with ascorbic acid and on glass fiber filters connected in series. The combined dichloromethane extracts were washed with a sodium hydroxide solution and cleaned by adsorption chromatography on aluminum oxide. The N-nitrosamines were determined by GC with a DB-5 column 30 m x 0.53 mm (ICT, Bad Homburg, Germany) and a thermal energy analyzer (TEA 543, Thermo Electron Corporation, via Isconlab, Heidelberg, Germany). For the determination of the metals, SS was collected on membrane filters with 0.22 µm pore size (GSWP 02500, Millipore). After the filters were digested with

nitric acid, the metals were determined by graphite furnace atomic absorption spectrometry by Henkel KGaA (Düsseldorf, Germany).

Animal Exposure System

The whole-body exposure chambers made of glass and stainless steel were equipped with 24 stainless steel wire mesh cages, which were mounted above stainless steel excretion pans. Each cage had a separate supply of test atmosphere. The flow rate through the chambers was 180 l/min. The position of the cages within the chamber was systematically changed on a weekly basis. Rats were exposed to RASS as well as to filtered, conditioned fresh air (sham-exposed control) for 12 h per day, 5 days per week, using two exposure chambers per group for WB0 and WB12 and 1 chamber for WB6.

Another group of rats (HO12) was head-only exposed to RASS for 7 h per day, 5 days per week. This exposure system was described previously (Haussmann et al., 1998). The position of the rats in the chamber was systematically changed on a daily basis.

In-Life Observations

The rats were observed daily for mortality, moribundity, signs of overt toxicity, or injuries. Detailed checks on general condition and behavior of the rats were performed on 3 rats/group per day shortly after the end of the daily exposure throughout the first 3 months, and two times per week in months 4 to 6. Starting with month 7, tumor checks were performed two times per week on all rats. The body weight of the individual rats was determined one day after their arrival, at the start of the inhalation period, and once per week during the inhalation period.

Biomonitoring

In order to provide an estimate of the amount of test atmosphere taken up by the rats, respiratory frequency and tidal volume were determined on at least 10 rats/group by whole-body plethysmography, three times during the inhalation period, as previously described (Haussmann et al., 1998). Rats were allowed to adapt to the plethysmographic tubes prior to data acquisition. During the adaptation and acquisition periods, they were continuously exposed to the test atmospheres.

To monitor exposure to CO, steady-state proportions of blood carboxyhemoglobin (HbCO) were determined in 5 rats/group according to Klimisch et al. (1974), three times during the inhalation period. The blood samples were collected after at least 5 h of exposure, a duration sufficient to obtain steady-state HbCO proportions (*cf.*, Tyuma et al., 1981). Rats were removed from the exposure chambers for a short period of time, and blood samples were taken under diethyl ether narcosis by puncturing the retro-orbital sinus with glass micropipettes.

To monitor exposure to aromatic amines, hemoglobin adducts of nine aromatic amines were determined in 7 to 8 rats/group following 12 months of RASS inhalation according to Kutzer et al. (1997). The hydrolyzed amines were derivatized with pentafluoropropionic anhydride (Aldrich, Steinheim, Germany) and analyzed using gas chromatography/mass spectrometry with negative chemical ionization in the SIM mode (DB-5MS column, J&W Scientific, Folsom, CA / TSQ 700, Finnigan, Bremen, Germany). For internal standardization, D_5-aniline (Aldrich) and D_9-2-aminobiphenyl (IC Chemikalien, Ismaning, Germany) were used.

To provide an estimate of the amount of nicotine taken up by the rats, nicotine metabolites were determined in urine collected from 6 rats/group over 24 h, three times during the inhalation period. During the 7-h head-only exposure period, the urine was collected using specially modified exposure tubes. During the postexposure period and for the whole-body

exposed groups, custom-made metabolism cages were used. All samples per rat and time point were combined, centrifuged, and stored frozen. The nicotine metabolites were determined by HPLC after derivatization with 1,3-diethyl-2-thiobarbituric acid (Rustemeier et al., 1993). The separation of all analytes in one chromatographic run at ambient temperature was enabled by modifying the derivatization conditions (0 °C and pH 3.3), the composition and pH of solvent A (54 mM 1-pentanesulphonic acid, 5 mM 1-heptanesulphonic acid, pH 5.0), and the solvent and flow program.

Gross Pathology and Organ Weight

Necropsy was performed without prior fasting. On the day following the last exposure at the end of the 12-month inhalation period, 8 rats/group were killed and examined as previously described (Haussmann et al., 1998). The weights of the lungs with larynx and trachea, liver, heart, adrenal glands, and kidneys were determined.

Histopathology

Histopathology of the respiratory tract was performed after 12 months of inhalation as previously described (Haussmann et al., 1998) with additional levels in the posterior nasal cavity (levels 3 and 4 according to Young, 1981). Processing and sectioning of the samples was performed at Huntingdon Research Centre (Huntingdon, Cambridgeshire, U.K.). The larynges of most of the rats were not reproducibly sectioned at the pre-defined levels needed for a semi-quantitative evaluation and a comparison to previous data. Thus, they could only be evaluated qualitatively. All slides were read by a veterinary pathologist in a blinded manner with experience in cigarette smoke-related changes in the respiratory tract of rodents. All pathological findings were scored according to a defined severity scale from 0 to 5 (marked effects). Mean severity scores were calculated based on all rats of a group.

Composition of Bronchoalveolar Lavage Cells

As a measure for possible inflammatory processes in the lungs, a differential count of bronchoalveolar lavage cells was performed using flow cytometry. For this purpose, 8 rats/group were killed as described following 6 (WB0 and WB12 only) and 12 *months* (all groups) of RASS inhalation. The lungs were lavaged via the trachea with 120 ml lavage medium per rat in 10 lavage cycles. The lavage medium was Dulbecco's phosphate buffered saline without calcium and magnesium (Biochrom, Berlin, Germany) supplemented with 3.25 g/l bovine serum albumin (Sigma) (pH 7.2). The viability of the lavaged cells of all groups was 95.5 % (SE: 0.4 %) as determined by the Trypan blue dye exclusion method. After fixation of the cells, they were incubated on ice for 48 h in a cell membrane permeabilization medium (according to Sander et al., 1991, with some modifications) to enable labeling of an intracellular epitope with the *pan* rat macrophage antibody ED1-fluoresceinisothiocyanate (FITC) conjugate (15 mg/l; Serotec, Kidlington, UK). Propidium iodide was added as a nucleic acid marker at 5 mg/l. The cells were analyzed using a Cytofluorograf 50H flow cytometer (Ortho Diagnostic Systems, Westwood, MA) in conjunction with the Cicero data acquisition and analysis workstation (Cytomation, Ft. Collins, CO). The cell populations (alveolar macrophages (AM), lymphocytes, and polymorphonuclear leukocytes (PMNL)) were identified by their characteristic appearance on a dot plot histogram of green (ED1-FITC; 515 - 530 nm) *vs.* red (nucleic acid content; ≥ 630 nm) fluorescence. From each sample, 30,000 counts were collected. Microscopic evaluation of Pappenheim-stained (Romeis, 1968) smears of bronchoalveolar lavage cells confirmed the flow cytometric results.

An aliquot of the above fixed lavaged cells was analyzed for spontaneous fluorescence. The system was internally calibrated by adding monodisperse (diameter: 1.95 µm) fluorescent latex beads (Duke Scientific, Palo Alto, CA) into the cell suspension. After gating the AM

population from a dot plot histogram of axial light loss *vs.* right angle light scatter, the green fluorescence (515 - 530 nm) was quantified in arbitrary units normalized to that of the admixed latex beads. From each sample, 15,000 counts were collected.

8-Hydroxy-Deoxyguanosine Determination

As a measure for possible oxidative DNA damage, 8-OHdG was determined in 8 rats/group after 6 (WB0 and WB12 only) and 12 months (all groups) of inhalation. During the last exposure day, rats were killed as described. The lungs were perfused in situ with isotonic saline to remove erythrocytes. The lungs as well as the nasal respiratory epithelium and the nasal olfactory epithelium were stored frozen at -70 ºC until further processing. DNA extraction was performed according to Gupta (1984) with emphasis on fast tissue processing to prevent both DNA repair and artificial DNA oxidation. Enzymatic digestion of DNA as well as chromatographic separation (Nucleosil 100-5 C18 precolumn, CS Chromatographie Service, Langerwehe, Germany; Novapack C18, Waters Millipore; HPLC HP 1050, Hewlett Packard) and electrochemical determination (HP 1049A, Hewlett Packard) of 8-OHdG were performed according to Shigenaga et al. (1990). The amount of deoxyguanosine (dG) present in the samples was determined by its absorbance at 245 nm.

As an integrative monitor for oxidative DNA damage without specification of the organs involved, 8-OHdG excretion in urine was determined in 5 to 6 rats/group at 5 and 12 months of inhalation. Twenty-four hour urine samples were collected as described for nicotine biomonitoring. The purification of 8-OHdG from the rat urine was performed according to Shigenaga et al. (1990) with some modifications. An internal standardization method was employed using ^{3}H-labeled 8-OHdG prepared from [1',2'-^{3}H]-dGTP (Amersham, Braunschweig, Germany) according to Shigenaga et al. (1990). Interfering substances in the

eluate were oxidized at +0.35 V before the actual 8-OHdG determination using an additional electrochemical detector.

Statistical Analysis

The following statistical tests were performed on the biological data: For the overall comparison of the whole-body RASS- and sham-exposed groups (WB0, WB6, and WB12), the one-way analysis of variance for continuous data (Sachs, 1982) and the generalized Cochran-Mantel-Haenszel test (Koch and Edwards, 1988) for ordinal data were used with the TPM concentration as the stratifying variable. If the overall comparison showed a significant difference, the Duncan test (Duncan, 1955) and the generalized Cochran-Mantel-Haenszel test were applied to continuous and ordinal data, respectively, for a pairwise comparison between the groups. All tests were conducted at the nominal level of significance of $\alpha = 0.05$ (2-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.

RESULTS

RASS Composition

Throughout the 12-month inhalation period, RASS was reproducibly generated and delivered to both types of exposure chambers. The analytical characterization of the sham and RASS exposure groups is shown in Table 2. As targeted, the TPM concentrations determined in the high concentration groups were the same for both exposure modes. The concentrations of most of the particulate phase constituents paralleled the TPM concentrations in the various groups. However, the catechol concentration in HO12 was almost twice as high as in WB12, for which there is no explanation. To achieve equal TPM

concentrations in the high concentration groups, the TPM losses in the whole-body chambers were compensated by dilution of the RASS entering the head-only chamber. This was reflected by a 20 % lower CO concentration in HO12 compared to WB12. Most of the other gas phase components paralleled CO, with the exception of nicotine and formaldehyde, which were 30 and 70 % lower, respectively, in WB12 than expected based on the HO12 data if a proportional dilution of all RASS components would be assumed. The carbon dioxide concentration was determined both at the inlet and outlet of the exposure chambers to assess its concentration in RASS as well as the concentration added by the exhalate of the rats, respectively.

The particle size distribution was the same for all RASS exposure groups regardless of the exposure mode, with an average median mass aerodynamic diameter of 0.42 µm and a geometrical standard deviation of 1.8.

The relative humidity in the sham-exposed group was 58 ± 8 % (mean ± SD); this is considered to be representative for the other exposure groups. The temperature within the exposure chambers were between 22 and 24 °C (SD: 1°C). These environmental conditions complied with the exposure conditions specified by the OECD (1981).

In-Life Observations

There was no RASS-related mortality. Shortly after the end of the daily exposure, detailed checks of the rats revealed findings that occurred more often in rats in one or both high RASS concentration groups than in those of the sham-exposed group, *i.e.*, secretion from the Harderian glands, slight yellow-brown discoloration of the fur, and impaired gripping ability. No other RASS-associated effects on the general condition and behavior of the rats were detected.

Signs of eye inflammation were found starting at 7 months peaking at 9 months of inhalation and were predominantly found in WB0 and HO12 (40 to 50 % of all rats) and less frequently in WB6 and WB12 (approximately 10 %). A cross-check of the data revealed no influence of this local inflammation on the other parameters of the study.

Biomonitoring

RASS Uptake Statistically significant decreases in respiratory frequency were seen in the whole-body exposed rats accompanied by a tendency to increased tidal volume resulting in slight decreases in the minute volume. For example, after 5 months of inhalation, the minute volume was 180 ± 7, 168 ± 8, and 162 ± 7 ml (mean ± SE) for WB0, WB6, and WB12, respectively. The respiratory minute volume for HO12 rats was 130 ± 6 ml. Daily RASS TPM doses were calculated taking into account these differences in respiratory minute volume, body weight, TPM concentrations, and daily exposure durations, the results being 2.8, 6.0, and 3.1 mg/kg body weight for WB6, WB12, and HO12, respectively. Thus, apart from the conventional comparison based on equivalent TPM concentrations (WB12 *vs.* HO12), a further comparison based on equivalent daily TPM doses is possible between WB6 and HO12.

Carboxyhemoglobin The steady-state HbCO proportions were in agreement with those expected based on the CO concentrations in the various test atmospheres (Figure 1A).

Hemoglobin Adducts Following 12 months of inhalation, a TPM dose dependent increase in hemoglobin adducts of 4-aminobiphenyl (4-AB) was found for WB0, WB6 / HO12, and WB12 (Figure 1B). The adduct levels for the other aromatic amines investigated (aniline, *o*-, *m*-, *p*-toluidine, 2-ethylaniline, 2,4-dimethylaniline, *o*-anisidine, and 3-aminobiphenyl) were

not statistically significantly different among the groups, although a notable increase (up to 6-fold) in the 3-aminobiphenyl adduct level was seen (data not shown).

Nicotine Uptake For the evaluation of the nicotine uptake, five major urinary nicotine metabolites were determined, *i.e.*, nicotine-N'-oxide, nornicotine, cotinine, *trans*-3'-hydroxycotinine, and norcotinine. Nicotine itself was also determined but not used in this evaluation, since control experiments showed that aerosol nicotine directly dissolved in the urine collected in the whole-body chambers. The sum of the five metabolites excreted over 24 h at 5 months of RASS inhalation is presented in Figure 2A. Similar data were obtained at 12 months of inhalation. At 3 weeks of inhalation, the total amount of metabolites excreted in the whole-body exposed groups was up to 50 % lower than at the later time points. The amount of nicotine metabolites excreted, and correspondingly the nicotine uptake, increased dose dependently in the whole-body exposed groups to levels which far exceeded that in the head-only exposed rats. This is partially due to the longer daily exposure duration and the higher respiratory minute volume for the rats in the WB groups: A theoretical uptake of inhaled nicotine was calculated using the nicotine concentrations in the test atmospheres, the exposure durations, the respiratory minute volume, the latter being calculated based on the body weights of the rats at the respective time points (Guyton, 1947), and assuming total absorption. Based on this theoretical uptake, the nicotine uptake in the whole-body exposed rats was still 2- to 3-fold higher than that in the head-only exposed rats (Figure 2B). The sum of the five metabolites excreted in HO12 accounted for almost 50 % of the estimated nicotine taken up. Overall, the pattern seen for the five nicotine metabolites relative to each other was similar at all three time points as well as for the different dose levels and exposure modes (data not shown).

Body Weight Development

The body weight of the rats increased throughout the inhalation period (Figure 3). After 3, 6, 9, and 12 months of inhalation, the body weight gain was statistically significantly lower by 5 to 8 % in WB12 compared to WB0; with the exception of the 12-month time point, the body weight gain in WB6 was statistically significantly lower by 4 to 6 % compared to WB0 (SE 1 to 2 % in all cases). The pronounced body weight effect seen in HO12 is considered to be mainly due to the exposure mode (Griffith and Standafer, 1985). A direct evaluation of the RASS effect on body weight development under head-only conditions was beyond the scope of the present study since a corresponding sham-exposed head-only control group had not been included.

Gross Pathology and Organ Weights

No RASS-related gross pathological changes were observed. The only statistically significant change in absolute organ weights in the whole-body exposed groups was a slightly decreased absolute heart weight in WB12 (-9 ± 2 %), which was no longer apparent when normalized to the respective body weight. As for the body weights, possible organ weight effects in HO12 could not be evaluated due to the lack of a corresponding sham-exposed head-only control group.

Histopathology

In the anterior level of the nose (level 1), patchy, slight to moderate reserve cell hyperplasia of the respiratory epithelium was observed (Table 3). This effect was concentration dependent in the whole-body exposed rats and similar in degree and incidence in HO12 and WB12. Slight squamous metaplasia of the respiratory epithelium was observed in WB12, this effect being seen in only a few rats in WB6 and HO12. Minimal goblet cell hyperplasia was also seen. At level 2, minimal epithelial changes were seen in the RASS-exposed groups, such as reserve cell hyperplasia and atrophy of the olfactory epithelium and reserve cell hyperplasia of the respiratory epithelium. At levels 2 and 3, slight eosinophilic globules were observed in the sustentacular cells of the olfactory epithelium of the RASS-exposed groups. There were no other histopathological findings in levels 3 and 4 of the nasal cavity.

The few larynges that could be evaluated as intended showed distinct squamous metaplasia of the pseudostratified epithelium and hyperplasia of the squamous epithelium at the base of epiglottis (Table 3). Those laryngeal sections that were not cut at the pre-defined levels could only be qualitatively evaluated. This evaluation confirmed epithelial hyperplasia

and squamous metaplasia to be the only findings observed in this organ. Further, the results given in Table 3 are representative for the findings in each of the respective groups. These effects were pronounced and seemed to be dose-dependent.

At the tracheal bifurcation, slight reserve cell hyperplasia of the respiratory epithelium was observed in only a few rats (not statistically significant; Table 3). In the pulmonary bronchial respiratory epithelium of the lungs, the degree of this finding was similar but the incidence was higher occurring most frequently in HO12. For both WB6 and WB12, this finding was statistically significantly different from WB0. The number of goblet cells was similar in all groups. A slight accumulation of pigmented AM was seen in WB12 and HO12.

The histopathological findings in the upper respiratory tract were less pronounced in HO12 compared to WB12. In the larynx at the base of epiglottis, the degree of the findings in HO12 was similar to that in WB6 (equal TPM doses). In the lower respiratory tract, the HO12 findings were closer to those seen in WB12, and the accumulation of pigmented AM was even seen at a higher incidence in HO12 than in WB12.

Composition of Bronchoalveolar Lavage Cells

There was a slight but statistically insignificant increase in the number of AM and thus of the total number of bronchoalveolar lavage cells with increasing TPM concentrations after 12 months of RASS inhalation (Table 4). No effect was seen for the number of lymphocytes and PMNL. Thus, no sign of a RASS-related inflammatory effect was observed. The same holds true for the comparison of WB12 and WB0 following 6 months of RASS inhalation (data not shown).

A green fluorescence was observed in the AM, the intensity of which increased with the RASS concentration (Table 5). For WB12, the intensity was similar following 6 or 12 months

of RASS inhalation indicating that a saturation or a steady-state equilibrium was already obtained after the shorter inhalation period.

8-Hydroxy-Deoxyguanosine Formation

Tissue Content Both after 6 and 12 months of RASS inhalation, the nasal 8-OHdG content increased in WB12 compared to WB0 (Figure 4A and B); this increase was statistically significant in two out of four cases. The most pronounced effect was seen in the respiratory epithelium after 12 months of inhalation (+158 %). Overall, a steady-state equilibrium between formation and repair of this modification seemed to be reached already by the 6-month time point. The results for the head-only exposed group are difficult to interpret, because a tube restraint-related effect cannot be excluded.

In the lungs, the 8-OHdG content did not change statistically significantly. However, a consistent trend to lower 8-OHdG levels (up to 30 %) was observed at both time points regardless of the exposure mode (Figure 4C).

Urinary Excretion At 5 months of RASS inhalation, an increased excretion of 8-OHdG was observed in the whole-body exposed groups with increasing RASS concentrations (Figure 5). Such an effect was not seen at 12 months of inhalation. Again, a contribution of a tube restraint-related effect cannot be excluded for the HO12 results.

DISCUSSION

The chemical characterization of the RASS used in the present study is the most comprehensive characterization of SS in an inhalation study to date. It allows a detailed comparison of RASS to ETS. The most coherent and representative recent publication on ETS composition involved volunteers in an environmental chamber who smoked the 50 top-selling US cigarette brands (Martin et al., 1997). Respirable suspended particles (RSP) as reported in this study can be considered to be solely particulate matter due to the controlled conditions employed, and would thus correspond to the definition of TPM in the present study. On the basis of TPM or RSP, the high RASS concentration in the present study was about 10-fold higher than the extreme ETS concentrations employed in the market cigarette study (Martin et al., 1997) (Figure 6). The ratios of the other SS constituents determined in both studies are in the same order of magnitude suggesting a proportional composition of 1R4F RASS and ETS generated from market cigarettes. In the most thorough recent ETS field study performed in 16 US cities, only those ETS constituents were determined which have generally been used as ETS markers (Jenkins et al., 1996). The comparison of the ETS concentrations of these constituents to those determined in RASS again supports the representative character of RASS for ETS (Figure 6).

The RASS concentrations in the present study were 2 to 3 orders of magnitude above the ETS concentration determined by Jenkins et al. (1996) in smokers' homes, and approximately 2 orders of magnitude above maximum average RSP concentrations contributed by ETS for residential (0.1 µg/l) and office (0.06 µg/l) environments reported by the US EPA (1992). Based on these RSP concentrations and a respiratory minute volume of 7 l/min for a 70 kg person, the daily RSP dose taken up in residences during 16 h and in offices during 8 h can

be estimated to be approximately 10 and 3 µg/kg, respectively. The daily TPM dose taken up by the rats in the present study was 3 orders of magnitude higher.

As a result of dilution, the concentrations of most RASS components decreased in proportion to the concentrations of either the particulate matter or gas phase markers. The major exceptions to this rule were the low concentrations of nicotine, formaldehyde, and catechol found in the whole-body compared to the head-only exposure chambers. Nicotine is known to adsorb with high affinity to all kinds of surface materials. The same lack of proportional dilution associated with the exposure mode was found for the nicotine concentration in MS (Chen et al. 1989). Formaldehyde is a highly reactive compound which, under whole-body exposure conditions, even reacts with rat excretion products and/or fur (unpublished results; Kewitz and Welsch, 1966). No explanation is available to date for the comparatively low catechol concentrations in the whole-body exposure groups.

Biomonitoring via the analysis of HbCO proportions in blood, hemoglobin adducts of 4-AB, and nicotine metabolites in urine confirmed exposure of the rats as planned. Further, valuable information was obtained on the feasibility of using these biomarkers in smoke inhalation studies and on their concentration/dose-responses.

After establishing a steady-state equilibrium, the blood HbCO proportion is directly proportional to the RASS CO concentration. However, the HbCO proportion does not reflect the overall dose of RASS taken up by the rats, especially when considering the differences in the daily whole-body and head-only exposure durations. The levels of the 4-AB hemoglobin adduct corresponded to the daily TPM doses for all groups. This adduct is considered to reflect the overall dose of metabolically activated 4-AB over the lifetime of the erythrocytes. However, for smoke inhalation studies, another explanation for the increase in this adduct with increasing TPM doses cannot be ruled out: The metabolic activation of 4-AB might be

dose-dependently induced, similar to the induction seen for benzo(a)pyrene metabolism in previous cigarette smoke inhalation studies (*e.g.*, Gairola, 1987; Haussmann et al., 1998). Indeed, pretreatment of rats with polycyclic aromatic hydrocarbons was shown to shift the hepatic metabolism of 4-AB towards activated metabolites and to proportionally increase the yield of protein adducts (Orzechowski et al., 1994). In any case, even if the increase in the 4-AB hemoglobin adduct seen in the present study is not a specific marker for 4-AB uptake, it might be a marker for the uptake of compounds capable of inducing 4-AB metabolism. The source of 4-AB responsible for the formation of the adduct level found in the sham-exposed group remains to be determined. A similar 4-AB background adduct level was reported by others (Bryant et al., 1987). High background adduct levels may also be responsible for the failure to detect possible increases in hemoglobin adduct levels of other aromatic amines associated with RASS inhalation.

The biomonitoring end points discussed above do not necessarily reflect the route of uptake of the respective compounds. This is especially important for whole-body exposure. Therefore, care has to be taken in determining the dose taken up by inhalation *vs.* the dose taken up by other routes. During whole-body exposure, aerosol is known to deposit on the fur of the rats and its constituents may be taken up either transdermally or orally by grooming (Langard and Nordhagen, 1980; Wolff et al., 1982; Iwasaki et al., 1988). This increases the total dose taken up and may exaggerate the associated toxicity. It should be kept in mind, however, that humans are exposed to ETS in a manner comparable to whole-body exposure. A certain degree of transdermal uptake of ETS constituents can thus be expected; its contribution to the total uptake remains to be determined. In rats, the gastrointestinal uptake of test material by grooming adds to the non-inhalative transdermal uptake, and this is certainly not representative for human ETS exposure. Chen et al. (1995) concluded that whole-body exposure increased the amount of cigarette smoke particles passing from the fur

into the gastrointestinal tract by about a factor of two compared to nose-only exposure. Mauderly et al. (1989) found nicotine concentrations in the plasma and urine of rats which were 5- to 6-fold higher with whole-body MS exposure than with nose-only exposure, based on equal nicotine concentrations in MS. Their corresponding urine cotinine concentrations were 2.6-fold higher. The latter factor fits well with the 2- to 3-fold higher amounts of nicotine metabolites excreted by whole body vs. head-only exposed rats in the present study, based on equal nicotine inhalation. Thus, for RASS, as is the case for other aerosols, whole-body exposure is associated with a significant uptake of test materials by routes other than inhalation.

The sum of the five nicotine metabolites determined in HO12 accounted for almost 50 % of the calculated inhaled nicotine dose per day. About the same percentage was obtained for the urinary excretion of these five metabolites following intravenous administration of nicotine to male Sprague-Dawley rats (Schepers et al., 1993). This percentage decreased to approximately 20 % in Aroclor 1254-induced Sprague-Dawley rats due to a different metabolic pattern in induced and non-induced rats. These data suggest that the nicotine metabolite pattern of the RASS-exposed rats was similar to that of non-induced rats, although this comparison includes an extrapolation between different rat strains and genders. This interpretation is supported by the lack of any change in the relative pattern of the five metabolites between 3 weeks and 12 months of inhalation.

The above mentioned biomonitoring end points cannot be used to determine the deposition of particulate matter in the lungs. The accumulation of pigmented AM may be an indication of particles accumulating in the lungs following smoke inhalation, since this phenomenon was found in rats following inhalation of whole MS but not in those only exposed to the vapor phase of MS (Davis et al., 1975a and b; Coggins et al., 1980). However, the

pigmentation may also result from lipid peroxidation or hemoprotein-derived iron incorporation in the AM as a secondary effect due to smoke particle inhalation. The number of AM staining positive for iron/hemosiderin was found to increase in rats following subchronic SS inhalation (CO concentration: 35 ppm; Escolar et al., 1995). In the present study, no iron-specific staining was used, and the slight accumulation of pigmented AM in the high concentration groups was seen in hematoxylin/eosin-stained sections. No such pigmentation was reported for subchronic SS inhalation studies on rats (von Meyerinck et al., 1989; Coggins et al., 1993; Lee et al., 1993; Teredesai and Prühs, 1994; Haussmann et al., 1998). It remains to be investigated whether this pigmentation is due to the higher daily particulate matter dose in the chronic compared to the subchronic studies and/or whether this is related to the chronic inhalation period. AM pigmentation was not seen in the chronic SS/MS inhalation study on A/J mice at 87 µg TPM/l (Witschi et al., 1997a). The discrepancy between the results of these two chronic inhalation studies remains to be resolved in view of a possible use of AM pigmentation as a pulmonary particulate biomonitor. To date, this discrepancy speaks for a species-specific secondary source for the pigmentation rather than for a simple endocytosis of TPM.

A dose-dependent increase in the fluorescence of AM from bronchoalveolar lavage was seen in the present study. This fluorescence has not been described in SS inhalation studies to date. Increased AM fluorescence was reported for MS-exposed rats (Coggins et al., 1980; Sköld et al., 1993) as well as for smokers compared to nonsmokers (Vassar et al., 1960; Sköld et al., 1989). There was no progression of this effect between 6 and 12 months of RASS inhalation, suggesting either a saturation of the effect as discussed for the AM fluorescence in smokers (Sköld et al., 1989) or the constitution of a steady-state equilibrium which would be dependent on the RASS concentration rather than the daily or accumulating dose. The nature of the MS-dependent fluorescence is still open, and similar causes have been discussed as for the histologically observed AM pigmentation (*e.g.*, Sköld et al., 1992).

A possible relationship between AM pigmentation and fluorescence remains to be investigated.

The degree of body weight gain reduction observed for WB12 can be interpreted as a sign of toxicity and demonstrates that the study design would satisfy the requirements set by the OECD (1981) or others for the highest dose level in a carcinogenicity study.

With few exceptions, the histopathological changes observed in the nose were qualitatively and quantitatively similar to those observed following subchronic RASS inhalation (Haussmann et al., 1998) indicating that there was no progression of these findings. This analogy further suggests that these effects, except for the minimal atrophy in the olfactory epithelium, could be considered adaptive responses to repeated irritation as shown for subchronic studies (von Meyerinck et al., 1989; Coggins et al., 1993; Haussmann et al., 1998). In addition to the reserve cell hyperplasia seen in the anterior part of the nose in the subchronic studies, slight squamous metaplasia of the respiratory epithelium in this part of the nose was seen in WB12. The appearance of this effect in WB12 might be due to the higher daily TPM dose compared to the subchronic studies and/or the chronic inhalation period. Some additional epithelial changes in nose level 2, although not seen in subchronic SS inhalation studies, are not considered relevant due to their very low incidence and degree. The eosinophilic material observed in olfactory epithelial sustentacular cells at levels 2 and 3 has not been reported in cigarette smoke inhalation studies. The significance of this finding is not clear.

In the larynx, the hyperplastic and metaplastic changes were the most pronounced. Those seen in HO12 were in line with those observed in the previous subchronic head-only exposure study (Haussmann et al., 1998) on the basis of an assumed linear concentration-response relationship (Figure 7A). However, the degree of the changes seen in WB6 and WB12

exceeded those observed in HO12 and also those seen in the previous subchronic head-only exposure studies. Normalizing these larynx effects to daily TPM doses reveals a dose-response relationship with no distinction between subchronic or chronic inhalation periods for the squamous metaplasia (Figure 7B) as well as the hyperplasia. Thus, there seems to be no progression with prolonged inhalation.

An association of most of the histopathological changes with the particulate matter phase of the SS aerosols was assumed in the previous subchronic study (Haussmann et al., 1998). The upper respiratory tract results of the present study support this interpretation and place emphasis on the daily particulate matter dose rather than on concentration (WB12 *vs.* HO12 / WB6). This contrasts with gaseous irritants, such as formaldehyde, which induced epithelial hyperplasia and metaplasia in the anterior part of the nose depending on the concentration rather than on the daily inhaled dose (Wilmer et al., 1989).

The base of epiglottis was the most sensitive site of the respiratory tract in this and the subchronic study (Haussmann et al., 1998). The hyperplastic and metaplastic changes seen there were not seen in SS inhalation studies performed by other laboratories (von Meyerinck et al., 1989; Coggins et al., 1993).

The only relevant histopathological finding in the lower respiratory tract following chronic RASS inhalation was slight reserve cell hyperplasia in the bronchial respiratory epithelium, which was not seen in the subchronic SS inhalation studies (von Meyerinck et al., 1989; Coggins et al., 1993; Teredesai and Prühs, 1994; Haussmann et al., 1998). It remains to be investigated whether this effect is due to the chronic inhalation period and/or the higher concentration or dose in the present study compared to the previous ones. Morphometric analyses to assess the pulmonary changes as reported by Escolar et al. (1995) were not performed in the present study.

The composition of the bronchoalveolar lavage cells did not indicate an inflammatory response to chronic RASS inhalation, although there was a slight but statistically insignificant increase in the number of AM, a response similar to that seen in rats following MS inhalation (Gairola, 1986; Bjermer et al., 1993; Miller et al., 1996). Also for MS, Mauderly et al. (1989) reported an increase in the concentration of PMNL in the bronchoalveolar lavage fluid of rats, whereas no statistically significant responses were found by others for this species (Gairola, 1986; Bjermer et al., 1993; Miller et al., 1996). Mice were considered to be more sensitive to MS inhalation with regard to PMNL responses (Gairola, 1986). However, no histopathological indication of an inflammatory response was seen by Witschi et al. (1997a) when A/J mice were chronically exposed to a mixture of SS and MS at a TPM concentration as high as 87 µg/l. On the other hand, pronounced effects on rat bronchoalveolar lavage cell composition indicating a strong inflammatory response were found following inhalation of aerosols including insoluble particles, such as Diesel engine exhaust, at particulate matter concentrations far below those used in cigarette smoke inhalation studies (Henderson et al., 1988). Pulmonary inflammation following particle inhalation has been associated with lung carcinogenesis in experimental animals (Dungworth et al., 1994).

An increase in the formation of 8-OHdG has been investigated in the present study as a marker for oxidative stress since this end point has been associated with carcinogenesis (Floyd, 1990) and with cigarette smoking (Asami et al., 1996, 1997). The observed increase of the 8-OHdG levels in the nasal epithelia at the highest RASS concentration was only in part statistically significant and consistent following 6 or 12 months of inhalation. The biological relevance of this effect remains to be investigated. With regard to its relation to carcinogenesis, it is interesting to note that Witschi et al. (1997a,b) have observed increased cell proliferation and epithelial lesions in the nose but did not specifically report nasal tumors

using SS/MS concentrations up to one order of magnitude higher than those in the present study.

In contrast to the results obtained for the nasal epithelia, in the lungs no statistically significant effect in the levels of 8-OHdG was found following 6 and 12 months of RASS inhalation; indeed, the levels tended to decrease. This might be plausible in view of several adaptive antioxidant responses reported for lungs following cigarette smoke exposure, *e.g.*, the accumulation of vitamins E and C or increased activities of glutathione peroxidase or superoxide dismutase, as reviewed by Chow (1993). Furthermore, the repair of oxidative DNA modifications might be induced as a consequence of chronic exposure as reported for the repair activity in the leukocytes of smokers (Asami et al., 1996). However, the above results do not preclude possible oxidative changes in particular pulmonary cell types not detectable by analyzing the homogenate of the whole organ. At the dose level used in the present study and within the limitations of the method, these data do not support the hypothesis of a free radical-mediated oxidative stress in the lungs of SS-exposed rodents (Witschi et al., 1997b).

The determination of the urinary excretion of 8-OHdG enables an overall assessment of oxidative DNA modifications and repair in the whole body (Shigenaga et al., 1990). A 50 % increase of the 8-OHdG excretion was reported for smokers (Loft et al., 1992). In the present study, a dose dependent increase of 8-OHdG excretion in the whole-body exposed rats was seen following 5 months of RASS inhalation. After 12 months, however, this effect was no longer seen. It is not known whether this can be explained by adaptive processes in the course of chronic RASS inhalation. Further, a distinction between effects related to the head-only RASS exposure and those related to the oxidative stress possibly associated with a certain degree of immobilization (Liu et al., 1996) imposed by the head-only exposure mode is not possible. Another point to consider is the higher catechol concentration in HO12

compared to WB12: Aqueous catechol solutions were capable of generating 8-OHdG in vitro (Leanderson and Tagesson, 1990), and a combined intraperitoneal administration of catechol and hydroquinone or phenol resulted in an increased level of 8-OHdG in mouse bone marrow (Kolachana et al., 1993).

Using the whole-body exposure mode, the rats could be exposed for a daily duration longer than that considered feasible for head-only exposure, thereby maximizing the daily RASS dose. This also enabled evaluations on the impact of the daily RASS dose *vs.* the RASS concentration as demonstrated, *e.g.*, for the histopathological changes in the larynx. For most of the end points investigated in this study, the exposure mode *per se* did not influence the RASS-induced changes, which were mainly local effects in the respiratory tract. It did, however, impact the evaluation of the body weight gain, which was essentially the only sign of systemic toxicity in the present study. In addition, the exposure mode might have also influenced the formation and excretion of 8-OHdG. For cigarette smoke carcinogenicity studies, whole-body exposure must be considered to be a substantial confounding factor: (1) The composition of the cigarette smoke aerosol, either RASS (present study) or MS (Chen et al., 1989), was found to artificially change in the whole-body situation. (2) There is a considerable non-inhalative uptake of smoke components which, based on the nicotine biomonitoring data for RASS (present study) and MS (Mauderly et al., 1989; Chen et al., 1995), might exceed the inhalative uptake by far. NNK, a compound structurally similar to nicotine, is a rodent lung carcinogen regardless of the route of administration, including dermal application (LaVoie et al., 1987), with a preferential formation of adenomas and adenocarcinomas (Hoffmann et al., 1996). Furthermore, pulmonary DNA adducts were found after topical application of MS condensate (Randerath et al., 1988) or Diesel engine exhaust extracts (Gallagher et al., 1990) to mouse skin. The extent of non-inhalative uptake might be different for nicotine and other compounds, since no influence of the exposure mode was

seen on the levels of the 4-AB hemoglobin adduct (RASS, this study) or those of pulmonary DNA adducts (MS, Mauderly et al., 1989) detected by the postlabeling method.

The role of particulate matter-related smoke constituents in the pulmonary carcinogenicity in A/J mice has recently been questioned by Witschi et al. (1997b), who reported that the carcinogenic potential of a HEPA-filtered mixture of SS and MS would be similar to that of the unfiltered smoke. However, the susceptibility as well as the relevance of this animal model remains to be clarified (Maronpot et al., 1986), especially at the dose/toxicity level employed.

In summary, for histopathological changes, pulmonary inflammation, or oxidative DNA damage, there was little indication of progression or occurrence of new effects following an extended inhalation period of 12 months. The two slight histopathological changes which were not seen in the subchronic studies, *i.e.*, squamous metaplasia in the anterior nasal respiratory epithelium and the bronchial reserve cell hyperplasia, might well be due to the higher concentration and daily dose in the chronic compared to the subchronic studies. However, it cannot be ruled out that these effects are due to the chronic inhalation period. Chronic head-only exposure to cigarette smoke was shown to be technically feasible and is considered preferable to whole-body exposure in order to avoid artificial changes in smoke composition and the considerable non-inhalative uptake of smoke constituents.

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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 6%	8.7 ± 0.3 10%	10.0 ± 1.0 27%*	8.4 ± 0.2 7%	8.9 ± 0.4 13%	8.5 ± 0.2 8%
Floor of the larynx	10.5 ± 0.4	11.2 ± 0.4 7%	11.3 ± 0.4 7%	12.2 ± 0.4 16%*	10.7 ± 0.4 2%	12.1 ± 1.0 15%	12.0 ± 0.9 14%
Vocal cords†	22.2 ± 1.2	22.6 ± 1.2 2%	24.5 ± 1.4 10%	26.1 ± 1.3 17%	24.2 ± 1.1 9%	27.5 ± 1.2 24%*	27.5 ± 1.1 24%*

Note. Epithelial thickness (μm) 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 μg TPM/liter for FSS and RASS, respectively. The no-observed-effect levels were 6 ppm CO, equivalent to 1.5 and 0.6 μg 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

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TABLE 1. Experimental Groups

Group Code	TPM Target Concentration (µg/l)	Exposure Mode, Daily Exposure Duration	Daily TPM Target Dose (µg/l x h)	Total Number of Rats Initially Exposed
WB0	0	whole-body, 12 h	0	96
WB6	6	"	72	48
WB12	12	"	144	96
HO12	12	head-only, 7 h	84	48

TABLE 2. Concentrations of Selected Smoke Components in the Test Atmospheres

Parameter	Unit of Measure	n	Exposure Groups			
			WB0	WB6	WB12	HO12
TPM	µg/l	257 to 259	n.d.	5.9 ± 0.5	11.9 ± 0.6	12.1 ± 0.7
carbon monoxide	ppm	259	n.d.	27 ± 2	51 ± 2	42 ± 2
carbon dioxide (chamber inlet)	"	11 to 12	270 ± 130	390 ± 150	510 ± 160	480 ± 150
" (chamber outlet)	"	11 to 12	1060 ± 90	1400 ± 240	1410 ± 260	2000 ± 650
nicotine	µg/l	56 to 60	n.d.	0.94 ± 0.38	1.95 ± 0.57	2.35 ± 0.56
3-ethenyl pyridine	"	52 to 53	-	0.20 ± 0.05	0.42 ± 0.06	0.37 ± 0.05
ammonia	"	11	1.3 ± 0.7	2.8 [a]	5.2 [b]	4.5 ± 1.2
solanesol	"	13	-	0.06 ± 0.01	0.12 ± 0.02	0.10 ± 0.02
formaldehyde	ppm	12	-	0.10 ± 0.03	0.17 ± 0.04	0.50 ± 0.07
acetaldehyde	"	12	-	0.67 ± 0.05	1.31 ± 0.06	1.11 ± 0.05
acrolein	"	12	-	0.13 ± 0.01	0.24 ± 0.01	0.22 ± 0.01
nitric oxide	"	11	0.01 [c]	0.84 ± 0.08	1.68 ± 0.09	1.42 ± 0.13
nitric oxides	"	11	0.03 ± 0.05	0.87 ± 0.10	1.73 ± 0.10	1.47 ± 0.13
1,3-butadiene	µg/l	5 to 6	-	0.07 ± 0.03	0.14 ± 0.06	0.13 ± 0.07
isoprene	"	5 to 6	-	1.1 ± 0.2	2.5 ± 0.6	2.1 ± 0.7
benzene	"	5 to 6	-	0.16 ± 0.02	0.33 ± 0.05	0.29 ± 0.04
toluene	"	5 to 6	-	0.47 ± 0.05	0.90 ± 0.15	0.73 ± 0.10

[a] median; quartiles: 2.3 and 4.0 µg/l
[b] median; quartiles: 4.7 and 6.2 µg/l
[c] median; quartiles: 0.00 and 0.03 ppm

TABLE 2. Concentrations of Selected Smoke Components in the Test Atmospheres (cont.)

Parameter	Unit of Measure	n	Exposure Groups			
			WB0	WB6	WB12	HO12
phenols						
phenol	ng/l	4	-	2.8 ± 0.5	6.9 ± 0.7	6.6 ± 0.9
o-cresol	"	4	-	0.48 ± 0.06	1.19 ± 0.10	1.12 ± 0.13
m-cresol	"	4	-	0.53 ± 0.08	1.32 ± 0.14	1.37 ± 0.17
p-cresol	"	4	-	1.1 ± 0.2	2.7 ± 0.3	2.6 ± 0.3
catechol	"	4	-	7.4 ± 2.2	16.7 ± 4.1	29.7 ± 4.9
resorcinol	"	4	-	0.24 ± 0.02	0.44 ± 0.04	0.43 ± 0.05
hydroquinone	"	4	-	32 ± 5	69 ± 8	71 ± 7
polycyclic aromatic hydrocarbons						
fluoranthene	pg/l	2	-	170/120	370/270	510/440
pyrene	"	2	-	160/120	320/260	430/370
benzo(a)anthracene	"	2	-	140/ 90	260/190	270/210
chrysene	"	2	-	320/220	610/490	560/510
benzo(-)fluoranthene	"	2	-	100/ 80	200/170	170/160
benzo(a)pyrene	"	2	-	65/ 55	132/123	112/121
indeno(1,2,3,-cd)pyrene	"	2	-	25/ 28	53/ 53	42/ 50
dibenzo(-)anthracenes	"	2	-	6/ 7	14/ 14	11/ 11
benzo(ghi)perylene	"	2	-	16/ 19	39/ 37	30/ 33

TABLE 2. Concentrations of Selected Smoke Components in the Test Atmospheres (cont.)

Parameter	Unit of Measure	n	Exposure Groups			
			WB0	WB6	WB12	HO12
N-nitrosamines						
N-nitrosodimethylamine	ng/l	2	n.d.	0.30/n.d.	0.58/n.d.	0.57/n.d.
N-nitrosopyrrolidine	"	2	n.d.	n.d.	n.d.	0.14/0.12
N-nitrosonornicotine	"	2	n.d.	n.d.	n.d.	0.21/0.25
N-nitrosoanatabine	"	2	n.d.	n.d.	n.d.	n.d.
N-nitrosoanabasine	"	2	n.d.	n.d.	n.d.	n.d.
NNK	"	2	n.d.	1.26/1.12	2.08/1.98	2.33/2.17
cadmium	pg/l	2	n.d.	280/310	470/590	460/520

Note. - Data are given as mean ± standard deviation (except when n = 2).
- WB0: whole-body sham-exposed control group;
- WB6 and WB12: whole-body exposure groups at 6 and 12 µg TPM/l, respectively;
- HO12: head-only exposure group at 12 µg TPM/l.
- "-": not determined.
- n.d.: not detectable.
- Detection limits: TPM: 0.1 µg/l, CO: 1.5 ppm, nicotine: 0.05 µg/l, N-nitrosodimethylamine: 0.16 ng/l, N-nitrosopyrrolidine: 0.11 ng/l, N-nitrosonornicotine: 0.21 ng/l, N-nitrosoanatabine and N-nitrosoanabasine: 0.19 ng/l, NNK: 0.36 ng/l, cadmium: 10 pg/l.
- Chromium, nickel, lead, and zinc: no RASS-dependent increase above blind filter contents of these metals.

TABLE 3. Histopathological Findings Following 12 Months of RASS Inhalation

Parameter	Exposure Groups			
	WB0	WB6	WB12	HO12
nasal cavity, level 1				
respiratory epithelium				
reserve cell hyperplasia	0 0/8	0.8 ± 0.2 * 6/8	1.9 ± 0.1 * 8/8	1.6 ± 0.3 7/8
squamous metaplasia	0 0/8	0.1 ± 0.1 1/8	1.0 ± 0.3 * 5/8	0.3 ± 0.2 2/8
goblet cell hyperplasia	0 0/8	0 0/8	0.4 ± 0.4 1/8	0.1 ± 0.1 1/8
nasal cavity, level 2				
respiratory epithelium				
reserve cell hyperplasia	0 0/8	0.1 ± 0.1 1/8	0.1 ± 0.1 1/8	0 0/8
olfactory epithelium				
reserve cell hyperplasia	0 0/8	0 0/8	0.1 ± 0.1 1/8	0 0/8
atrophy	0 0/8	0 0/8	0.3 ± 0.2 2/8	0.3 ± 0.2 2/8
eosinophilic globules	0.1 ± 0.1 1/8	1.1 ± 0.5 3/8	1.0 ± 0.5 4/8	1.3 ± 0.5 4/8
nasal cavity, level 3				
olfactory epithelium				
eosinophilic globules	0 0/8	0.6 ± 0.4 2/8	0.9 ± 0.5 3/8	1.5 ± 0.6 4/8
larynx				
base of epiglottis				
squamous metaplasia of pseudostratified epithelium	0 0/1	2.8 ± 0.3 4/4	4.0 ± 0.0 3/3	2.6 ± 0.2 5/5
hyperplasia of squamous epithelium	0 0/1	3.3 ± 0.3 4/4	4.0 ± 0.0 3/3	2.8 ± 0.2 5/5

TABLE 3. Histopathological Findings Following 12 Months of RASS Inhalation (cont.)

Parameter	Exposure Groups			
	WB0	WB6	WB12	HO12
trachea				
bifurcation				
reserve cell hyperplasia of respiratory epithelium	0 0/7	0 0/7	0.4 ± 0.2 3/7	0.1 ± 0.1 1/7
lungs				
bronchi, respiratory epithelium				
reserve cell hyperplasia	0 0/8	0.6 ± 0.3 * 4/8	0.9 ± 0.3 * 5/8	0.9 ± 0.1 7/8
goblet cell hyperplasia	1.1 ± 0.5 4/8	0.3 ± 0.2 2/8	1.1 ± 0.5 4/8	0.9 ± 0.5 3/8
alveoli				
accumulation of pigmented macrophages	0 0/8	0 0/8	0.6 ± 0.4 3/8	0.9 ± 0.1 7/8

Note. Histopathological findings are given as mean score ± SE and incidence. Due to the sectioning problems, only few larynges per group could be evaluated, thereby precluding statistical analysis.

* For whole-body exposed groups: statistically significantly different from WB0.

TABLE 4. Bronchoalveolar Lavage Cell Composition Following 12 Months of RASS Inhalation

	Exposure Groups			
Cell Type	WB0	WB6	WB12	HO12
AM	5.3 ± 0.5	5.6 ± 0.6	6.4 ± 0.4	6.4 ± 0.6
lymphocytes	0.074 ± 0.009	0.100 ± 0.013	0.079 ± 0.006	0.136 ± 0.019
PMNL	0.075 ± 0.017	0.048 ± 0.014	0.036 ± 0.007	0.040 ± 0.014

Note. Bronchoalveolar lavage cells are given in absolute numbers (10^6), mean ± SE (no statistically significant differences).

Table 5. Alveolar macrophage fluorescence (relative units).

	Exposure Groups			
Inhalation Period	WB0	WB6	WB12	HO12
6 months	100 ± 5	-	315 ± 14*	-
12 months	100 ± 5	211 ± 19*	271 ± 15*	260 ± 16

Note: Results are expressed as percentages of WB0 (means ± SE). *For whole-body exposed groups: statistically significantly different from WB0."

FIGURE LEGENDS

Figure 1 Biomonitoring of RASS exposure: A: Blood carboxyhemoglobin determined at three time points (means ± SE), B: Hemoglobin adduct of 4-aminobiphenyl after 12 months of RASS inhalation; daily TPM dose calculated from the respiratory minute volume averaged over the duration of the inhalation period (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

Figure 2 Biomonitoring nicotine uptake: A: sum of five nicotine metabolites (nicotine-N'-oxide, nornicotine, cotinine, trans-3'-hydroxycotinine, norcotinine) excreted in 24-h urine in month 5 of the inhalation period (means ± SE), B: Data from A, normalized to the inhaled nicotine dose (based on the nicotine concentration in the test atmospheres, the respiratory minute volume calculated according to Guyton (1947), and the body weight of the rats; means ± SE).

Figure 3 Body weight development (means).

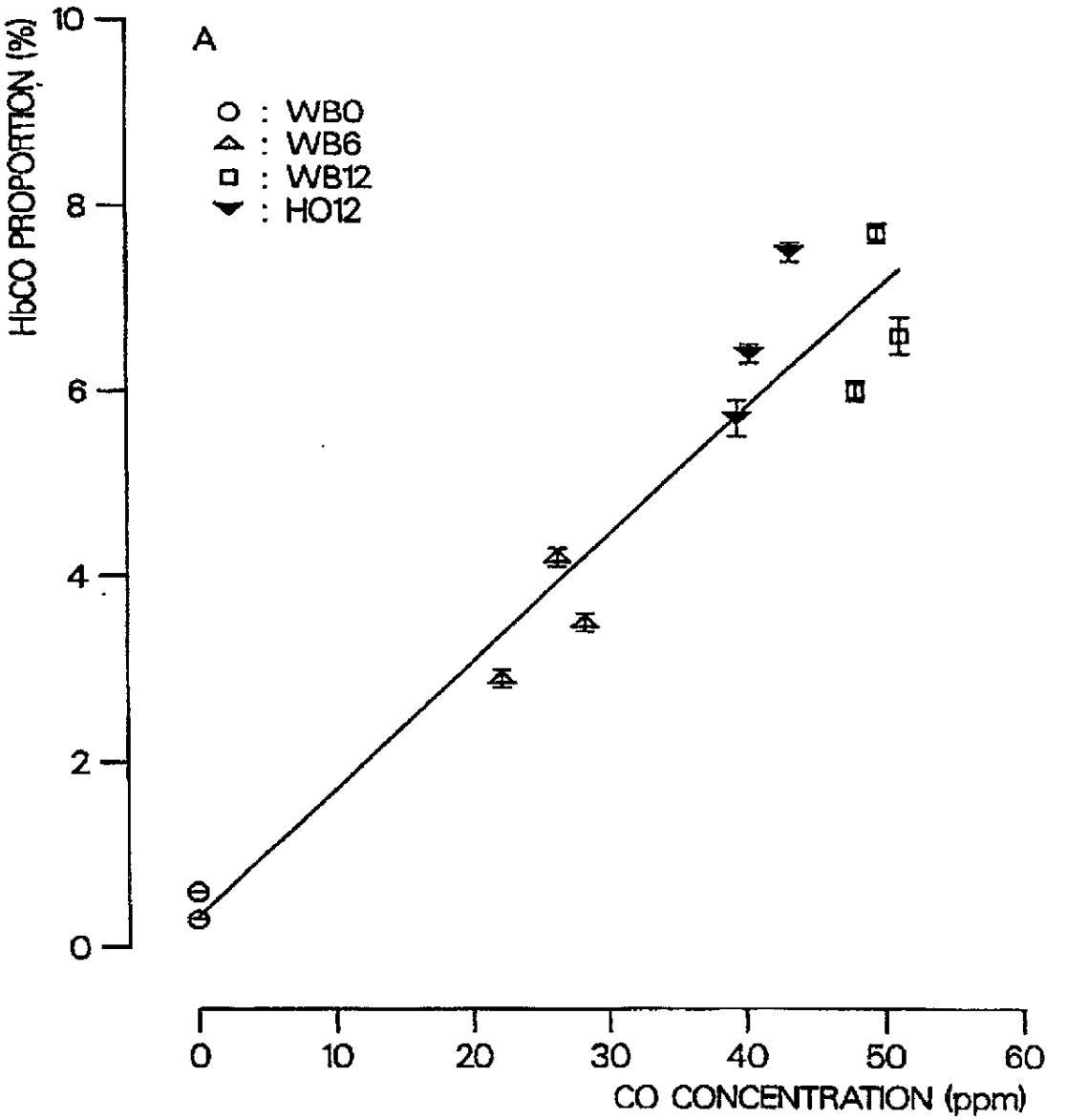
Figure 4 Tissue levels of 8-hydroxy-deoxyguanosine: A: nasal respiratory epithelium, B: nasal olfactory epithelium, C: lungs (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

Figure 5 Urinary excretion of 8-hydroxy-deoxyguanosine (means ± SE; *: for whole-body exposed groups: statistically significantly different from WB0).

Figure 6 Comparison of RASS in present study (HO12) with ETS either generated at an extreme concentration under experimental conditions (Martin et al., 1997) or as determined in a large field study (Jenkins et al., 1996) (abbreviations: CO: carbon monoxide, TPM: total particulate matter, AMM: ammonia, NIC: nicotine, ISO: isoprene, AA: acetaldehyde, NO, nitric

oxide, TOL: toluene, FA: formaldehyde, EP: 3-ethenyl-pyridine, BEN: benzene, BUT: 1,3 butadiene, SOL: solanesol, CAT: catechol).

Figure 7 Squamous metaplasia of the pseudostratified epithelium in the larynx, base of epiglottis, in the present and the previous subchronic inhalation study (Haussmann et al., in press) (mean scores ± SE): A: Dependence on the TPM concentration, B: Dependence on the daily TPM dose calculated from the respiratory minute volume averaged over the duration of the inhalation period (n = 4 to 10 rats/group).



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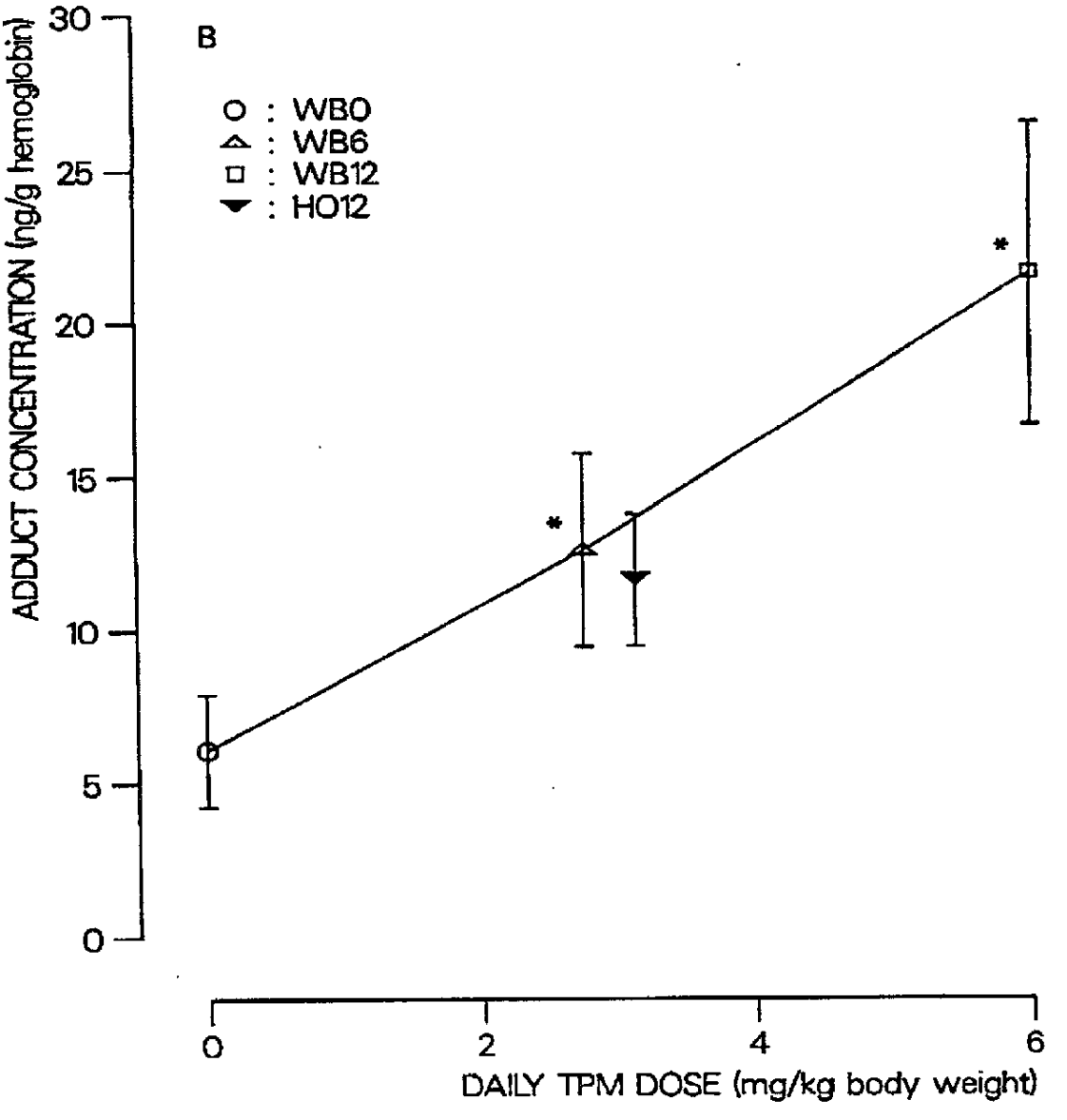

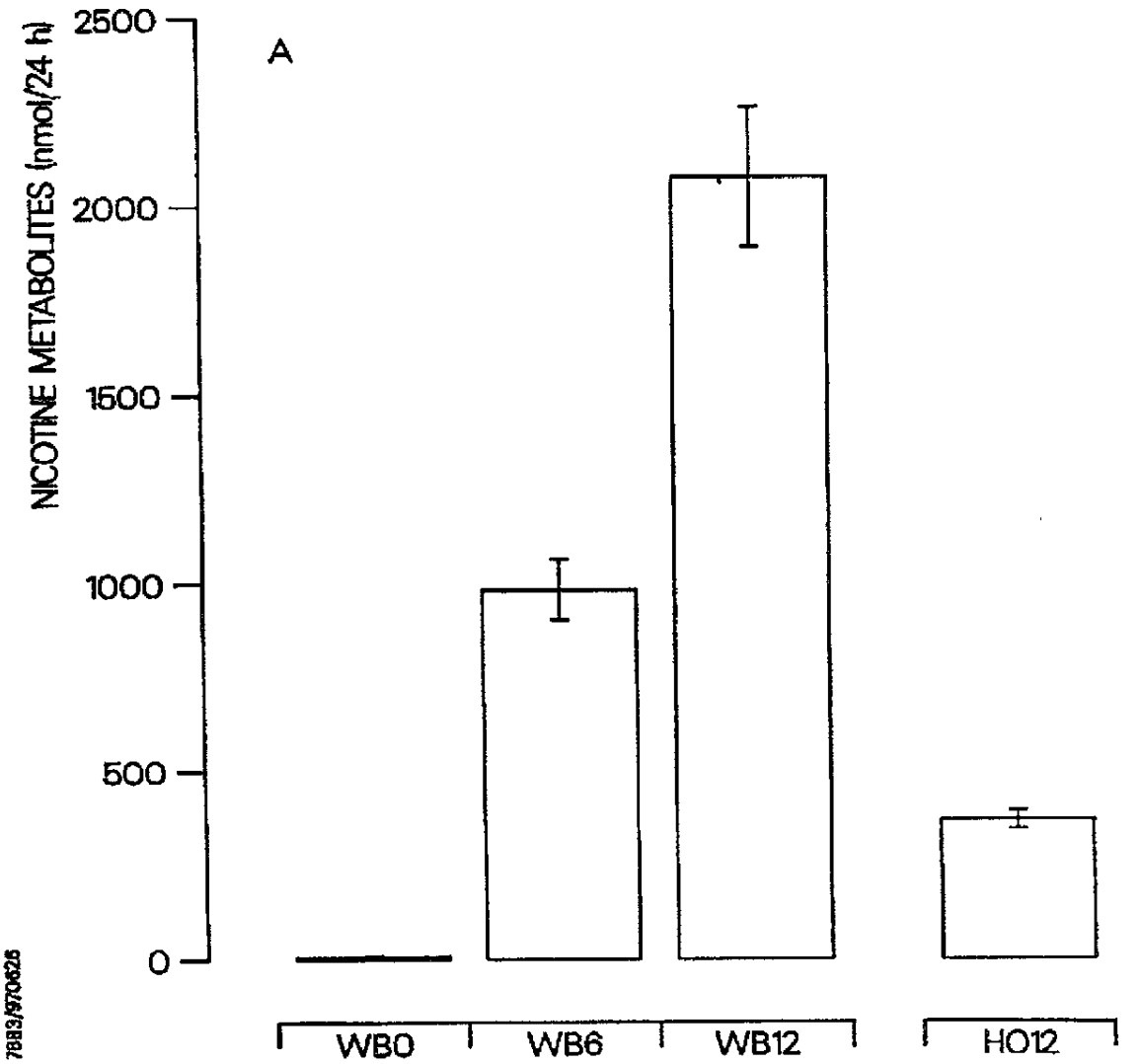

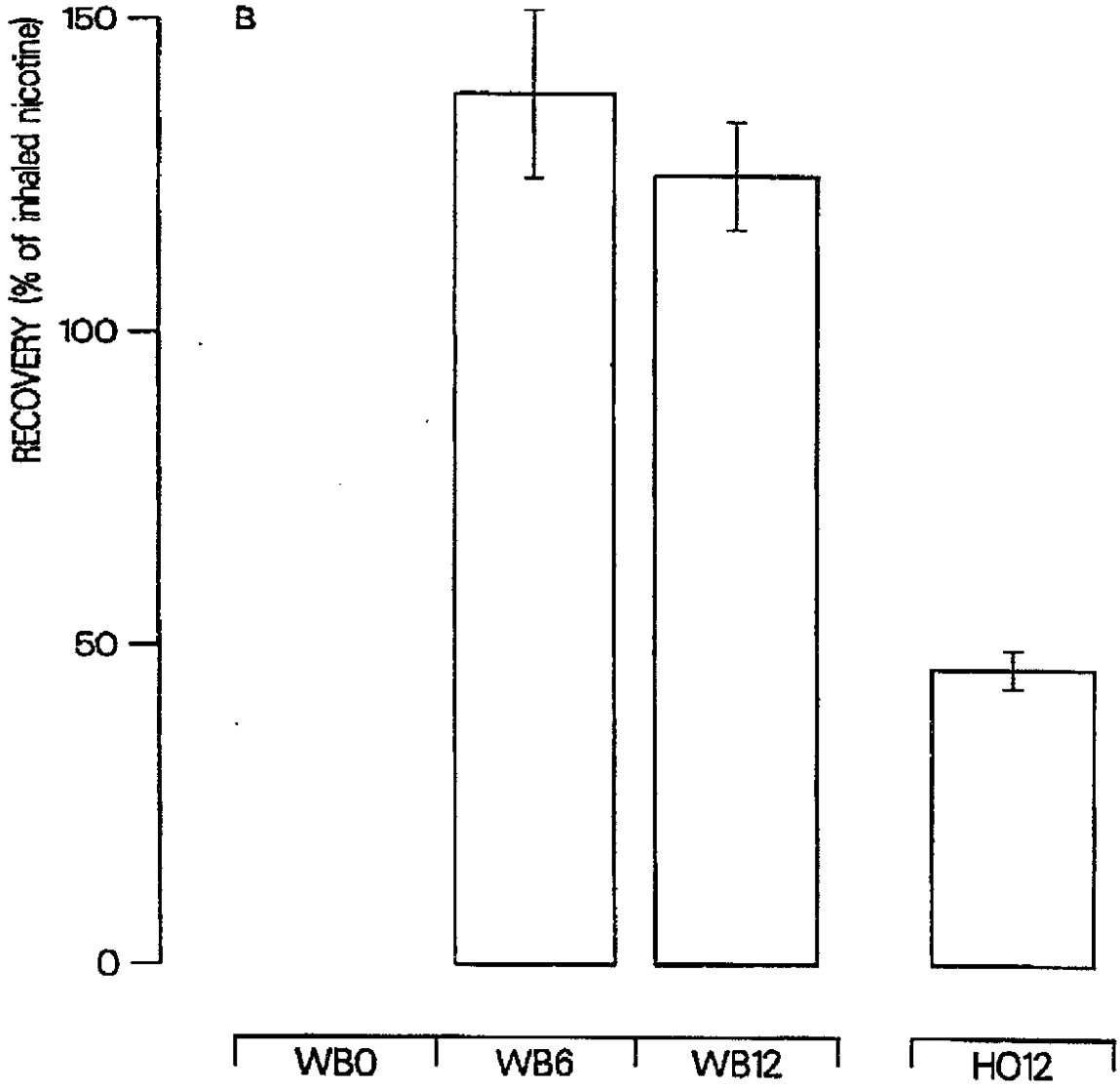

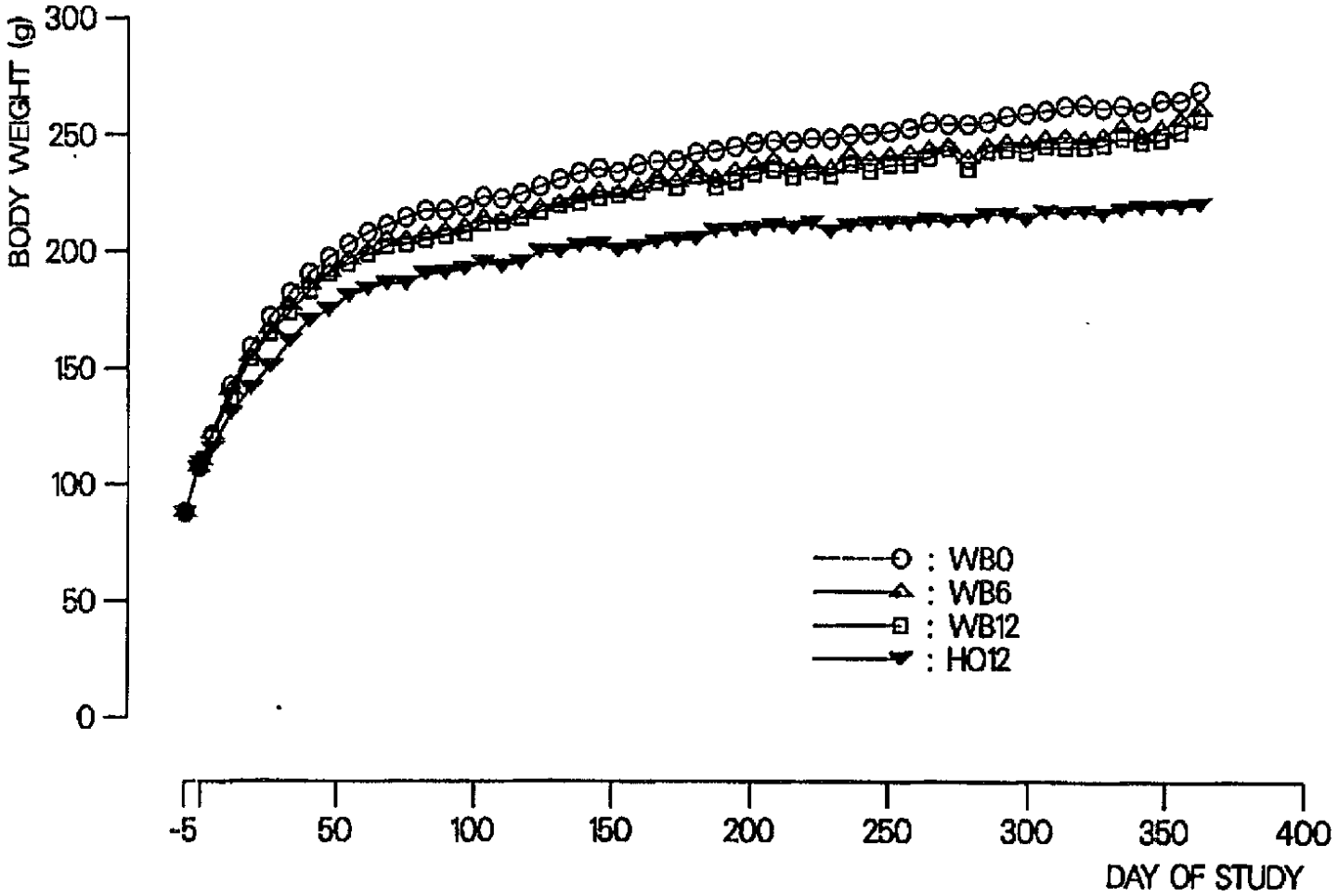

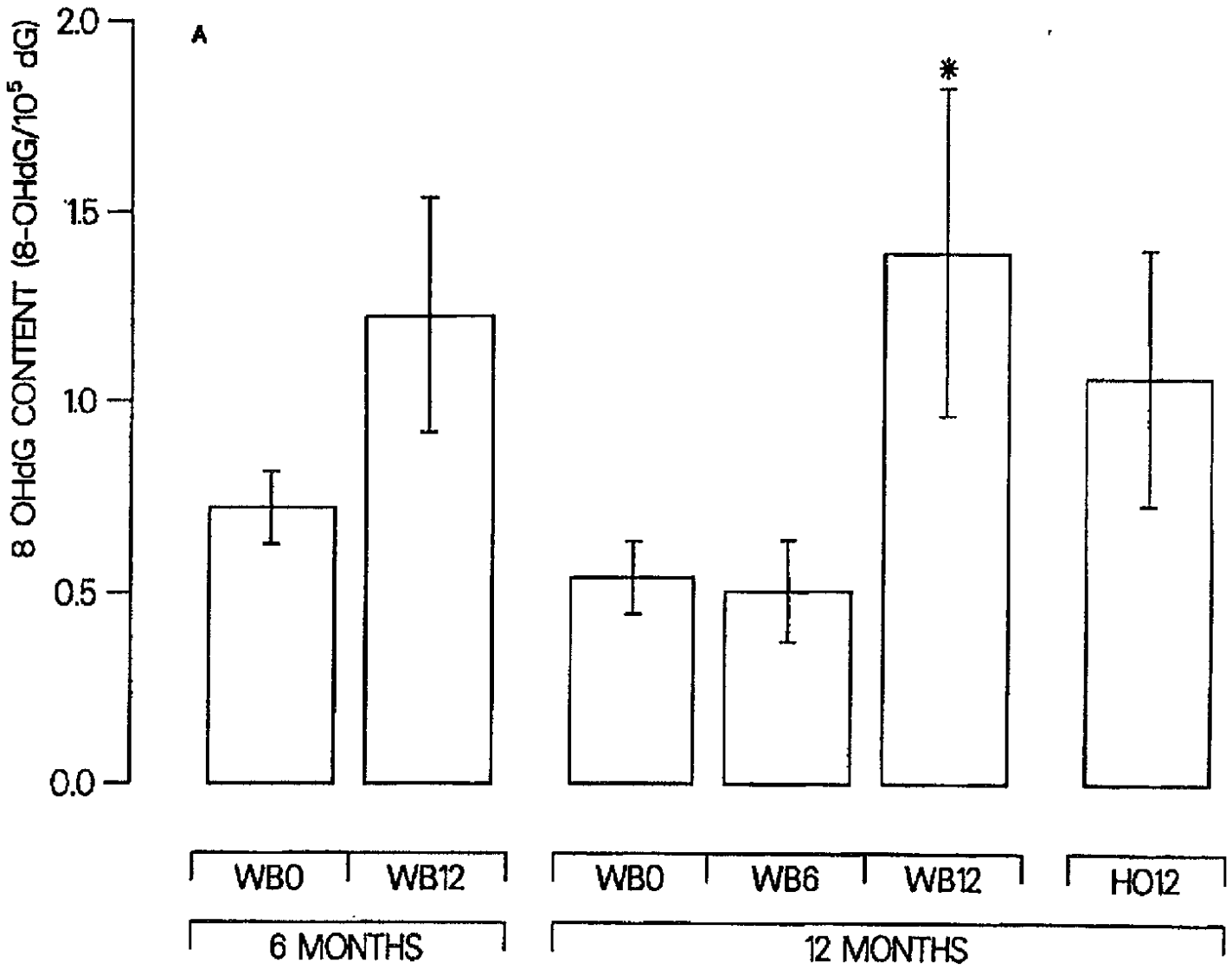

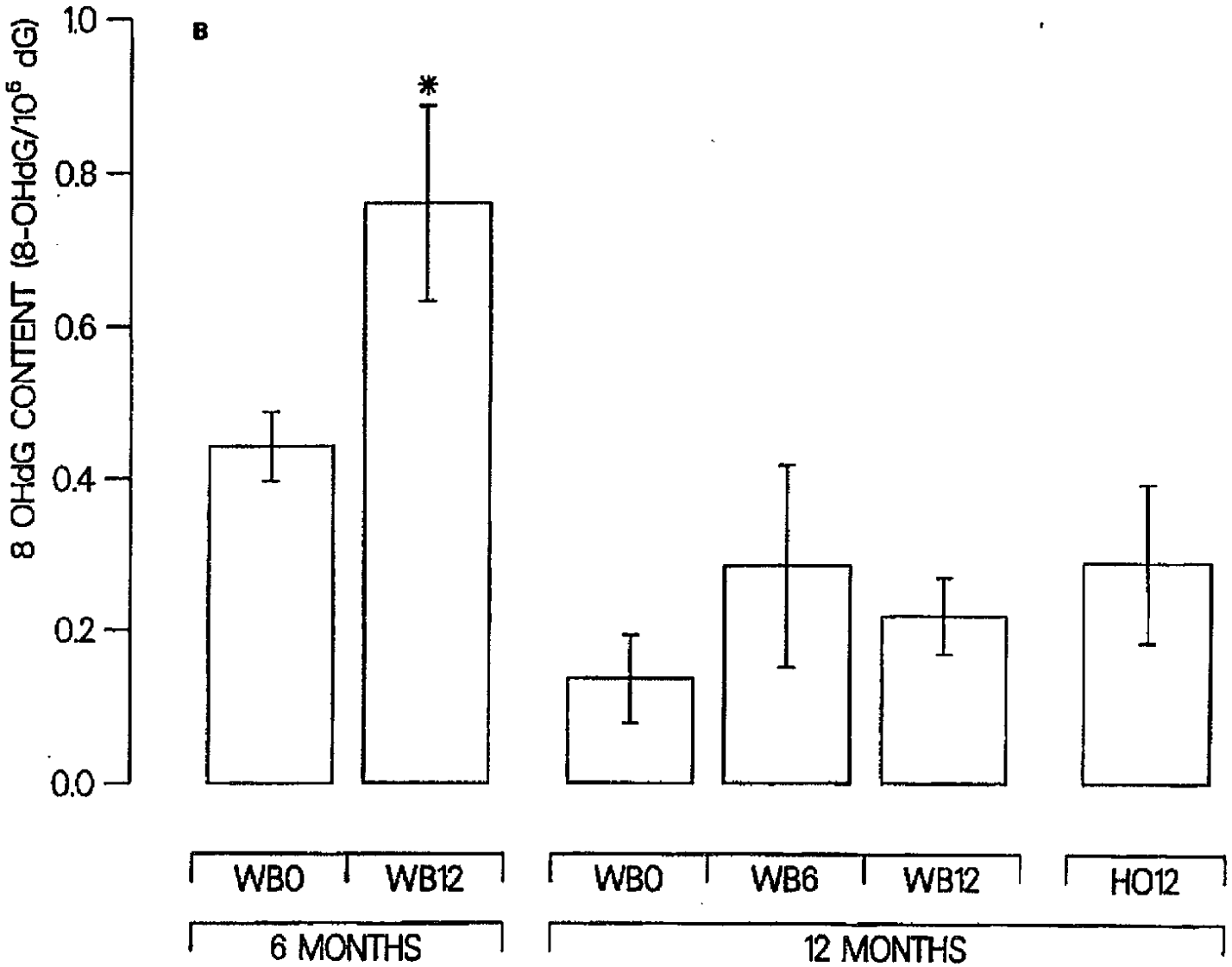

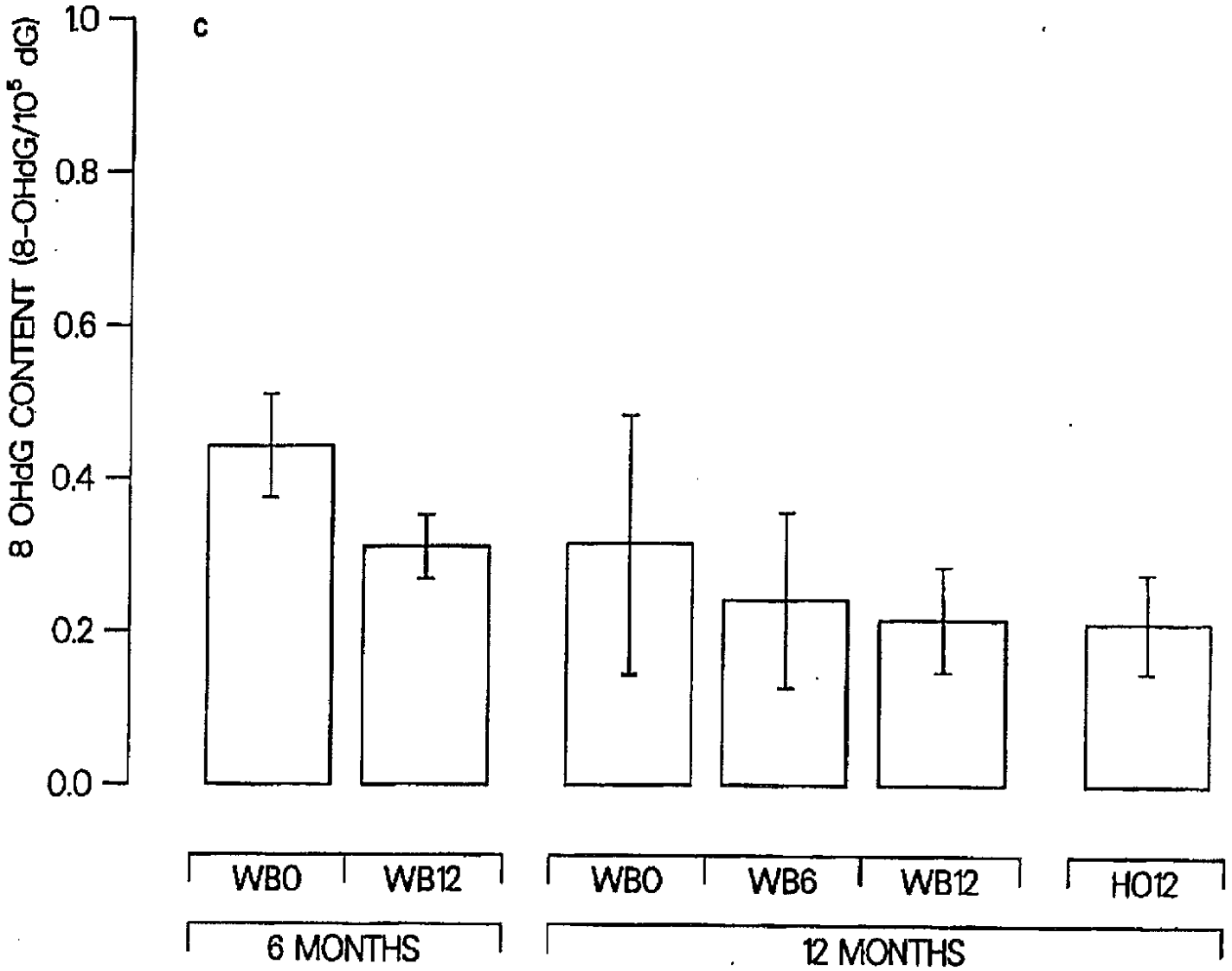

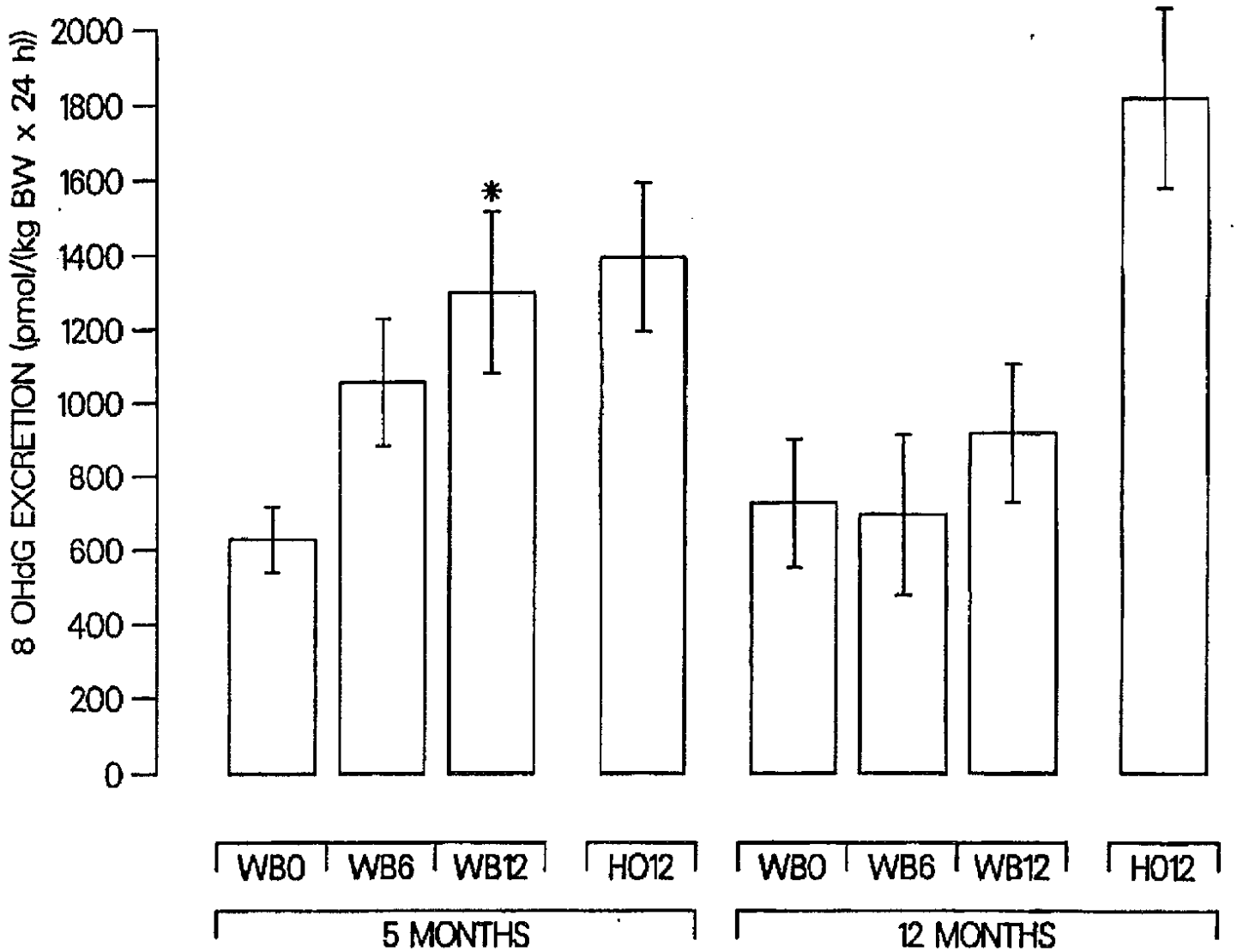

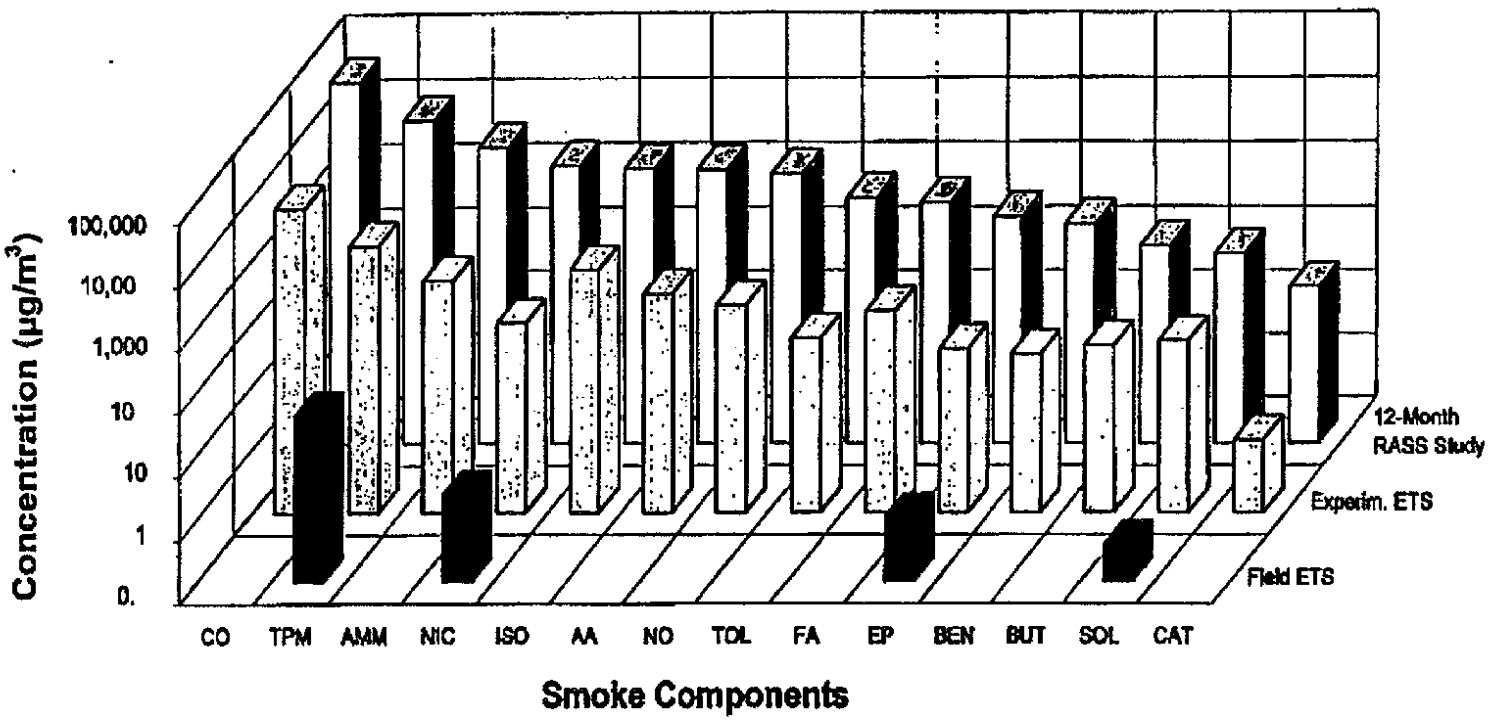

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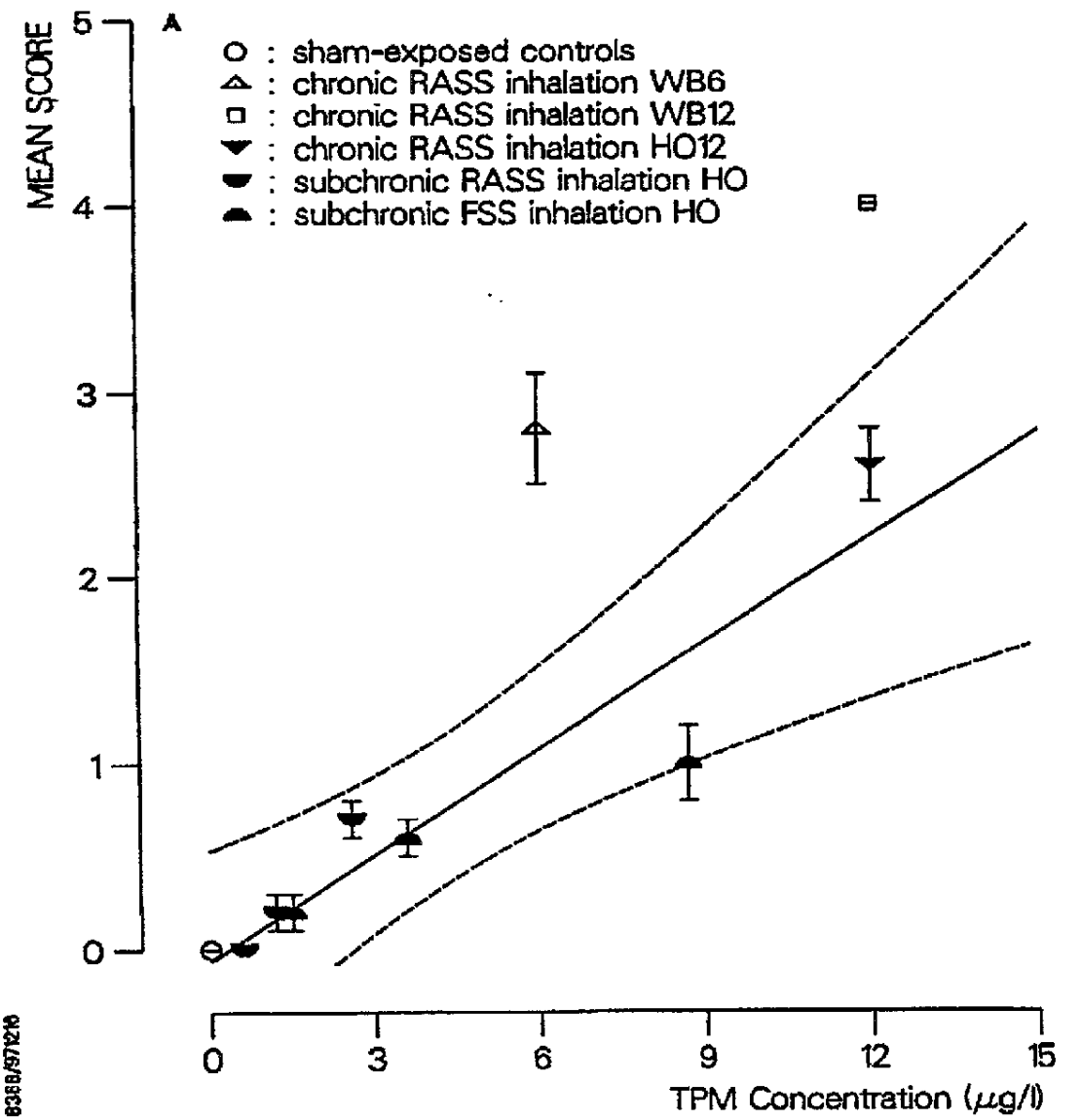

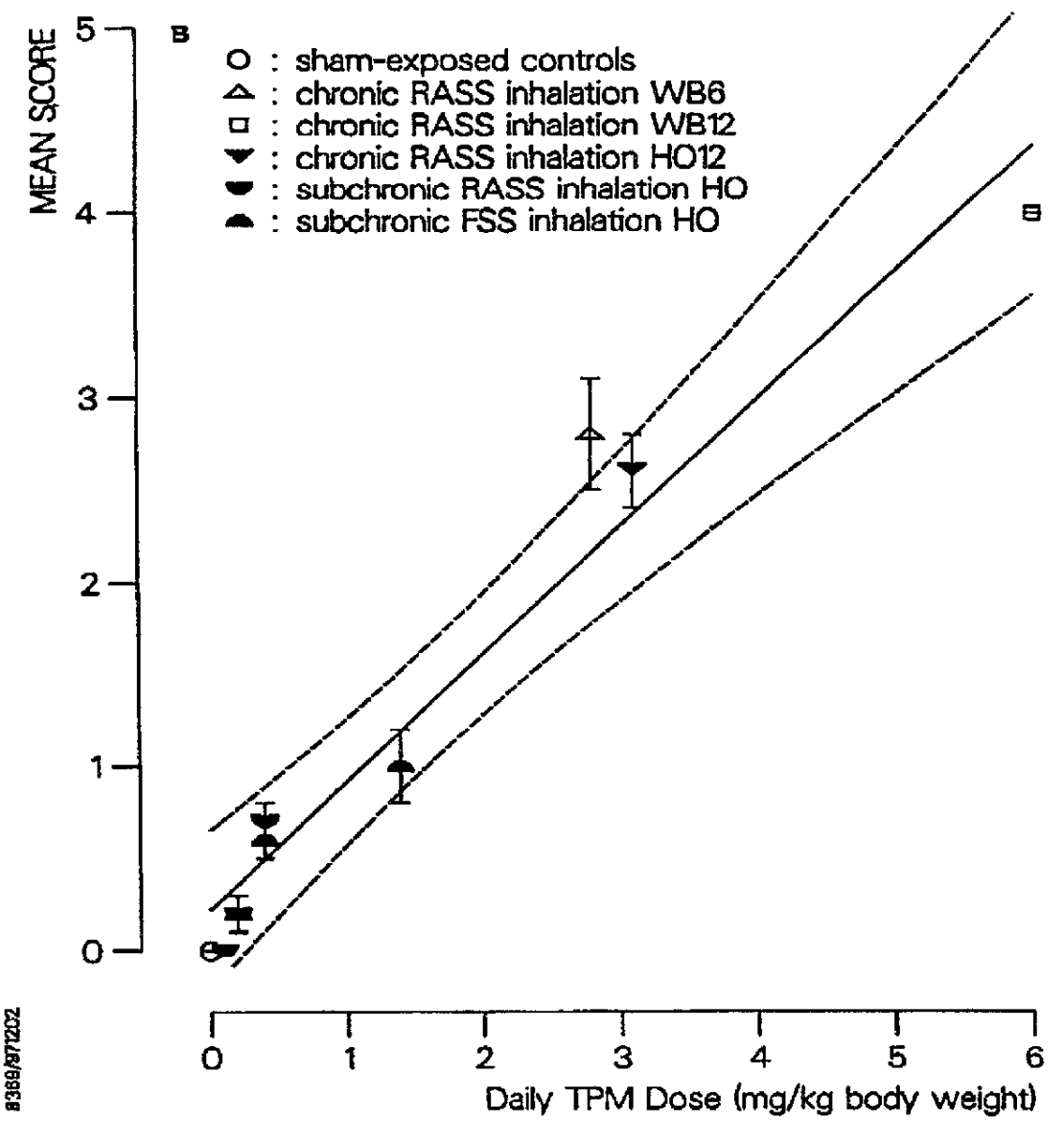

Influence of Aging and Surface Contact on the Composition of Cigarette Sidestream Smoke. Models for Environmental Tobacco Smoke

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Introduction

Environmental tobacco smoke (ETS) is a complex mixture derived from sidestream smoke of burning cigarettes and mainstream smoke exhaled by smokers. Its composition is highly variable in indoor environments as it undergoes chemical/physical changes due to dilution and aging influenced by contact with materials. Due to the discussed health impact of ETS on nonsmokers, numerous studies have been performed (Eatough et al. 1990, Guerin et al. 1992), for example, to chemically characterize ETS or to determine its irritative potential. Since it is practically impossible to reproducibly generate "real" ETS, only models of ETS can be used for analytical and biological testing. However, a major problem in developing ETS models is the lack of generally accepted standards for reproducible generation. To obtain information about the influence of aging and surface contact on the composition of cigarette sidestream smoke, fresh sidestream smoke (FSS) was room-aged under various conditions, because FSS is not considered to be an adequate model for ETS.

Method

Smoke Generation

Diluted FSS, age approximately 1 second, was continuously generated via a 30-port automatic smoking machine using 2R1 University of Kentucky standard reference cigarettes. Room-aged sidestream smoke (RASS) was generated by continuously passing diluted FSS at a rate of 20 m^3/hour through a 30-m^3 empty experimental room (epoxy-coated walls and ceiling, PVC floor, door, window pane, fluorescent lights, heat exchangers, ceiling fan). The mean age of RASS was 1.5 hours.

Experiments

In a series of 1-day experiments, the influence on smoke composition of aging alone (empty room) and aging in combination with materials found in rooms was investigated. The following materials were used individually or altogether: wool curtain (22 m^2), wool carpet (11 m^2), wooden book shelf, painted wallpaper (31 m^2). To check the long-term stability of the smoke composition, FSS and RASS (all materials present in the room) were generated over 90 days, 7 days/week, 6 hours/day followed by air-flushing for 18 hours.

Smoke Analyses

Total particulate matter (TPM) was determined gravimetrically using glass fiber filters. Carbon monoxide was monitored continuously by nondispersive infrared photometry of the gas phase. Nicotine was determined by gas chromatography, and aldehydes and ammonia were determined by high-performance liquid chromatography after derivatization. Hydrogen cyanide was determined by headspace gas chromatography. For each 1-day experiment, these parameters were determined at least three times consecutively. During the 90-day experiment the parameters were determined at regular intervals.

Results

One-Day Experiments

Table 1 gives the analytical data for FSS and RASS generated to an equal CO concentration of approximately 30 ppm. In Figure 1, the changes in composition of RASS relative to FSS are shown for those components affected by aging and materials.

Aging alone reduced nicotine and TPM to approximately 30% and 70% of their respective FSS concentrations, whereas other components were not affected or were only slightly affected. The individual material together with aging resulting in the most pronounced overall reductions was a wool curtain, with the final smoke component concentrations ranging between 10% and 60% of those seen for FSS. Aging with all materials resulted in the strongest reductions, with the final smoke component concentrations ranging between 5% and 45% of those seen for FSS. Of all components, nicotine decreased most strongly due to aging alone and ammonia most strongly by aging together with materials.

90-Day Stability

Over a period of 90 days, FSS and RASS (all materials present in the room)

Table 1. Concentration of smoke components in FSS and RASS.

Parameter	FSS	RASS					
		Empty room/ aging	Wool curtain	Wool carpet	Wooden book shelf	Painted wallpaper	All materials together
TPM (µg/L)	10.8	7.1	4.1	6.0	6.4	6.7	3.9
Nicotine (µg/L)	2.49	0.77	0.39	0.81	0.61	0.39	0.23
Ammonia (µg/L)	3.95	3.73	0.46	2.07	1.32	1.49	0.24
Hydrogen cyanide (µg/L)	0.18	0.15	0.11	0.09	0.12	0.10	0.08
Formaldehyde (ppm)	0.51	0.49	0.21	0.25	0.35	0.23	0.11
Acetaldehyde (ppm)	0.75	0.70	0.68	0.71	0.68	0.72	0.68
Acrolein (ppm)	0.11	0.08	0.09	0.08	0.09	0.10	0.09

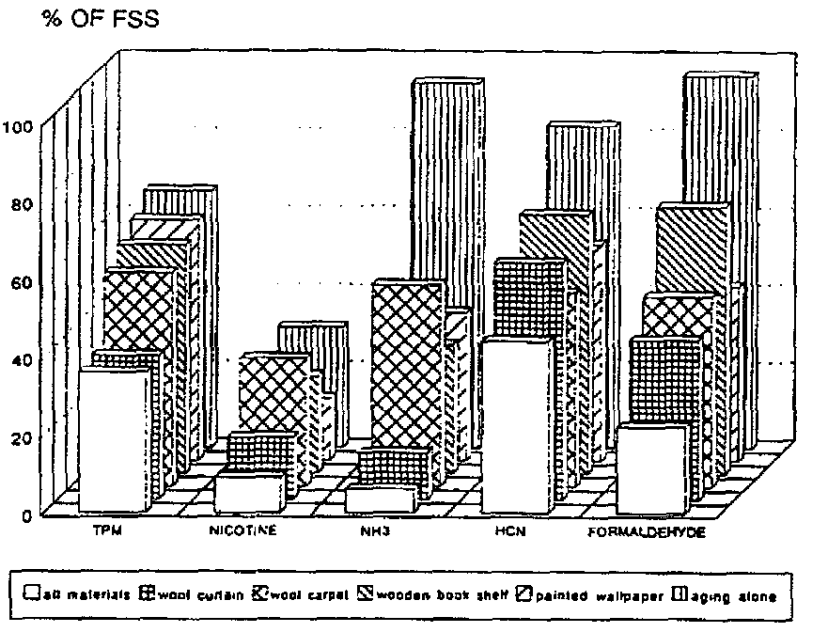

Figure 1. Influence of aging and materials on FSS.

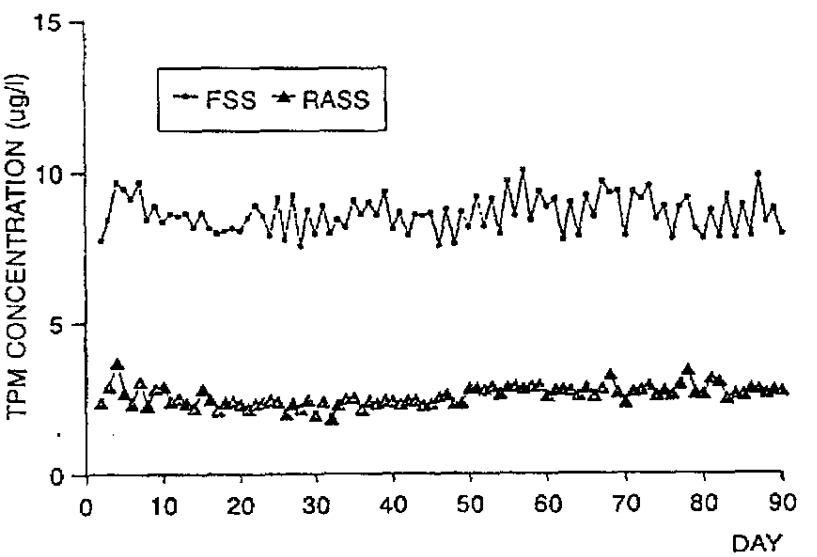

Figure 2. TPM concentration.

were reproducibly generated. No notable trends for the concentrations of all components determined were observed (see Figure 2 for TPM).

Summary

Large differences in the composition of FSS and RASS were found. The concentrations of RASS components vary considerably depending on the material present. With the setups used, significantly different ETS models were reproducibly generated.

Acknowledgment. This work was sponsored by Philip Morris, USA.

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- Appendix 3 -

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific *N*-nitrosamine, and funded research on the metabolism of NNK

March 20, 1998

- Current data do not support the assumption that NNK, present in tobacco products and

Background

Based on experimental data available prior to 1984, the International Agency for Research on Cancer (IARC) concluded that "There is *sufficient evidence* for the carcinogenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone to experimental animals," but "No data on humans were available" (IARC, 1985). In a footnote it was stated: "In the absence of adequate data on humans, it is reasonable, for practical purposes, to regard chemicals for which there is *sufficient evidence* of carcinogenicity in animals as if they presented a carcinogenic risk to humans." As far as we are aware, no regulatory agency has further evaluated the carcinogenicity of this compound to humans.

Following internal company review of all scientific literature published after the IARC assessment on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), it was concluded that:

- NNK is a carcinogen in the A/J mouse, F344 rat, and Syrian golden hamster, inducing mainly tumors of the lung, and to a lesser extent, tumors of the liver.

- Extensive evidence exists to demonstrate that human metabolism of NNK differs significantly from that observed in laboratory animals. However, relevant data have not been obtained under identical experimental conditions to allow a valid comparison of the metabolism of NNK in laboratory animal and human tissues.

are considered to occur only in tobacco and tobacco smoke (Hecht and Hoffmann, 1989). To date seven different TSNA derived from the nitrosation of nicotine and other minor tobacco alkaloids have been identified (Amin et al., 1995). **[Figure 1]** (The following abbreviations will be used in addition to NNK: NNN, *N*-nitrosonornicotine; NAB, *N*-nitrosoanabasine; NAT, *N*-nitrosoanatabine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; and iso-NNAC, 4-(methylnitrosamino)-1-(3-pyridyl)butyric acid.)

TSNA Present in Mainstream Cigarette Smoke and Indoor Air

The U.S. National Academy of Sciences has previously estimated that a smoker of domestic filter cigarettes has a total *N*-nitrosamine exposure of 16.2 μg/day (6.1 μg NNN and 2.9 μg NNK), based on the assumption that the average smoker consumes 20 cigarettes/day (Assembly of Life Sciences, 1981). This exposure estimate was based on unpublished analytical data provided by Hecht and Hoffmann of the American Health Foundation, Valhalla, NY. More recent data for filter cigarettes **[Table 1]** yields a lower exposure estimate of 3.4 μg/day (1.5 μg NNN and 1.0 μg NNK) (Tricker, 1997).

Since TSNA are also transferred to sidestream cigarette smoke (Adams et al., 1987), and presumably exhaled by smokers, trace levels occur in environmental tobacco smoke (ETS) present in indoor air (Brunnemann et al., 1992; Klus et al., 1992; Tricker et al., 1994). Extensive smoking under poor ventilation conditions results in mean ETS concentrations of 2.8±1.6 (range n.d.-6.0) ng/m^3 NNN and 4.9±9.6 (range n.d.-13.5) ng/m^3 NNK (Klus et al., 1992). Similar levels

Figure 1. **Proposed formation of tobacco-specific *N*-nitrosamines.**

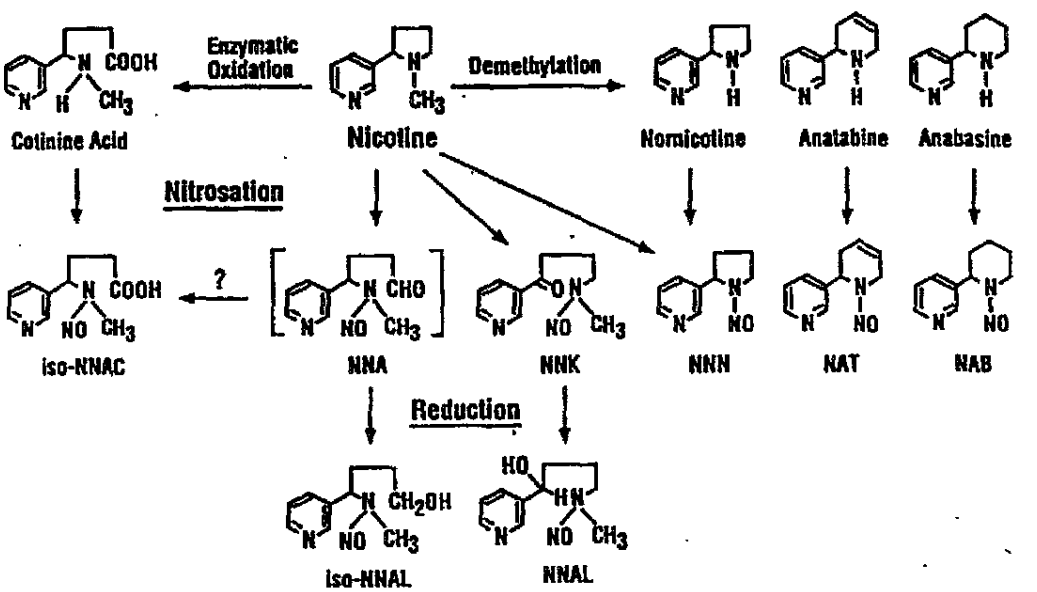

Table 1. Estimates of exposure to TSNA in smokers.

	Exposure estimate (μg/day) For a smoker of 20 filter cigarettes		
TSNA	1981[1]	1991[2]	1994[3]
NNK	3.0	1.0	1.6
NNN	6.2	1.5	1.0
NAB	--	--	0.2
NAT	7.4	--	1.8
NAB/NAT	--	1.5	--

1. Academy of Life Sciences (1981).
2. Tricker et al. (1991).
3. Hoffmann et al. (1994).

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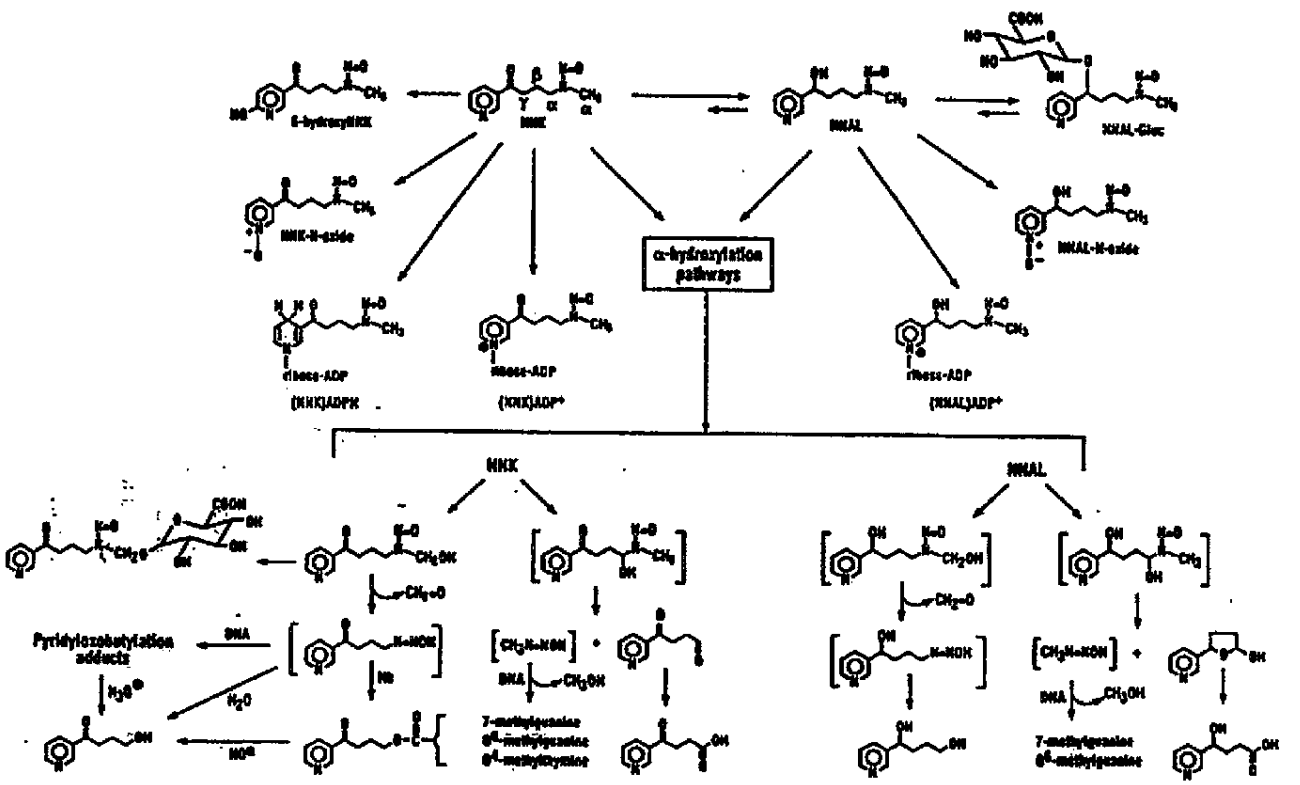
occur in the home; 0.8±1.2 (range n.d.-3.3) ng/m^3 NNN and 4.0±4.6 (range n.d.-14.3) ng/m^3 NNK (Tricker et al., 1994), and other venues (Brunnemann et al., 1992).

Putative Metabolism of NNK in Laboratory Animals

The major reported pathways of NNK metabolism in experimental animals involve carbonyl reduction, α-hydroxylation of the methylene and methyl groups adjacent to the *N*-nitroso group, and pyridine-N-oxidation **[Figure 2]**.

Carbonyl reduction of NNK to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol **(NNAL)** is probably a detoxification pathway of NNK metabolism since it provides the functional hydroxy moiety necessary for glucuronidation to [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-O-D-glucosiduronic acid **(NNAL-Gluc)** (Maser et al., 1996; Kim and Wells, 1996). α-Hydroxylation of the NNK methyl carbon results in the formation of an unstable intermediate which spontaneously decomposes to yield formaldehyde and 4-(3-pyridyl)-4-oxobutanediazohydroxide, a potential pyridyloxobutylating agent, which can react with water to yield 4-hydroxy-1-(3-pyridyl)-1-butanone **(keto alcohol)**. α-Hydroxylation of the methylene group in NNK produces 4-hydroxy-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone which spontaneously decomposes to methane diazohydroxide, a potential methylating species, and stable 4-oxo-1-(3-pyridyl)-1-butanone **(keto aldehyde)**, which is further oxidized to 4-oxo-4-(3-pyridyl)butyric acid **(keto acid)**. Glucuronidation of 4-((hydroxymethyl)nitrosamino)-1-(3-pyridyl)-1-butanone, the initial

Figure 2. **Putative metabolism of NNK in laboratory animals (Hecht, 1996).**



intermediate formed in the "keto alcohol" pathway, may be a detoxification route (Murphy et al., 1995).

α-Hydroxylation of the methylene and methyl groups adjacent to the *N*-nitroso group in NNAL produces unstable metabolic intermediates before ultimately forming stable 4-hydroxy-4-(3-pyridyl)butric acid (**hydroxy acid**) and 4-hydroxy-1-(3-pyridyl)-1-butanol (**diol**). α-Hydroxylation of the methyl group and ultimate formation of the diol is a putative detoxification pathway since this pathway has not been reported to result in adduct formation, while α-hydroxylation of the methylene group ("hydroxy acid" pathway) can potentially result in methylation of cellular macromolecules. NNAL is a poor substrate for α-hydroxylation compared to NNK (Hecht and Trushin, 1988; Belinsky et al. 1989; Staretz et al. 1997a), and consequently exhibits less biological activity than NNK (Liu et al., 1990; Castonguay et al., 1983a; Hoffmann et al., 1993).

Pyridyl-N-oxidation of both NNK and NNAL to yield 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanone (**NNK-N-oxide**) and 4-(methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol (**NNAL-N-oxide**), respectively, are considered to be detoxification pathways of NNK metabolism (Liu et al., 1990; Castonguay et al., 1983a; Hecht, 1994; Staretz et al., 1997a).

The determination of hydroxy acid, keto acid and keto alcohol as stable end products of NNK metabolism by α-hydroxylation pathways represents the metabolic activation of the host to produce reactive intermediates with the potential to form adducts with DNA or other cellular macromolecules.

Urinary NNK Metabolite Excretion in Laboratory Animals and Man

Urinary excretion profiles provide evidence of species-dependent differences in metabolic activation of NNK by α-hydroxylation and detoxification **[Table 2]**.

Urinary NNK metabolite profiles in the A/J mouse and F344 rat (Morse et al., 1990) are highly dose dependent **[Table 3]**. Similar studies have not been performed in other animal species. The data in **Table 3** indicate that α-hydroxylation pathways account for about 50% of NNK metabolism in the A/J mouse and F344 rat, regardless of the administered dose. At the lowest administered dose of NNK (1 μg/kg body weight), excretion of NNAL and NNAL-Gluc does not occur in either species, although NNAL is apparently formed and further metabolized by pyridyl-N-oxidation. NNK-N-oxide excretion increases with decreasing administration of NNK.

Human biomonitoring studies report the presence of NNAL, NNAL-Gluc and NNAL-N-oxide in 24-h urine of smokers maintaining constant smoking habits (Hecht et al. 1995; Carmella et al., 1997). NNK-N-oxide is not a urinary metabolite of NNK in smokers (Carmella et al., 1997). Other metabolites of NNK detected in experimental animal excretion studies, such as stable end products of α-hydroxylation , are not specific to NNK metabolism since they are also formed during metabolism of NNN and nicotine. Combined urinary excretion of NNAL, NNAL-Gluc and NNAL-N-oxide in urine of smokers (Hecht et al., 1995; Carmella et al., 1997), when calculated in molar equivalents of NNK, balances well with predicted total NNK exposure **[Table 4]**. These data

Table 2. Urinary excretion of NNK metabolites in different species.

Metabolite	% urinary excretion of NNK metabolites			
	A/J mice[1] 0.1 mg/kg i.p. 48-h excretion	F344 rat[2] 0.1 mg/kg i.p. 24-h excretion	F344 rat[1] 0.1 mg/kg i.p. 48-h excretion	Patas monkey[3] 0.1 mg/kg i.v. 24-h excretion
Hydroxy acid*	34	16.0	28	41.9-42.9
Keto acid*	19	37.8	21	24.6-26.6
Keto alcohol	--	<0.06	--	--
NNAL	1	3.3	7	n.d.-2.0
NNAL-Gluc	3	0.6	2	19.1-19.9
NNK-N-oxide	8	11.7	7	13.6-15.7
NNAL-N-oxide	14	11.4	16	7.7-15.7
NNK	--	0.8	--	n.d.-0.1
6-hydroxy-NNK	--	1.0	--	--
Total α-hydroxylation*	53	54	49	58.1

1. Morse et al. (1990).
2. Murphy et al. (1995).
3. Hecht et al. (1993a).

Table 3. Dose response for excretion of NNK metabolites.[1]

Metabolite	% 24-h excretion of NNK metabolites at different dose levels (mg/kg i.p.)					
	103.5	10.35	1.035	0.103	0.010	0.001
A/J mouse:						
Hydroxy acid*	18	32	37	34	27	35
Keto acid*	11	16	26	19	27	23
NNAL	29	11	2	1	--	--
NNAL-Gluc	22	8	4	3	--	--
NNK-N-oxide	--	--	5	8	10	7
NNAL-N-oxide	11	13	8	14	7	6
Total α-hydroxylation*	29	48	63	53	54	58
F344 rat:						
Hydroxy acid*	26	24	20	28	16	14
Keto acid*	24	39	45	21	38	40
NNAL	25	12	6	7	4	--
NNAL-Gluc	8	3	2	2	2	--
NNK-N-oxide	3	3	6	7	14	14
NNAL-N-oxide	6	6	8	16	13	12
Total α-hydroxylation*	50	63	66	49	54	54

1. Morse et al. (1990).

Table 4. Biomonitoring of NNK metabolites in human urine.

Smoker	Cigarettes/day	Total excretion of NNK Metabolites (nmol/day)[1]	Theoretical NNK exposure and excretion Exposure (μg/day)[2]	Excretion (μg/day)
1	18.2±2.2	7.32	0.91-1.46	1.52
2	16.7±1.1	6.24	0.85-1.34	1.29
3	16.8±1.1	4.63	0.84-1.34	0.96
4	15.0±1.1	4.68	0.75-1.20	0.97
5	15.8±0.4	4.75	0.79-1.26	0.98
6	9.5±1.2	Data incomplete	--	--
7	14.2±1.1	2.41	0.71-1.14	0.50
8	13.6±0.7	Data incomplete	--	--
9	19.1±1.7	4.30	0.95-1.53	0.89
10	8.0±1.4	3.74	0.40-0.64	0.77
11	15.9±1.0	Data incomplete	--	--
Mean			0.78-1.24	0.99
S.D.				0.31

1. Total excretion of NNAL, NNAL-Gluc and NNAL-N-oxide. To convert to μg NNK multiply by 207.

2. Estimated exposure range based on mainstream cigarette smoke NNK concentrations (Tricker et al., 1991; Hoffman et al., 1994).

provide evidence that NNK metabolism and excretion in man differs significantly from that observed in laboratory animals **[Table 2]**.

Total NNAL plus NNAL-Gluc excretion in 5 nonsmokers measured on 2 occasions is reported to be 8.4±11.2 ng/day, and increases after experimental exposure to sidestream smoke used as a surrogate for ETS (Hecht et al., 1993b). Another study reported 17 of 29 nonsmokers to have a mean excretion of 8.8±9.4 (range 0.8-31) ng/day total NNAL plus NNAL-Gluc compared to 0.68±0.41 (range 0.08-1.68) μg/day in smokers (Meger et al., 1998). Twelve of 29 nonsmokers had no detectable levels of NNAL plus NNAL-Gluc in urine.

Metabolism of NNK by Laboratory Animal and Human Microsomes

Microsomes from lung and liver have been extensively used to investigate metabolism of NNK under various experimental conditions **[Appendix 1]**. Despite differences in the individual study protocols (NNK substrate concentration and time of incubation), rodent lung and liver microsomes metabolize NNK to yield significant levels of α-hydroxylation products. Contrary to this, lung and liver microsomes of human origin primarily metabolize NNK by keto reduction to NNAL in the absence of significant α-hydroxylation. Only limited kinetic data are available to document interspecies differences in NNK metabolism by lung and liver microsomes.

The available kinetic parameters for NNK metabolism by lung microsomes **[Table 5]** provide further indication that laboratory animals (A/J mouse and patas monkey) primarily metabolize NNK by α-hydroxylation with no significant formation of NNAL. Human lung microsomes primarily metabolize NNK to NNAL, with no significant formation of α-hydroxylation products. The biological relevance of data for metabolism of NNK by human lung microsomes is partially comprised by the high experimental substrate range compared to actual human exposure to NNK (19-135 ng/filter cigarette; 70-650 pmol [Tricker et al., 1991]). No data are available for microsomes isolated from rat lung.

Metabolism of NNK by hepatic microsomes is less well documented **[Table 6]**. Liver microsomes from the patas monkey primarily metabolize NNK via α-hydroxylation with no significant formation of NNAL at low NNK substrate concentrations, while at high substrate concentrations metabolism to NNAL would be predicted to occur. Human liver microsomes primarily metabolize NNK to NNAL, and to a lesser extent NNAL-N-oxide, at low substrate concentrations, with no significant formation of α-hydroxylation products. The formation of NNAL-N-oxide at low substrate concentrations supports the presence of this metabolite in human urine (Carmella et al., 1997). Only kinetic parameters for NNK α-hydroxylation pathways have been reported for A/J mouse and F344 rat liver microsomes.

In conclusion, kinetic parameters of NNK metabolism in lung and liver microsomes provide strong evidence that significant differences occur in metabolism between laboratory animals and man. At low levels of exposure to NNK, human metabolism is characterized by reduction of

Table 5. Metabolism of NNK by lung microsomes.

Kinetic parameters: *Km* (μM), *Vmax* (pmol/min/mg protein)

Metabolite	Mouse[1]		Rat		Patas monkey[2]		Human[3]	
	Km	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*
Keto alcohol	5.6	56			4.9	19.1	--	--
Keto aldehyde	--	--			10.3	5.3	653	4.6
Keto acid	9.2	4.2	No published		--	--	--	--
Hydroxy acid	--	--	data		--	--	526	2.9
NNAL	2541	1322			902	479	573	335
NNAL-N-oxide	4.7	54			--	--	--	--
NNK-N-oxide	--	--			5.4	19.1	531	7.7
Substrate range	1-100 μM NNK				1-20 μM NNK		7-200 μM NNK	

1. Smith et al. (1990).
2. Smith et al. (1997).
3. Smith et al. (1992).

Table 6. Metabolism of NNK by liver microsomes.

Kinetic parameters: *Km* (μM), *Vmax* (pmol/min/mg protein)

Metabolite	Mouse[1]		Rat[2]		Patas monkey[3]		Human[4]	
	Km	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*
Keto alcohol	73.8	239	211	156	474	37.7	1200	500
Keto aldehyde	19.1	173	234	153	8.2	37.4	367	60
NNAL	--	--	--	--	474	3470	56	282
							1600	3300
NNAL-N-oxide	--	--	--	--	--	--	53	19
							4500	560
NNK-N-oxide	--	--	--	--	--	--	--	--
Substrate range	1-100 μM NNK		1-200 μM NNK		1-50 μM NNK		5-2000 μM NNK	

1. Peterson et al. (1991).
2. Guo et al. (1992).
3. Smith et al. (1997).
4. Patten et al. (1996).

NNK to NNAL, in the absence of significant α-hydroxylation, while laboratory animals metabolize NNK to yield significant levels of α-hydroxylation products.

Metabolism of NNK by Laboratory Animal and Human Tissues

Similar profiles are observed for metabolism of NNK by microsomes and tissue samples **[Appendix 1]**. The data confirm that significant differences occur between laboratory animal and human metabolism of NNK. The major pathways of NNK metabolism in rodent tissues yield significant levels of α-hydroxylation products while human tissues primarily reduce NNK to NNAL.

DNA and Hemoglobin Adduct Formation by NNK

Metabolism of NNK by α-hydroxylation is assumed to be a critical event resulting in DNA-reactive intermediates; however, the exact role of methylating and pyridyloxobutylating species in different animal species and organs remains unclear. Metabolism of NNK to the keto acid via a methylating intermediate is thought to be a critical determinant of NNK-induced tumorigenesis in the A/J mouse lung (Peterson and Hecht, 1991; Belinsky et al., 1992) and hamster liver (Liu et al., 1992). Although NNK-induced methylation to yield O^6-methylguanine in Clara cells appears to be a suitable indicator of the carcinogenic potency of NNK in the rat lung (Belinsky et al., 1990), pyridyloxobutylation is thought to be the critical event in tumor induction in rat lung (Staretz et al., 1997b) and liver (Liu et al., 1992). Both NNK and NNN can be metabolized to a

pyridyoxobutylating species in the rat lung (Hecht et al., 1988) to yield a N^2-(pyridyloxobutyl)deoxyguanosine (HPB) adduct with DNA (Spratt et al., 1989). Linear dose-response relationships are not observed for either NNK-induced methylation (Belinsky et al., 1990; Murphy et al., 1990a) or NNK-induced pyridyloxobutylation in the rat lung (Murphy et al., 1990a). An HPB-releasing adduct with hemoglobin has been proposed as a surrogate marker for DNA adduct formation by both NNK and NNN (Carmella and Hecht, 1987; Peterson et al., 1990). However, the exact mechanism of adduct formation still remains to be determined (Murphy and Coletta, 1993).

Only two studies have investigated the presence of HPB-releasing DNA adducts in human lung (Foiles et al., 1991; Blömeke et al., 1996). Mean levels of 11±16 and 9.0±2.3 fmol HPB/mg DNA have been reported in smokers and nonsmokers, respectively (Foiles et al., 1991). Since the response for the analytical reagent blank was equivalent to 38±16 fmol HPB and 1-2 mg DNA were used for analysis, the reported levels are well below the blank response and could easily be due to an analytical artifact. No HPB-releasing DNA adducts were detected in 16 lung tissue samples from current smokers and 16 from nonsmokers (Blömeke et al., 1996). The levels of 7-methylguanine in 80 lung tissue samples could not be explained by differences in tobacco exposure (measured by serum cotinine), gender, age, or ethnicity (Blömeke et al., 1996). These data suggest that exposure to NNK via smoking does not result in HPB adduction to lung DNA or increase the background level of lung DNA methylation.

Determination of hemoglobin adducts as a possible surrogate for tissue DNA adducts shows less than a 3-fold difference in the mean level of HPB-releasing hemoglobin adducts in

smokers (163 fmol HPB/g hemoglobin) compared to nonsmokers (68 fmol HPB/g hemoglobin) (Hecht, 1994). A smaller difference was observed in another study (54.7±8.9 vs. 26.7±4.1 fmol/g hemoglobin) and self-reported exposure of nonsmokers to environmental tobacco smoke did not increase the background level of HPB-releasing hemoglobin adducts (Richter et al., 1995). Both studies indicated elevated hemoglobin adduct levels were only apparent in about 10% of smokers compared to all subjects.

Concluding Remarks

The above studies provide intensive evidence that human metabolism of NNK differs significantly from that observed in laboratory animals. These differences are evident from *in vitro* studies of NNK metabolism in microsomes and tissue samples, excretion profiles of NNK metabolites, and absence of a clear differentiation in NNK/NNN-derived DNA and hemoglobin adducts in smokers and nonsmokers. These data provide little support for the assumption that NNK in tobacco products and cigarette smoke induces similar biological effects in the lung and liver as reported in laboratory animals.

Research Funded by Philip Morris on the Metabolism of NNK

A research project has been funded at the Walther-Straub Institute for Pharmacology and Toxicology, University of Munich, Germany. The research has two major objectives:

1. To define the major routes of *in vitro* NNK and NNAL metabolism under identical experimental conditions in lung and liver of the A/J mouse, F344 rat, Syrian golden hamster, and man. The three animal species chosen represent those most often used for chronic bioassays of NNK.

2. To determine pharmacokinetic constants (*Km* and *Vmax*) for each pathway of NNK and NNAL metabolism in both organs of all four species.

Experimental Design

Lung and liver are removed from laboratory animals killed by decapitation. Human peripheral lung and liver tissue are collected from excess material removed at surgery from patients undergoing routine surgical procedures. Human tissues are rejected from subjects having current treatment with immuno-suppressants or other drugs known to induce or suppress metabolism, or a history of alcohol abuse. Undamaged macroscopically normal tissue is received in the laboratory stored in ice-cold Hanks medium within 30 min of removal.

Metabolic studies are performed using precision cut liver slices and lung tissue in dynamic culture (Vickers, 1994). Compared to using microsomes or isolated cells (extensively used in previous studies as summarized in **Appendix I**), tissue samples maintain structural heterogeneity with intact phase I and phase II metabolism, and cell interaction and communication are preserved to a certain extent, thus resembling the situation in the intact organ.

Radiolabeled [5-^{3}H]NNK or [5-^{3}H]NNAL (sp. act. 25-30 Ci/mmol) are incubated over a substrate concentration range of 0.01-100.0 μM with precision cut liver slices and lung tissue for 6 h in Krebs-Henseleit buffer (pH 7.4) under standard laboratory conditions for dynamic culture (Vickers, 1994). The substrate concentration range was selected to include the lowest possible concentration to approach the physiologically relevant concentration in man, and substrate concentrations predicted to occur in animal bioassays. Each incubation is performed in triplicate using lung and liver samples from at least 5 different laboratory animals. Metabolite profiles are determined by reversed-phase HPLC with radioflow detection (Richter and Tricker, 1994). Pharmacokinetic parameters (*Km* and *Vmax*) are calculated from Lineweaver-Burk plots with reaction velocities showing linear response to time. Pharmacokinetic parameters will be determined for lung and liver from different human donors.

Current Results

Preliminary results from this research have already been presented at two scientific meetings:

1. AACR Special Conference in Cancer Research "DNA methylation, imprinting, and the epigenetics of cancer," Las Croabas, Puerto Rico, December 12-16, 1997.

2. 37th Annual Meeting of the Society of Toxicology, Seattle, March 1-5, 1998.

Further results will be presented at the 89th Annual Meeting of the American Association for Cancer Research, New Orleans, March 28-April 1, 1998.

The data currently available from this research project **[Table 7, Table 8]** demonstrate that NNK metabolism in lung of the A/J mouse and F344 rat results in significant levels of α-hydroxylation products at high tissue substrate concentrations predicted to occur in animal bioassay protocols. The experimental conditions and data support the reported biological activity of NNK in the A/J mouse and F344 rat lung; metabolism of NNK at high substrate concentrations yields the keto acid via a methylation pathway in the A/J mouse lung, while formation of the keto alcohol via the pyridyloxobutylation pathway occurs in the F344 rat lung. These two pathways are considered critical for lung tumorigenesis in A/J mouse lung (Peterson and Hecht, 1991; Belinsky et al., 1992) and F344 rat lung (Staretz et al., 1997b), respectively. Metabolism of NNK by human lung results primarily in the formation of NNAL in the absence of formation of significant levels of α-hydroxylation products **[Figure 3]**. The kinetic data **[Table 7]** provide support for pyridyl-N-oxidation of NNAL, but not NNK, and are consistent with the reported occurrence of NNAL-N-oxide but not NNK-N-oxide in human urine (Carmella et al., 1997). The kinetic data do not support significant formation of keto alcohol via the pyridyloxobutylation pathway at low NNK substrate

Table 7. Metabolism of NNK in lung tissue.

Kinetic parameters: *Km* (μM), *Vmax* (pmol/min/mg protein)

Metabolite	A/J Mouse		SG Hamster		F344 Rat		Human	
	Km	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*
0.01-1.0 μM NNK								
NNAL	1.7	168	No	No	3.6	309	1.0	311
Keto acid	0.3	45	current	current	5.2	182	0.4	11
Keto alcohol	0.6	141	data	data	2.7	177	1.2	26
Hydroxy alcohol	1.0	56			n.d.	--	0.7	25
NNK-N-oxide	0.7	184			6.8	498	n.d.	n.d.
NNAL-N-oxide	n.d.	n.d.			n.d.	--	0.6	19
0.01-100μM NNK								
NNAL	39.0	4309			--	--	239	65640
Keto acid	10.1	317			317.0	15620	--	--
Keto alcohol	7.5	556			90.0	8398	--	--
Hydroxy alcohol	160.0	3405			2.4	107	--	--
NNK-N-oxide	25.1	25.1			68.0	7995	41240	413500
NNAL-N-oxide	n.d.	n.d.			0.9	21	--	--

Substrate range: 0.01-100.0μM NNK

n.d., no detectable formation; --, Michaelis-Menton kinetics could not be fitted.

Table 8. Metabolism of NNK in liver tissue.

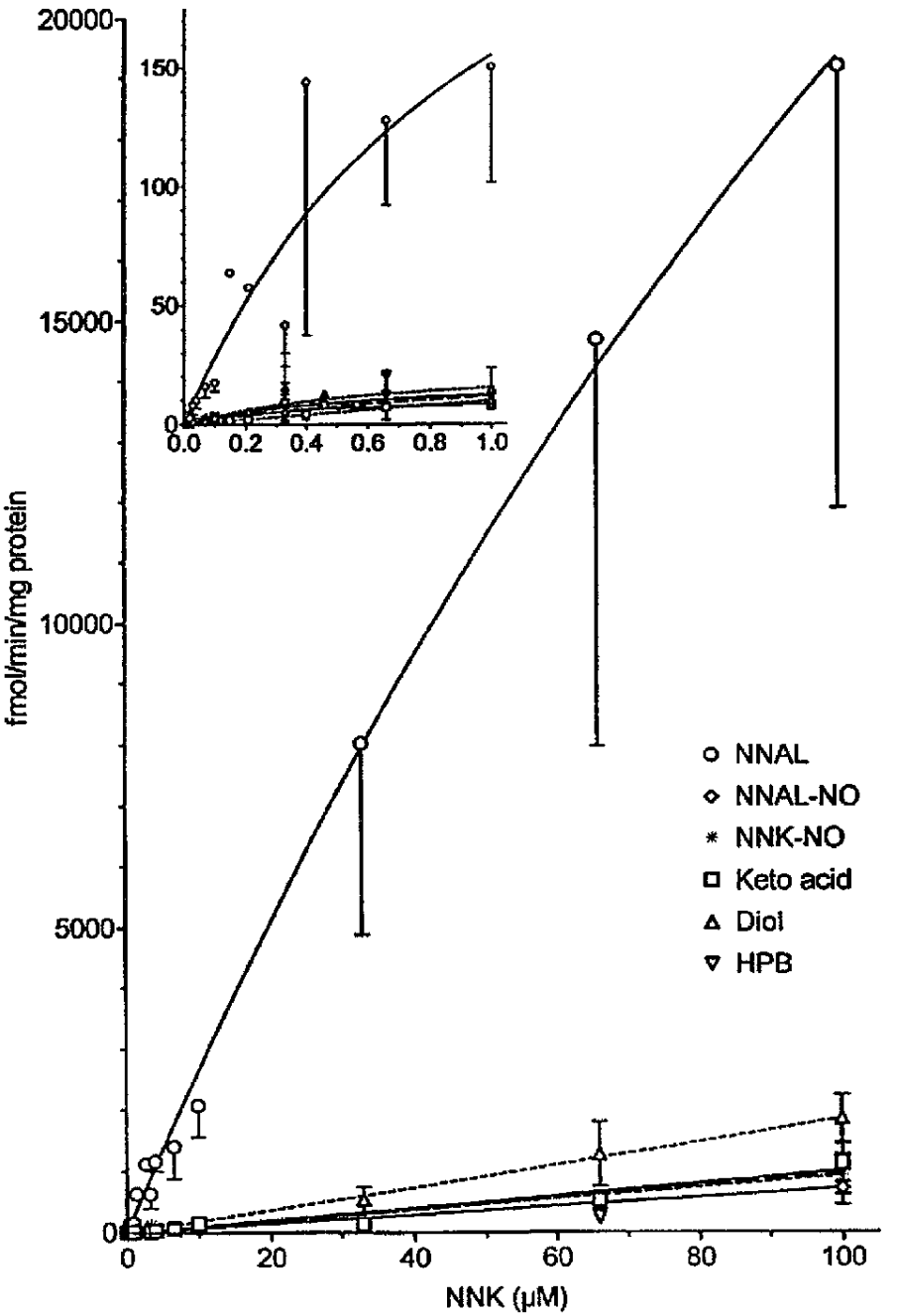
Kinetic parameters: *Km* (μM), *Vmax* (pmol/min/mg protein)

Metabolite	A/J Mouse		SG Hamster		F344 Rat		Human	
	Km	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*	*Km*	*Vmax*
0.01-1.0 μM NNK								
NNAL	--	--	No current data	No current data	26.0	4640	0.6	254
Keto acid	--	--			0.9	18	0.2	9.6
Keto alcohol	--	--			1.9	62	n.d.	n.d.
Hydroxy alcohol	--	--			n.d.	n.d.	--	--
0.01-100μM NNK								
NNAL	174.0	4623			96.0	19220	43.6	6805
Keto acid	677.0	5818			154.0	1159	690.0	8036
Keto alcohol	141.0	2609			260.0	8357	12760	144500
Hydroxy alcohol	199.0	3153			16.2	253	n.d.	n.d.

Substrate range: 0.01-100.0μM NNK

n.d., no detectable formation; --, Michaelis-Menton kinetics could not be fitted.

Figure 3. **Metabolism of NNK by 7 different human lungs.**



concentrations in human lung. These data suggest that HPB-releasing adducts derived from NNK are unlikely to be formed in the human lung and support data for their absence in human lung tissue (Blömeke et al., 1996).

Michaelis-Menton kinetics could not be fitted to NNK metabolism at low substrate concentrations in the A/J mouse liver. At high substrate concentrations (1.0-100 μM), significant metabolism of NNK by α-hydroxylation pathways occurs in addition to NNAL formation. NNK metabolism in the liver of the F344 rat suggested that hydroxy acid formation is the most favorable pathway for NNK metabolism; however, under conditions used in rat bioassay protocols, significant α-hydroxylation of NNK still occurs. At low physiological NNK substrate concentrations in the human liver, NNK metabolism is predicted to result primarily in the formation of NNAL.

In summary, these results obtained under identical experimental conditions provide further evidence that NNK metabolism in human lung and liver primarily yields NNAL. In contrast to this, NNK metabolism in lung and liver of the A/J mouse and F344 rat results in significant α-hydroxylation to DNA-reactive intermediates thought to be involved in NNK-induced tumorigenesis in these two organs.

The research program is predicted to be completed in April 1998.

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Appendix I. *In vitro* metabolism of NNK in tissues, cultured cells and microsomes from laboratory animals and man

Species	Tissue[1]	Conditions		Percentage formation of major metabolites[2]								Percentage α-hydroxylation[3]	Reference
		NNK (μM)	Time (h)	NNAL	NNK-N-oxide	NNAL-N oxide	NNAL-Gluc	Keto alcohol	Keto acid[4]	Hydroxy acid	Diol		
A/J mouse	Lung (t)	4.7	4.0	1.2	49.8	0.8	--	27.1	19.5	0.8	0.8	47.4	Pepin et al., 1992
A/J mouse	Lung (t)	4.7	0.8	0.6	53.7	1.3	--	27.6	13.4	1.3	2.1	42.3	Pepin et al., 1992
A/J mouse	Lung (t)	2.36	2.0	1.6	53.9	0.6	--	29.7	12.4	0.4	1.4	42.5	Bouchard & Castonguay, 1993
A/J mouse	Lung (t)	0.24	24	68.4	7.5	3.7	--	3.1	10.6	1.5	5.2	15.2	Castonguay et al., 1983a
A/J mouse	Lung (m)	200.0	0.5	76.3	10.4	--	--	--	2.5	--	10.8	2.5	Peterson et al., 1991
A/J mouse	Lung (m)	10.0	0.5	12.5	43.0	2.0	--	24.7	(17.1)	--	--	41.8	Smith et al., 1993
A/J mouse	Lung (m)	10.0	0.5	16.3	31.8	--	--	49.7	2.2	--	--	51.9	Smith et al., 1990
A/J mouse	Lung (m)	10.0	0.25	13.7	39.1	--	--	32.7	14.5	--	--	47.2	Lin et al., 1992
A/J mouse	Lung (m)	10.0	0.25	17.8	42.2	--	--	26.2	(13.8)	--	--	40.0	Desai et al., 1995
A/J mouse	Lung (m)	10.0	0.16	5.6	45.6	--	--	29.6	(19.2)	--	--	48.8	Hong et al., 1992
A/J mouse	Lung (m)	--	0.6	29.4	21.7	--	--	48.7	--	--	--	48.7	Morse et al., 1995
A/J mouse	Liver (m)	10.0	0.5	17.7	13.1	--	--	28.9	(40.3)	--	--	69.2	Hong et al., 1992
A/J mouse	Liver (m)	10.0	0.25	53.3	--	--	--	16.3	30.4	--	--	46.7	Lin et al., 1992
A/J mouse	Liver (m)	10.0	0.25	24.0	--	--	--	23.2	(52.8)	--	--	47.2	Desai et al., 1995
A/J mouse	Liver (m)	10.0	0.16	26.6	4.7	3.1	--	24.4	(41.1)	--	--	65.5	Smith et al., 1993
A/J mouse	Liver (m)	--	0.25	22.9	--	--	--	27.8	49.3	--	--	77.1	Morse et al., 1995
A/J mouse	Intestine (t)	14.0	0.75	87.8	2.9	0.8	--	2.7	5.4	0.3	0.1	8.4	Pepin et al., 1990
A/J mouse	Stomach (t)	14.0	2.0	67.2	8.0	0.3	--	5.7	18.2	0.3	0.3	24.2	Pepin et al., 1990
NMRI (f)	Intestine (t)	1.0	2.0	4.8	28.9	8.1	--	2.4	44.9	10.8	--	58.1	Schulze et al., 1996
NMRI (m)	Intestine (t)	1.0	2.0	7.1	29.5	4.7	--	4.2	50.6	3.9	--	58.7	Schulze et al., 1996
F344 rat	Lung (m)	1.0	0.5	22.9	38.3	--	--	34.4	4.4	--	--	38.8	El-Bayoumy et al., 1996
F344 rat	Liver (h)	5.0	2.0	41.7	6.1	0.5	--	6.4	35.4	9.0	0.9	50.8	Murphy & Coletta, 1993
F344 rat	Liver (h)	1.0	18.0	11.4	2.8	7.9	12.5	3.7	44.7	17.0	--	65.4	Murphy et al., 1995
F344 rat	Liver (m)	1.33	0.5	99.6	0.2	0.004	--	--	0.2	0.01	0.04	0.21	Hamilton & Teel, 1994
F344 rat	Liver (m)	1.0	0.5	98.9	0.1	--	--	0.7	0.3	--	--	1.0	El-Bayoumy et al., 1996
F344 rat	Esoph. (t)	40.0	24.0	91.3	4.5	--	--	0.7	3.5	--	--	4.2	Murphy et al., 1990b
F344 rat	Nasal (t)	23.8	3.0	5.4	--	--	--	21.3	64.0	5.3	4.0	70.6	Brittebo et al., 1983
F344 rat	Nasal (t)	23.8	24.0	4.9	--	--	--	5.6	69.9	11.2	8.4	86.7	Brittebo et al., 1983
F344 rat	Oral (t)	1.0	24.0	19.5	51.0	2.0	--	16.3	11.2	--	--	27.5	Murphy et al., 1990b
F344 rat	Oral (t)	1.0	24.0	26.2	36.3	8.8	--	6.9	20.7	0.6	0.5	28.2	Murphy et al., 1990b

F344 rat	Oral (t)	1.0	24.0	55.0	25.2	--	--	9.0	8.3	0.4	2.2	17.7	Murphy et al., 1991
F344 rat	Oral (t)	1.0	24.0	49.9	21.5	5.5	--	9.1	11.5	0.7	1.8	21.3	Murphy & Heiblum, 1991
F344 rat	Oral (t)	1.0	4.0	35.8	3.9	30.6	--	14.6	13.6	1.5	--	29.7	Murphy et al., 1990b
F344 rat	Oral (t)	1.0	4.0	50.8	20.5	5.1	--	8.7	14.3	0.6	--	23.6	Murphy et al., 1990b
F344 rat	Oral (t)	11.0	4.0	81.9	5.5	--	--	3.1	9.5	--	--	12.6	Murphy et al., 1990b
SD rat	Lung (m)	10.0	0.5	28.6	24.4	0.5	--	46.0	0.5	--	--	46.5	Yoo et al., 1992
SD rat	Lung (m)	0.47	0.33	25.7	46.3	--	--	15.5	12.5	--	--	28.0	Ardries et al., 1996
SD rat	Liver (m)	10.0	0.5	93.6	1.3	0.2	--	3.9	1.0	--	--	4.9	Yoo et al., 1992
SD rat	Nasal (m)	10.0	0.5	--	1.0	1.0	--	58.5	36.6	0.9	2.0	96.0	Hong et al., 1991
SD rat (f)	Intestine (t)	1.0	2.0	6.6	29.4	6.9	--	10.3	4.2	2.7	--	17.2	Schulze et al., 1996
SD rat (f)	Intestine (t)	1.0	2.0	78.2	11.2	1.1	--	3.2	6.0	0.4	--	9.6	Schulze et al., 1996
SG Hamster	Lung (t)	4.2	3.0	20.4	32.3	1.8	--	17.9	25.5	0.3	1.8	43.7	Charest et al., 1989
SG Hamster	Lung (t)	0.66	1.0	37.4	27.4	4.7	--	12.0	16.5	0.5	1.5	29.0	Tjälve & Castonguay, 1983
SG Hamster	Lung (t)	0.24	3.0	22.1	33.2	5.5	--	17.7	20.7	0.7	--	39.1	Charest et al., 1989
SG Hamster	Lung (m)	10.0	0.5	50.5	19.6	1.9	--	24.3	3.7	--	--	28.5	Jorquera et al., 1993
SG Hamster	Lung (m)	--	0.5	13.0	16.5	7.2	--	40.6	9.8[5]	--	12.9	50.4	Zhang et al., 1997
SG Hamster	Liver (t)	0.66	1.0	86.0	1.2	3.6	--	--	2.9	3.1	3.2	6.0	Tjälve & Castonguay, 1983
SG Hamster	Liver (m)	10.0	0.5	60.2	9.0	0.7	--	24.1	6.0	0.03	--	30.1	Jorquera et al., 1993
SG Hamster	Liver (m)	8.0	0.5	44.5	8.7	3.2	--	35.5	4.0[5]	--	4.1	59.5	Miller et al., 1994
SG Hamster	Liver (m)	5.1	0.5	69.9	12.7	0.9	--	6.8	1.4[5]	--	1.3	8.2	Miller et al., 1993
SG Hamster	Liver (m)	5.1	0.5	67.7	8.3	4.6	--	12.0	6.1[5]	0.1	1.2	18.2	Miller et al., 1996
SG Hamster	Liver (m)	1.33	0.5	55.4	19.6	2.9	--	17.5	0.8[5]	--	3.9	18.3	Hamilton & Teel, 1994
SG Hamster	Liver (m)	1.0	1.0	62.7	2.4	--	--	30.6	3.3	0.1	0.9	34.0	Castonguay & Rossignol, 1992
SG Hamster	Liver (m)	--	0.5	31.1	6.1	6.4	--	24.9	22.1[5]	0.4	9.0	47.4	Zhang et al., 1997
SG Hamster	Nasal (t)	0.66	1.0	21.0	1.8	2.0	--	13.2	51.3	2.2	8.5	66.7	Charest et al., 1989
SG Hamster	Trachea (t)	0.66	1.0	31.1	11.5	--	--	18.2	38.0	--	1.2	56.2	Charest et al., 1989
SG Hamster	Intestine (t)	1.0	2.0	11.0	25.8	31.1	--	5.8	14.6	11.7	--	32.1	Schulze et al., 1996
Man	Lung (t)	238.0	24.0	96.4	2.7	--	--	--	--	0.9	--	0.9	Castonguay et al., 1983b
Man	Lung (m)	10.0	1.0	96.4	1.2	<0.2	--	<0.2	(1.4)	0.7	--	2.3	Smith et al., 1992
Man	Lung (m)	10.0	0.17	96.1	--	--	--	------(3.0)------		0.9	--	3.9	Smith et al., 1995
Man	Liver (m)	3.0	0.17	83.5	1.0	--	--	7.5	7.0[5]	--	1.0	14.5	Staretz et al., 1997a
Man	Liver (m)	10.0	1.0	92.4	1.2	0.7	--	3.3	(1.5)	0.9	--	5.7	Smith et al., 1992
Man	Bladder (t)	238.0	24.0	99.9	--	--	--	--	--	0.1	--	0.1	Castonguay et al., 1983b
Man	Bronchus (t)	238.0	24.0	95.4	4.5	--	--	--	--	0.1	--	0.1	Castonguay et al., 1983b
Man	Oral (t)	238.0	24.0	99.2	0.1	--	--	--	--	0.1	--	0.1	Castonguay et al., 1983b
Man	Oral (t)	6.0	24.0	94.8	0.7	1.5	--	0.2	0.5	1.8	0.6	2.5	Liu et al., 1993
Man	Oral (t)	100.0	24.0	99.5	0.2	0.1	--	0.1	0.3	0.1	0.1	0.5	Liu et al., 1993
Man	Oral ©	6.0	24.0	94.7	1.2	0.4	--	0.8	2.1	0.4	0.5	3.3	Liu et al., 1993

Man	Oral ©	100.0	24.0	96.2	0.6	0.2	--	0.3	2.0	0.2	0.1	2.5	Liu et al., 1993
Man	Esophagus (t)	238.0	24.0	98.2	1.7	--	--	--	--	0.1	--	0.1	Castonguay et al., 1983b
Man	Kidney ©	2.3	50.0	99.8	0.03	--	--	--	0.05	0.06	0.03	0.11	Lacroix et al., 1992
Man	Trachea (t)	238.0	24.0	94.2	0.1	--	--	--	--	5.7	--	5.7	Castonguay et al., 1983b

1. Abbreviation in brackets: (t), tissue; ©, cultured cell; (h), hepatocyte; (m), microsome; --, not detected.

2. Calculated on the basis of total metabolites detected.

3. % α-hydroxylation calculated from the sum of keto alcohol, keto acid (keto aldehyde) and hydroxy acid; metabolic pathways known to produce reactive intermediates which bind to DNA.

4. Keto aldehyde measured instead of keto acid, value for keto aldehyde presented in brackets.

5. Joint determination of keto aldehyde and keto acid.

Submission by Philip Morris U.S.A.

to

The National Toxicology Program

- Appendix 4 -

Commentary on the Results of the IARC Multi-Center Study, "Lung Cancer and Exposure to Environmental Tobacco Smoke," Published in the IARC Biennial (1996-1997) Report

March 20, 1998

Commentary on the Results of the IARC Multi-Center Study, "Lung Cancer and Exposure to Environmental Tobacco Smoke," Published in the IARC Biennial (1996-1997) Report

Results from the International Agency for Research on Cancer (IARC) multi-center epidemiologic study of environmental tobacco smoke (ETS) exposure and lung cancer risk were recently published for the first time in the IARC Biennial Report for 1996-1997 (IARC, 1997; see Tab A, section 3.7.2). This study, which began in 1988, represents the largest study in Europe and the second largest study ever conducted of its kind.

In 1986, IARC made the following comments regarding the epidemiological evidence for the reported association between ETS exposure and lung cancer (IARC, 1986): "Several epidemiological studies have reported an increased risk of lung cancer in nonsmoking spouses of smokers, although some others have not . . . The resulting errors could arguably have artifactually depressed or raised estimated risks, and, as a consequence, each is compatible either with an increase or with an absence of risk." It was on the basis of that conclusion that IARC decided to undertake its multi-center study. The results, based on an analysis of 650 cases and 1,542 controls in 12 centers in 7 European countries, were as follows:

- RR for spousal exposure -- 1.16 (95% CI, 0.93-1.44)
- RR for workplace exposure -- 1.17 (95% CI, 0.94-1.45)
- RR for combined exposure -- 1.14 (95% CI, 0.88-1.47)

The report also states that there was no evidence of an association between lung cancer risk and ETS exposure during childhood.

The two immediate conclusions that can be drawn from these results are that both indices of ETS exposure, spousal and workplace, suggest a small but positive association with lung cancer, but neither association is statistically significant. What cannot be determined from the results as presented is the extent to which they have been corrected for known systematic biases -- in particular, misclassification of smoking habit and confounding by diet (spousal exposure only). The most recent meta-analysis carried out on the possible association of ETS exposure and lung cancer (Hackshaw et al., 1997) arrived at a combined relative risk of 1.24, which the authors estimated should have been reduced by 0.06 for misclassification of smoking habit and by 0.02 for dietary confounding. Although their suggested reduction is almost certainly inadequate, accepting even these values would yield a reduction of 33% for the spousal exposure risk estimate in the IARC study, resulting in a relative risk of 1.10, and a reduction of 25% for the workplace exposure estimate, resulting in a relative risk of 1.13. Although these values are, of course, still greater than 1.0, it is as likely that they suggest no association whatsoever, as it is likely that they suggest an association.

However, it should be noted that the results reported by IARC are in line with other studies and, in particular, in line with a number of meta-analyses. Therefore, clearly, a possible interpretation of these results is that they confirm an association between reports of ETS exposure and lung cancer, although the association is extremely weak. It is worth noting that the Introduction

to the brief text in the IARC Biennial Report states that ETS exposure is a "likely cause" of lung cancer. This is a far less conclusive statement than made by some other agencies and scientists.

There are several interesting points that were made in the discussion of these results in the IARC Biennial Report. The first deals with dose-response trends, where the claim is made that "several quantitative indicators of ETS exposure showed a dose-response relationship with lung cancer risk." It is impossible to analyze this statement from the data provided in the summary; however, the fact that not all such indicators showed a dose-response relationship suggests that such an analysis would be of great importance.

Secondly, IARC states that: "A further study of non-smoking women in Moscow, Russian Federation, confirmed the results on ETS of the larger international investigation and suggested a role of environmental air pollution independent of the effect of ETS." In actuality, the results of this study (Zaridze et al., 1998) "confirm" only the result for spousal exposure. The authors report no increase in lung cancer risk for workplace exposure; moreover, the reported dose-response effects were negative, whether years of exposure or number of cigarettes smoked by the spouse was used as the metameter of spousal exposure.

Lastly, the IARC summary states that: "In a separate exercise, the number of lung cancers occurring in the countries of the European Union that can be attributed to spousal ETS exposure was estimated to be about 800 among women and 300 among men." The reader is likely to assume that the calculation referred to utilized the relative risk of 1.16 obtained from the IARC

multi-center study. However, in actuality the authors of the paper used a relative risk for spousal exposure of 1.30, almost two times the uncorrected IARC result (Trédaniel et al. 1997).

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A

Germany; V. Constantinescu, Bucharest, Romania; P. Forastiere and C. Fortes, Rome, Italy; B. Gabriel, Poznan, Poland; K. Husgafvel-Pursiainen, Helsinki, Finland; A. Menezes, Pelotas, Brazil; F. Merletti, Turin, Italy; G. Pershagen and F. Nyberg, Stockholm, Sweden; L. Simonato, Padua, Italy; and D.G. Zaridze, Moscow, Russian Federation

Among lung cancer cases, non-smokers have been exposed on average to lower levels of carcinogens than smokers; genetic susceptibility may play a greater role in risk of lung cancer in the former group of cases.

In ten centres from Brazil, France, Germany, Italy, Poland, Romania, the Russian Federation and Sweden, blood samples have being collected from about 150 non-smoking lung cancer cases, 150 smoking lung cancer cases and 200 non-smoking control subjects, in order to determine (i) genetic polymorphism of glutathione *S*-transferase M1 and T1, (ii) the levels of the DNA repair enzyme O^6-methylguanine-DNA methyltransferase, (iii) the formation of haemoglobin adducts with 4-hydroxy-1-(3-pyridyl)-1-butanone (a metabolite of tobacco-specific nitrosamines), and (iv) genetic alterations in the *p53* gene and K-*ras* mutations in lung neoplastic tissue of cases. Cases and controls have been interviewed about their smoking habits and exposure to environmental tobacco smoke. Enrolment of patients and laboratory analyses have been completed and statistical analysis will take place in 1998.

3.8 *Head and neck cancer*

Cancers of the head and neck comprise an important group of neoplasms that are showing increasing incidence in many parts of the world. Although alcohol drinking and tobacco smoking are established causes of these cancers, infection with the human papillomavirus may represent an additional important risk factor, as do some occupational exposures. In addition, patients with head and neck cancer are at increased risk of developing a second tobacco-related neoplasm, making them an important population in which to explore genetic susceptibility.

3.8.1 Multicentric case–control study of laryngeal cancer in Brazil, Argentina and Uruguay

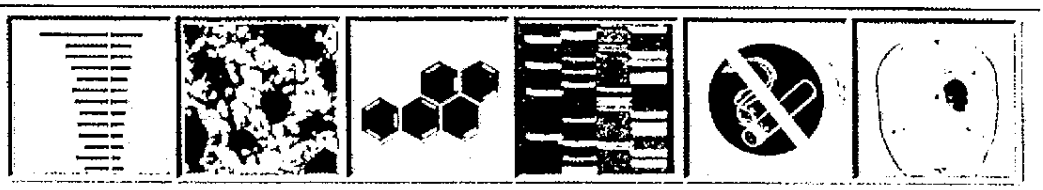
P. Boffetta, P. Brennan and R. Herrero; in collaboration with E. de Stefani, Montevideo, Uruguay; M. Kogevinas, Barcelona, Spain; S. Koifman, Rio de Janeiro, Brazil; E. Matos, Buenos Aires, Argentina; A. Menezes, Pelotas, Brazil; J. Siemiatycki, Montreal, Canada; and V. Wunsch, São Paulo, Brazil

Argentina, Uruguay and southern Brazil have high incidence rates of laryngeal cancer, that do not seem to be explained only by exposure to known carcinogens such as tobacco smoking and alcohol drinking. Following a series of studies of lung cancer (see Section 3.7.6), a multicentric study of laryngeal cancer has been initiated in three areas of Brazil (Rio de Janeiro, São Paulo and Pelotas and Porto Alegre), in Buenos Aires and in Montevideo. The study aims to identify occupational risk factors of this disease; additional aims are the assessment of the role of HPV infection, quantification of the contribution of tobacco smoking and alcohol drinking, and clarification of the role of other possible lifestyle risk factors, such as diet and mate drinking. Collection of interview data and biological samples started in 1997 and will be completed in 1999. In some of the centres, the study is being conducted in parallel with an investigation of the role of human papillomavirus infection in oral cancer (see Section 3.8.4).

3.8.2 Combined analysis of case–control studies of sinonasal cancer

P. Boffetta, E. Merler and D. Colin; in collaboration with R.B. Hayes, and L.A. Brinton, Bethesda, MD,

http://www.iarc.fr/



INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

WORLD HEALTH ORGANIZATION

ANNUAL REPORT 1998

Cancer Research for Cancer Control

SC/34/2
GC/39/2

WORLD HEALTH ORGANIZATION

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

BIENNIAL REPORT

1996–1997

International Agency for Research on Cancer
Lyon, France

1997

3.7 *Cancer of the lung*

Lung cancer is the most frequent malignant neoplasm worldwide: tobacco smoking is responsible for most cases, and the control of smoking represents the most important approach to prevent lung cancer (see Section 2.4). Among the important research questions still to be answered are the contributions of other risk factors (occupation, diet, environmental pollution) in both smokers and non-smokers and the role of genetic predisposition: these questions are being addressed in a series of studies conducted in areas of high and low risk for lung cancer.

3.7.1

Case–control study of lung cancer in northern Thailand

D.M. Parkin and P. Pisani; in collaboration with P. Srivatanakul, Bangkok, Thailand; N. Martin, Chiang Mai, Thailand; V. Saensingkaew, Bangkok, Thailand; and T. Bishop, Leeds, UK

This study is investigating the reasons for the relatively high incidence of lung cancer, particularly in women, in northern Thailand. Age-standardized incidence rates in Lampang province are 41.8 per 10 000 in men and 20.1 per 10 000 in women. A case–control study comparing 196 cases of lung cancer with two groups of controls (217 hospital controls and 156 community controls drawn at random from the population of this province) was carried out from 1993 to 1995 and data analysis began in 1996.

Because one hypothesis under investigation is the role of air pollution from numerous coal-fired electricity generating plants, place of residence is an important variable of interest, linked to corresponding environmental measurements of arsenic and cadmium. Other factors investigated include tobacco habits, exposure to domestic smoke, and cooking practices. Blood samples from all subjects have been stored for analysis of heavy metals and of metabolites and adducts of components of tobacco smoke. DNA is being extracted from white blood cells of cases and controls to study metabolic polymorphism at the GSTM1 and CYP1A1 loci.

3.7.2

Lung cancer and exposure to environmental tobacco smoke

P. Boffetta, P. Brennan, S. Lea and G. Ferro; in collaboration with W. Ahrens, Bremen, Germany; E. Benhamou and S. Benhamou, Villejuif, France; S.C. Darby, Oxford, UK; F. Forastiere and C. Fortes, Rome, Italy; C.A. González and A. Agudo, Barcelona, Spain; J. Trédaniel, Paris, France; S.K. Jindal, Chandigarh, India; K. H. Jöckel, Essen, Germany; A. Mendes, Lisbon, Portugal; F. Merletti, Turin, Italy; G. Pershagen and F. Nyberg, Stockholm, Sweden; R. Saracci, Pisa, Italy; L. Simonato, Padua, Italy; H. Wichmann, Munich, Germany; C. Winck, Porto, Portugal; and D. Zaridze, Moscow, Russian Federation

Environmental tobacco smoke (ETS) is a likely cause of lung cancer [27, 37], while evidence of an association with other neoplasms is inconclusive. However, the quantitative aspects of the association between ETS exposure and lung cancer risk are not yet well established, nor is the interaction between exposure to ETS and exposure to other carcinogens.

An IARC-coordinated international collaborative case–control study was aimed at investigating the relationship between exposure to ETS and to other environmental and occupational risk factors and the risk of lung cancer in subjects who have never smoked tobacco. A total of 650 cases and 1542 controls have been enrolled in 12 centres in seven European countries. Information on exposure to occupational carcinogens, urban air pollution, background radiation and dietary habits, as well as lifelong exposure to ETS, has been collected by personal interview of cases and controls. Self-reported (non-)smoking status was

confirmed by interviews of relatives. The relative risk (RR) of lung cancer risk was 1.16 (95% CI 0.93–1.44) for exposure to ETS from the spouse, 1.17 (0.94–1.45) for workplace ETS exposure and 1.14 (0.88–1.47) for combined spousal and workplace exposure. Several quantitative indicators of ETS exposure showed a dose–response relationship with lung cancer risk; RRs were higher for squamous cell carcinoma and small cell carcinoma than for adencarcinoma (Figure 20). There was no association between lung cancer risk and ETS exposure during childhood. Additional analyses are continuing on risk factors other than ETS.

A parallel study was conducted in Chandigarh, India, where ETS exposure comes mainly from bidi smoking. The statistical analysis will be completed in 1998. A further study of non-smoking women in Moscow, Russian Federation, confirmed the results on ETS of the larger international investigation and suggested a role of environmental air pollution independent of the effect of ETS [517]. In a separate exercise, the number of lung cancers occurring in the countries of the European Union that can be attributed to spousal ETS exposure was estimated to be about 800 among women and 300 among men [461].

3.7.3

Combined analysis of case–control studies of lung cancer in western Europe

P. Boffetta, P. Brennan and V. Gaborieau; in collaboration with W. Ahrens and H. Pohlabeln, Bremen, Germany; E. Benhamou and S. Benhamou, Villejuif, France; S.C. Darby, Oxford, UK; F. Forastiere and C. Fortes, Rome, Italy; C.A. González and A. Agudo, Barcelona, Spain; K. H. Jöckel, Essen, Germany; F. Merletti, Turin, Italy; G. Pershagen and F. Nyberg, Stockholm, Sweden; R. Saracci, Pisa, Italy; J. Siemiatycki, Montreal, Canada; L. Simonato, Padua, Italy; and H. Wichmann, Munich, Germany

In parallel to the study on non-smokers described in Section 3.7.2, cases of lung cancer and controls have been enrolled in a series of studies in 10 centres in western Europe, irrespective of their smoking habits. Comparable information on tobacco smoking, exposure to occupational carcinogens and urban air pollution has been collected from about 9000 cases and 10 000 controls. The analysis focuses on detailed aspects of tobacco carcinogenesis that cannot be addressed in smaller studies, such as the effect of very light smoking, long-term quitting and smoking of products other than cigarettes (Figure 21). These analyses will be completed in 1998. In parallel, information on exposure to occupational carcinogens

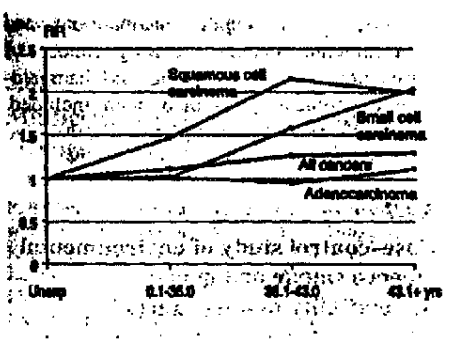


Figure 20. Relative risk of lung cancer by years of exposure to environmental tobacco smoke from spouse or workplace and by histological

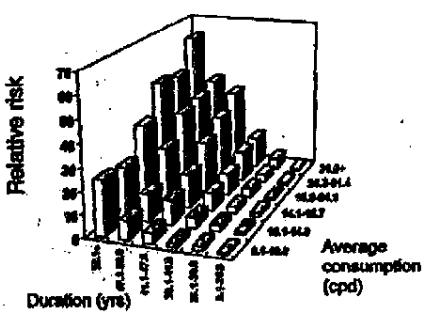


Figure 21. Relative risk of lung cancer by fine categories of cigarette consumption and duration of smoking. Reference category: never smokers.

and urban air pollution will be integrated into the common database.

3.7.4

Multicentric case–control study of lung cancer in central and eastern Europe

P. Brennan and P. Boffetta; in collaboration with E. Fabianova, Banska Bystrica, Slovakia; J. Fevotte, Lyon, France; A. Fletcher, London, UK; D. Mates, Bucharest, Romania; P. Rudnai, Budapest, Hungary; J. Siemiatycki, Montreal, Canada; N. Szeszenia-Dabrowska, Lodz, Poland; D.G. Zaridze, Moscow, Russian Federation; and W. Zatonski, Warsaw, Poland

Countries of central and eastern Europe have the highest incidence and mortality of lung cancer ever recorded. Air pollution is often blamed as the main contributor to this excess, but evidence for its role is limited. A study has been initiated in six areas of Hungary, Poland, Romania, the Russian Federation and Slovakia, to assess the relative contributions of tobacco smoking, occupational exposures and outdoor air pollution in lung carcinogenesis. Enrolment of a total of 3000 cases and a comparable number of controls has started. Special efforts are being made to assess past occupational exposures using detailed employment histories evaluated by panels of local experts. Blood samples will also be collected, to investigate polymorphisms of metabolic enzymes.

3.7.5

Case–control studies of lung cancer in Brazil, Uruguay and Argentina

P. Boffetta; in collaboration with E. de Stefani, Montevideo, Uruguay; E. Matos, Buenos Aires, Argentina; and V. Wunsch, São Paulo, Brazil

The urban areas of Brazil, Uruguay and Argentina have among the highest death rates in the Americas for cancer of all sites and of the lung in particular. Three similar studies have been designed to identify associations between environmental and occupational exposures and risk of lung cancer in São Paulo, Brazil, in Uruguay and in Buenos Aires, Argentina, and to examine the synergistic effect of selected occupational exposures and tobacco smoking. The study in Uruguay confirmed the important role of known carcinogens, such as tobacco smoking and asbestos, and suggested an increased risk among workers of the meat industry and workers exposed to pesticides [86]; it is also addressing the risks for other cancer sites. The study in São Paulo suggested a smaller role than expected for occupational exposures, with increased risks in only a few categories, such as machinery and pottery workers. Data collection for the study in Argentina was completed in 1997 and analysis will be carried out in 1998.

3.7.6

Multicentric case–control study of lung cancer in India

P. Boffetta and R. Sankaranarayanan; in collaboration with M. K. Nair, Trivandrum, India; D.N. Rao, Bombay, India; and V. Shanta, Madras, India

Although the industrial population in India is very large and many hazardous industries are present, virtually no information exists on occupational risk factors for cancer. The presence of a network of well organized cancer registries is a favourable condition for conducting multicentric case–control studies, and therefore such a study has been started in Bombay, Trivandrum and Madras, to investigate occupational and environmental factors for lung cancer. A series of cases of lymphatic and haematopoietic neoplasms has also been included. Data collection was completed in 1997 and the analysis will be completed in 1998.

3.7.7

Case–control study of environmental tobacco smoke and genetic susceptibility to lung cancer

P. Boffetta, M. Lang, N. Malats, M. Friesen, S. Atawodi, S. Lea and J. Hall; in collaboration with W. Ahrens, Bremen, Germany; S. Benhamou, Villejuif, France; I. Brüske-Hohlfeld and H. Wichmann, Munich,

Submission by Philip Morris U.S.A.

to

The National Toxicology Program

- Appendix 5 -

A Probabilistic Risk Analysis of Lung Cancer Mortality Associated with ETS Exposure

March 20, 1998

Introduction

For the purpose of demonstrating the fragility of the United States Environmental Protection Agency's (U.S. EPA) attributable death calculations in its Risk Assessment on ETS (1992), we utilized U.S. EPA's relative risk point estimate. It is important to note that we do not believe that this point estimate is scientifically justified. In fact, the data indicate that one cannot distinguish the association between reported exposure to ETS and lung cancer described by the EPA from no association.

The statistical analysis provided by Philip Morris in this section examines the influence of just two of several possible modifications to some of the assumptions employed by the U.S. EPA in its Risk Assessment on ETS (1992). The technique of Monte Carlo simulation is used to construct a probabilistic model of estimated mortality purportedly associated with ETS exposure; it emphasizes the uncertainty associated with the single estimate reported by the U.S. EPA. The reduction in the number of so-called attributable deaths that follows from changes in only two factors reinforces our position that the U.S. EPA failed to utilize reasonable statistical assumptions in its estimation of the number of attributable deaths in its report.

A Probabilistic Risk Analysis of Lung Cancer Mortality Associated with ETS Exposure

The U.S. EPA's (1992) estimates of female and male annual lung cancer mortality in nonsmokers (never smokers plus former smokers who have quit for 5+ years) purportedly

attributable to ETS sources for the United States are shown in **Table 1.** EPA's total estimate of 3,060 deaths among nonsmokers attributable to ETS exposure has two main components: (i) 2,200 deaths attributed to "Background" (non-spousal) exposure to ETS, primarily in the workplace and other away-from-home settings, and (ii) 860 deaths attributed to "Spousal" exposure to ETS from a spouse who smokes.

The estimated mortality associated with Background and Spousal ETS exposure is a function of two key parameters: (i) the Relative Risk (RR) of lung cancer for nonsmokers exposed to spousal ETS relative to nonsmokers not exposed to spousal ETS (but who are exposed to background ETS), and (ii) the Z-factor, which is the ratio between the mean dose level in the "exposed" (spousal ETS) group and the mean dose level in the "unexposed" (non-spousal or background ETS) group. The value of Relative Risk assumed by the EPA is RR = 1.19 (90% CI: 1.04-1.35), based on EPA's meta-analysis of 11 U.S. epidemiological studies of never-smoking females. The RRs from the 11 studies were corrected for smoker misclassification prior to the meta-analysis using a 1.09% misclassification rate. However, the RRs were not corrected for other likely sources of bias, such as confounding due to dietary and other lifestyle factors inherent in the spousal smoking design, as well as recall bias. The value of the Z-factor assumed by EPA is Z = 1.75, and is based on U.S. urinary cotinine studies cited by EPA.

As stated in EPA's report *Respiratory Health Effects of Passive Smoking* (EPA, 1992), the estimated mortality attributed to Background ETS exposure is proportional to (RR - 1) / (Z - RR), and can be computed as

Table 1. U.S. EPA's estimates of annual lung cancer mortality.

Smoking status	Sex	Exposed to spousal ETS	Population (in millions)	Estimated annual lung cancer mortality: Background ETS	Spousal ETS	Total ETS
Never-Smoker	F	No	12.92	410		410
Never-Smoker	F	Yes	19.38	620	470	1090
Never-Smoker	M	No	9.93	320		320
Never-Smoker	M	Yes	3.13	100	80	180
Former Smoker	F	No	2.0	60		60
Former Smoker	F	Yes	6.7	210	160	370
Former Smoker	M	No	8.8	280		280
Former Smoker	M	Yes	6.2	200	150	350
TOTAL			69.07	2200 (71.9%)	860 (28.1%)	3060

$$\text{Estimated Mortality Attributed to Background ETS} = 6484.21 \times \frac{RR - 1}{Z - RR}$$

where 6484.21 is a proportionality constant such that EPA's estimate of 2,200 is obtained when RR = 1.19 and Z = 1.75. Similarly, the estimated mortality attributed to Spousal ETS exposure is proportional to (RR - 1) (Z - 1) / (Z - RR), and can be computed as

$$\text{Estimated Mortality Attributed to Spousal ETS} = 3379.65 \times \frac{(RR-1)(Z-1)}{Z - RR}$$

where 3379.65 is a proportionality constant such as that EPA's estimate of 860 is obtained when RR = 1.19 and Z = 1.75.

Based on the above formulas, the estimated mortality attributed to Background, Spousal, and Total (Background + Spousal) ETS exposure as a function of the assumed value of Z is shown in **Figures 1, 2, and 3** for values of RR equal to 1.19, 1.15, and 1.10, respectively.

As shown by Figures 1, 2, and 3, the estimated mortality is very sensitive to the assumed values of RR and Z, both of which are subject to considerable uncertainty. A Monte Carlo analysis was performed to help understand the distribution of possible values of the mortality estimates as a function of the uncertainty in RR and Z. For this analysis, the uncertainty in RR incorporates both the uncertainty in the smoker misclassification rate as well as the sampling distribution for the "true" Relative Risk. The uncertainty in the smoker misclassification rate is modeled as a compound distribution, consisting of a uniform distribution from 1% to 3% and a

EPA Estimate of Total (Spousal + Background) ETS-Attributable Lung Cancer Deaths
By Z-Factor

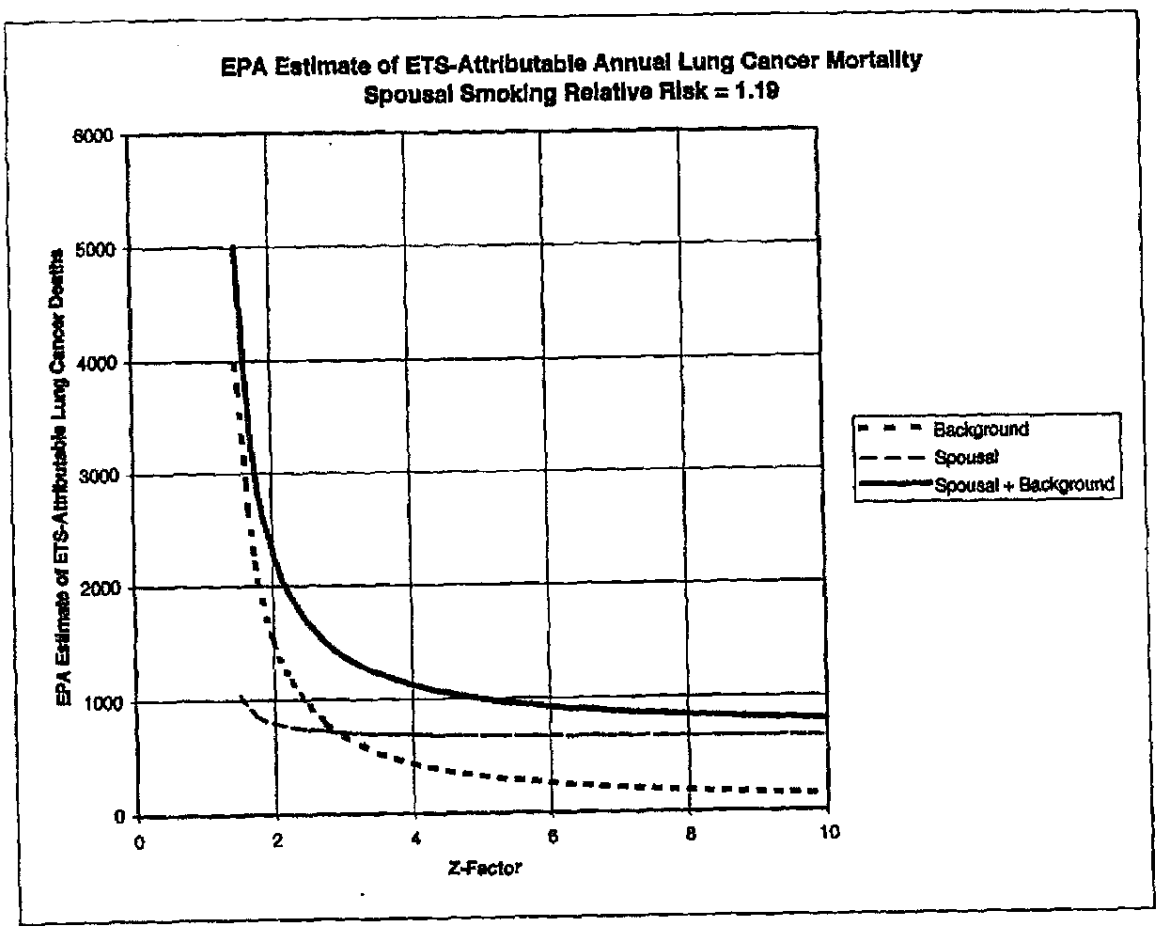


Figure 1.

EPA Estimate of Total (Spousal + Background) ETS-Attributable Lung Cancer Deaths
By Z-Factor

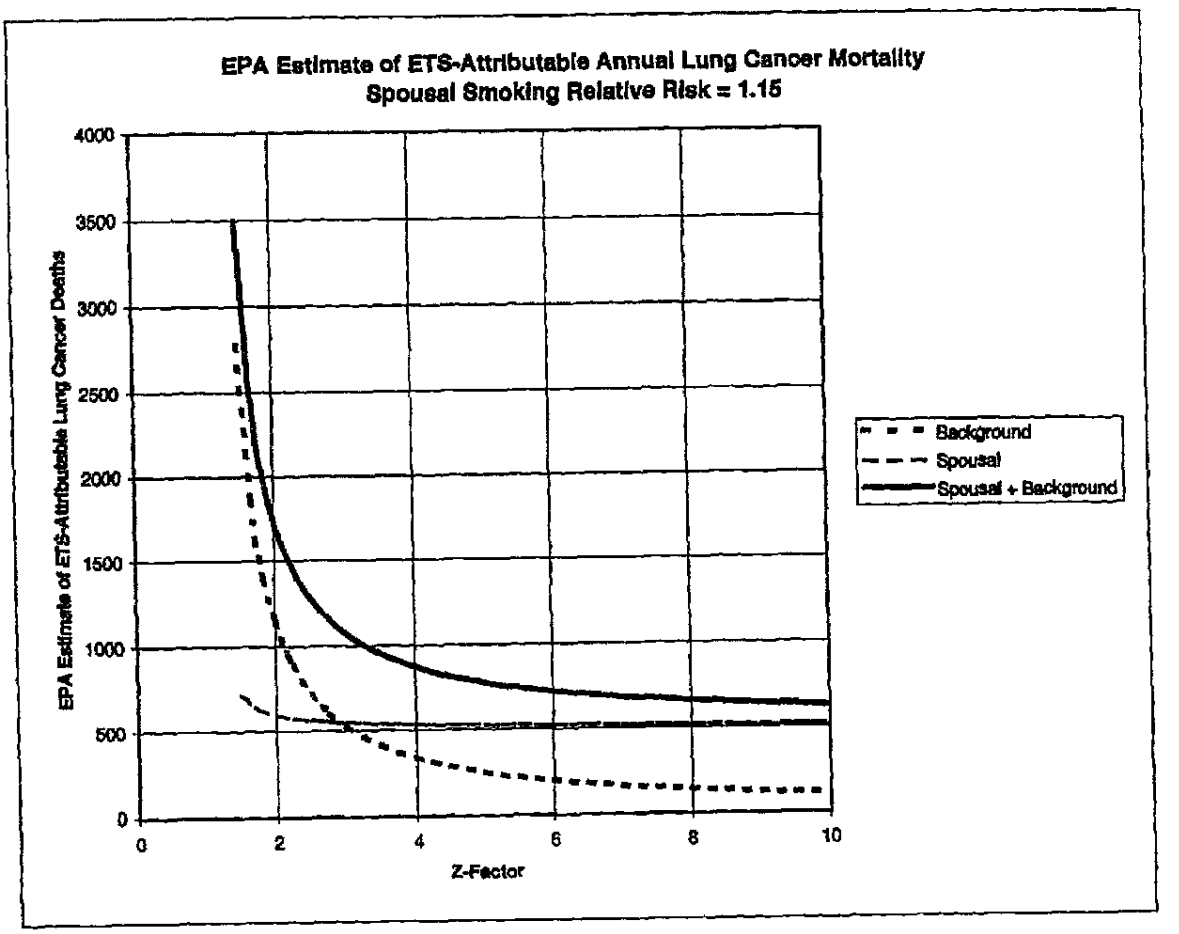


Figure 2.

EPA Estimate of Total (Spousal + Background) ETS-Attributable Lung Cancer Deaths
By Z-Factor

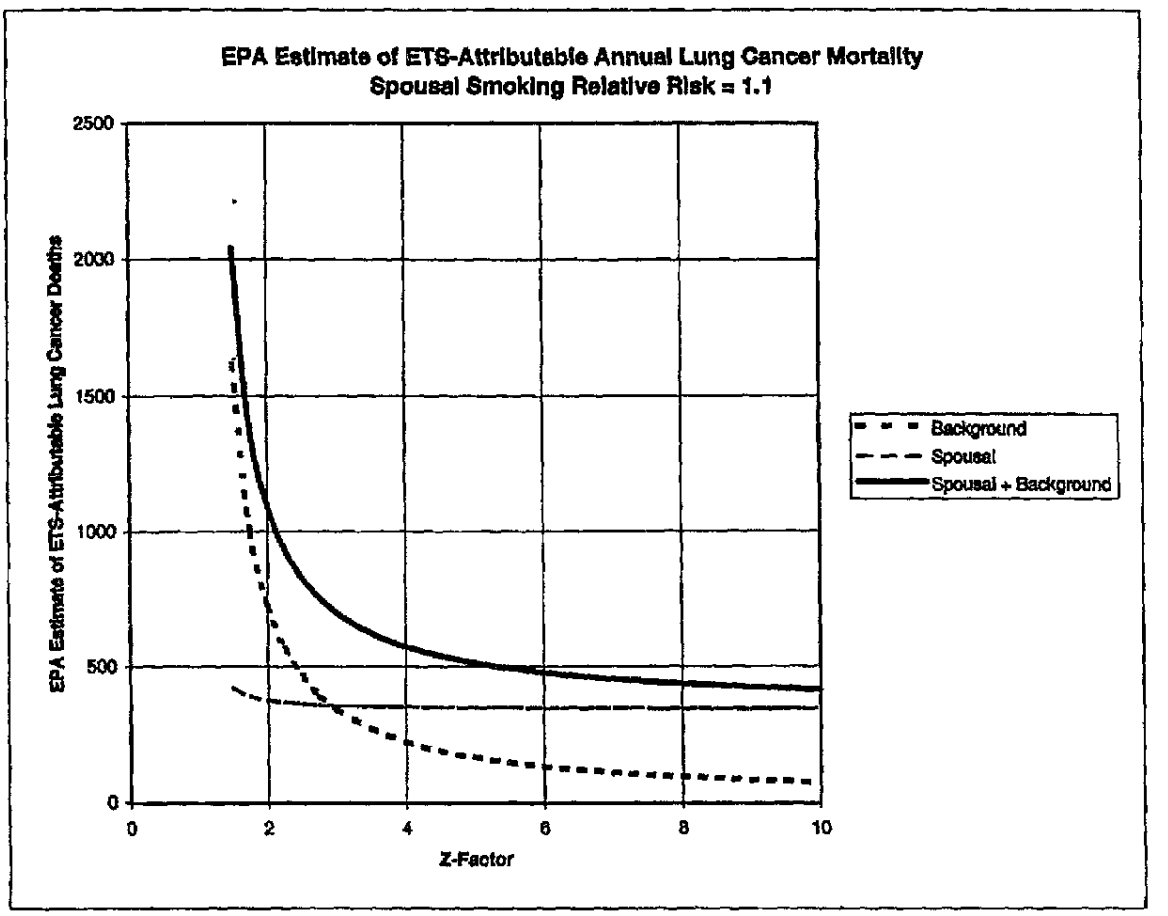


Figure 3.

decreasing triangular distribution from 3% to 5%. Based on results by Ogden et al. (1997), the uncertainty in the smoker misclassification rate is simulated by using the distribution in **Figure 4** for the geometric mean of the Relative Risk distribution. The value of the "true" Relative Risk is estimated from a lognormal distribution with the randomly selected value of the geometric mean and a geometric standard deviation equal to 1.0825 (determined from the 90% confidence interval 1.04-1.35 for the "true" Relative Risk).

The uncertainty in the value of the Z-factor is represented by a compound distribution, consisting of a uniform distribution from 1.75 to 10, followed by a declining triangular distribution from 10 to 20. This distribution is shown in **Figure 5**.

Based on the preceding uncertainty assumptions for Relative Risk and Z-factor, the Monte Carlo analysis of estimated mortality was performed for 10,000 iterations using Crystal Ball® Version 4.0. Again, we reaffirm our strong view that the Relative Risk point estimate reported by U.S. EPA is scientifically unjustified and that our use of this value is solely illustrative. With this in mind, the resulting simulated distribution for mortality associated with Total (Background + Spousal) ETS exposure is shown in **Figure 6**. The mean and median of this distribution are 804 and 702 respectively, which are far below the EPA's estimate of 3,060. In fact, 3,060 is at the 98.7th percentile of this distribution.

In this simulation, uncertainty in the Relative Risk and Z-factor are represented by independent probability distributions. As stated in the EPA's report, however, the parameters RR

Figure 4.

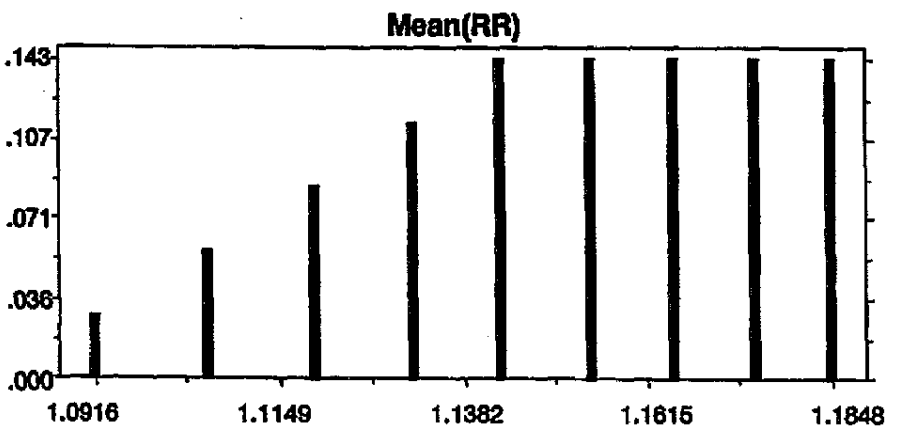


Figure 5.

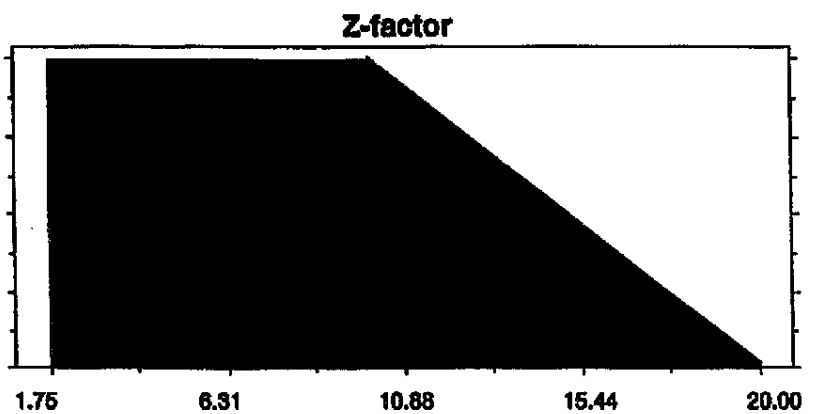
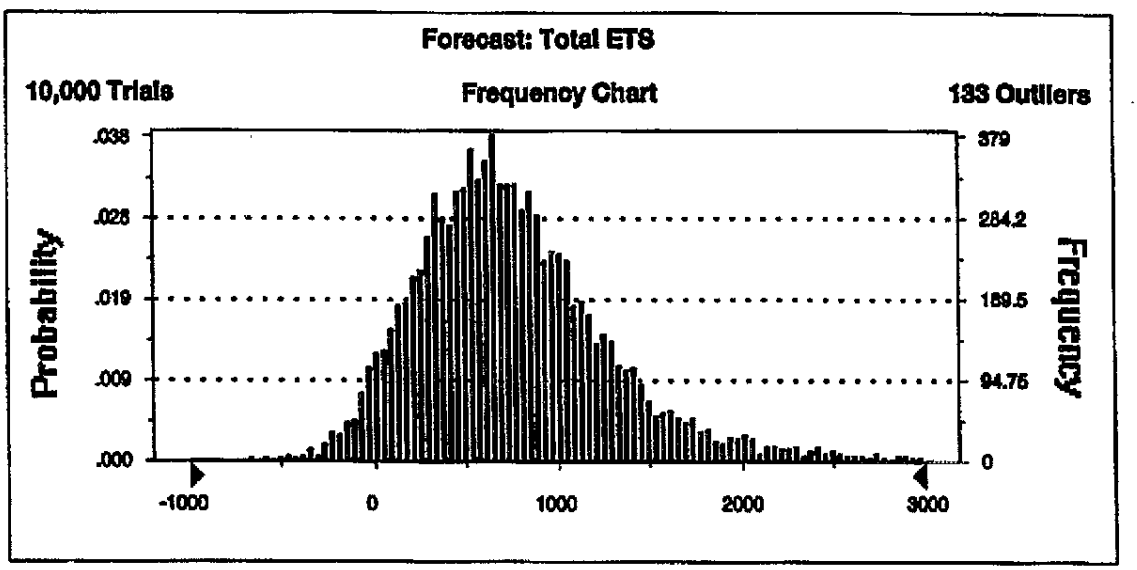


Figure 6.



and Z are not actually independent, but would be expected to covary in the same direction (to be positively correlated). For example, if the contributions of background to total ETS exposure decrease, Z would increase, and the observable relative risk from spousal exposure would tend to increase as well. Sensitivity analyses reveal that the effect of increasing correlation between RR and Z decreases the mean and standard deviation of the resulting simulated distribution of estimated mortality. Hence, estimates of mortality as high as 3,060 are even less likely than represented by the distribution in **Figure 6.**

References

Ogden, M.W., Morgan, W.T., Heavner, D.L., et al., National incidence of smoking and misclassification among the U.S. married female population, *Clin Epidemiol* 50: 253-263 (1997).

United States Environmental Protection Agency, *Respiratory Health Effects of Passive Smoking: Lung Cancer and Other Disorders*, Washington, D.C., Office of Research and Development, U.S. Environmental Protection Agency, EPA/600/6-90/006F (1992).