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Report nr. 658603 010

Tumorigenic effects in Wistar rats orally administered benzo[a]pyrene for two years (gavage studies). Implications for human cancer risks associated with oral exposure to polycyclic aromatic hydrocarbons.

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Erratum

Due to a mistranslation, the word "rumen" in the summary should be replaced by "forestomach"(p.8).

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CONTENTS

ABBREVIATIONS

ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
BPDE	Benzo[a]Pyrene-Diol-Epoxyde
BrdU	5-Bromodeoxyuridine
bw	body weight
DNA	Deoxyribonucleic acid
EROD	7-Ethoxyresorufin O-deethylase
GGT	Gamma-gultamyltranspeptidase
Hb	Haemoglobin
HCN	Health Council of The Netherlands
Ht	Haematocrite
ICD	Integrated Criteria Document
LDH	Lactate dehydrogenase
LED_{10}	95% Lower confidence value of the effective dose inducing a 10% extra tumor indence
LMS	Linearized Multistage Model
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular haemoglobin
MTD	Maximum tolerated dose
PAH	Polycyclic aromatic hydrocarbons
PLT	Platelets
RBC	Red blood cells
TBA	Tumour-bearing animals
TLC	Thin Layer Cromatography
VCP	Food consumption survey
VSD	Virtually safe dose
WBC	White blood cells

Abbreviations used for the various PAH referred to in this report

Ace	Acenaphtene	DB[el]Py	Dibenzo[el]pyrene
Acen	Acenaphthylene	DBT	Dibenzothiophene
An	Antanthrene	3,6-DMPh	3,6-Dimethylphenanthrene
A	Anthracene	F	Fluoranthene
B[a]A	Benzo[a]anthracene	f	Fluorene
B[e]AA	Benzo[e]aceanthrylene	I	Indan
B[j]AA	Benzo[j]aceanthrylene	IP	Indeno[1,2,3-cd]pyrene
B[l]AA	Benzo[l]aceanthrylene	x-MB[a]A	..-Methylbenzo[a]anthracene
B[a]Ac	Benzo[a]acridine	1-MCH	1-Methylchrysene
B[c]Ac	Benzo[c]acridine	2-MCH	2-Methylchrysene
B[a]f	Benzo[a]fluorene	3-MCH	3-Methylchrysene
B[b]f	Benzo[b]fluorene	5-MCH	5-Methylchrysene
B[a]F	Benzo[a]fluoranthene	6-MCH	6-Methylchrysene
B[b]F	Benzo[b]fluoranthene	3-MC	3-Methylcholanthrene
B[k]F	Benzo[k]fluoranthene	1-MN	1-Methylnaphtalene
B[ghi]F	Benzo[ghi]fluoranthene	2-MN	2-Methylnaphtalene
B[j]F	Benzo[j]fluoranthene	N	Naphthalene
BNT	Benzo[b]naphto[2,1-d]thiophene	Ph	Phenanthrene
B[ghi]Pe	Benzo[ghi]perylene	Pe	Perylene
B[rst]pp	Benzo[rst]pentaphene	Py	Pyrene
B[a]P	Benzo[a]pyrene	Pi	Picene (Benzo[a]chrysene)
B[e]P	Benzo[e]pyrene	T	Triphenylene
B[c]Ph	Benzo[c]phenanthrene		
B[b]tp	Benzo[b]triphenylene	***nitro-PAH***	
C	Coronene	9-NA	9-Nitroanthracene
Ca	Carbazole	6-NB[a]P	6-Nitro-Benzo[a]pyrene
CH	Chrysene	1-NCH	1-Nitrochrysene
CPP	Cyclopenta[cd]pyrene	2-NCH	2-Nitrochrysene
DB[ac]A	Dibenzo[ac]anthracene	3-NCH	3-Nitrochrysene
DB[ah]A	Dibenzo[ah]anthracene	6-NCH	6-Nitrochrysene
DB[aj]A	Dibenzo[aj]anthracene	2-NF	2-Nitrofluoranthene
DB[ah]Ac	Dibenzo[ah]acridine	3-NF	3-Nitrofluoranthene
DB[aj]Ac	Dibenzo[aj]acridine	1-NPy	1-Nitropyrene
7H-DB[cg]C	*7H*-Dibenzo[cg]carbazole	2-NPy	2-Nitropyrene
DBF	Dibenzofuran	3-NPy	3-Nitropyrene
DB[ae]Py	Dibenzo[ae]pyrene	1,3-DNPy	1,3-Dinitropyrene
DB[ai]Py	Dibenzo[ai]pyrene	1,6-DNPy	1,6-Dinitropyrene
DB[ah]Py	Dibenzo[ah]pyrene	1,8-DNPy	1,8-Dinitropyrene
DB[al]Py	Dibenzo[al]pyrene (DB[1,2,3,4]P)	DNFs	Dinitrofluoranthenes

GLP-compliance Statement

This report describes the results of the following studies:

- Range-finding study (dossier study no. T90-22)
- Verification forestomach hyperplasia by BrdU-incorporation (dossier study no. T91-181)
- 90-day study (dossier study no. T92-216)
- DNA adduct study (dossier study no. T92-216)
- Chronic carcinogenicity study (dossier study no. T92-216)

All animal experiments within this project were performed at the Animal Facility (Central Animal Laboratory (CDL/BES) according to Good Laboratory Practice (GLP) principles as laid down by OECD.

All necropsies as well as histopathological examinations are performed under responsibility of the Laboratory of Pathology and Immunology (LPI; formerly Laboratory of Pathology (PAT)) according to the OECD-principles for GLP.

All haematological, clinical chemistry, and urine analysis are performed at the former Laboratory of Toxicology according to the OECD-principles for GLP.

The DNA adduct analysis conducted within the context of the carcinogenicity study (i.e. dossier study no. T92-216) is performed at the former Laboratory for Carcinogenesis and Mutagenesis according to the OECD-principles for GLP.

The present report is based on the raw data and internal reports from these Laboratories. Raw data are stored in the institute's archives and may be consulted upon request

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SUMMARY

Polycyclic Aromatic Hydrocarbons (PAH) are widespread environmental and food contaminants introduced predominantly by anthropogenic processes. On the basis of both experimental and epidemiological studies, these PAH mixtures are considered carcinogenic to humans (IARC, 1983). Humans are continuously exposed to compounds of this group through the inhalatory and oral (food consumption) routes, and occasionally also via the skin. Results from a quantitative cancer risk assessment for the inhalatory route of exposure, based on available epidemiological and experimental data, suggest that from a regulatory point of view, cancer risks associated with environmental exposures clearly exceed acceptable levels, i.e. one per million on lifetime exposure. The estimation of cancer risks associated with oral exposure to PAH is hampered by the absence of adequate epidemiological and experimental data. The need for experimental data for this route is substantiated by the large daily dose received via the oral route (one order of magnitude larger than the dose via inhalation) for several potent carcinogenic PAH compounds e.g. benzo[a]pyrene (B[a]P), and the fact that epidemiological studies are not expected to provide useful data in this respect.

This last point prompted us to perform a carcinogenicity study in rats orally exposed to B[a]P, considered to adequately represent the carcinogenic fraction of PAH. House-bred Riv:TOX rats of the Wistar strain (52 animals per dose and per sex were used. B[a]P dissolved in soy oil was applied by gavage at 0 (control), 3, 10 and 30 mg/kg bodyweight, five days a week. The B[a]P treatment, lasting for two years, resulted in a dose-dependent increase in tumour incidence in a wide spectrum of organs and tissues, the most prominent clearly being the liver and rumen, both being organs with a low spontaneous tumour-incidence in this strain. Liver tumours were also responsible for the high mortality rate at the highest dose level in both sexes. Tumour development / prevalence at this site in female rats was used as a starting point for quantitative cancer risk estimations via the methodology adopted by the Dutch Health Council (HCN, 1994-1996), and resulted in a Virtually Safe Dose (VSD) of 5 ng B[a]P/kg bodyweight per day, i.e. a one per million risk on lifetime exposure. On the basis of the available data on occurrence and carcinogenic potency of PAH in the Dutch diet, it is suggested to apply a correction-factor of 10 for conversion to a VSD for all dietary PAH, i.e. a VSD of 0.5 ng B[a]P/kg bodyweight per day, taking B[a]P as PAH indicator. Cancer risks associated with the Dutch diet are estimated to be around acceptable

risk levels at this VSD. This ‘unexpected’ low risk (i.e. when compared to the above-mentioned risks), associated with relatively lower inhalatory exposures, is discussed, along with the uncertainties in the database and applied methodology.

The formation of DNA adducts by B[a]P was also investigated in a similar parallel study. DNA adducts (analysed by ^{32}P-postlabeling, enabling a very sensitive detection of stable DNA adducts), were found in all investigated organs and tissues. As tumours appeared in just a few organs, it may be concluded that the formation of stable DNA adducts by B[a]P as such is not sufficient for tumour induction. Neither did the data support the possibility of either the total number of adducts (i.e. the density) or the presence of specific adducts being responsible for tumourigenesis. Observations in the pre- and subchronic studies suggest rather that local cell proliferation may be the additional critical factor for tumour development. The possible implications of this finding are also discussed.

SAMENVATTING

Polycyclische Aromatische Koolwaterstoffen (PAK) komen zowel wijdverbreid in het milieu als in voedsel voor, beide als gevolg van menselijk handelen. PAK worden beschouwd als kankerverwekkend voor de mens (IARC, 1983). Dit is gebaseerd op zowel dierexperimenteel werk als op epidemiologische studies. De mens staat continu bloot aan deze groep verbindingen via de inhalatoire alsook de orale route (via voedselconsumptie), en in sommige gevallen via de huid. Een kwantitatieve schatting van het risico op kanker als gevolg van de inhalatoire blootstelling aan PAK in het milieu laat zien dat deze de acceptabel geachte grens, één extra kankergeval per miljoen levenslang blootgestelden, ruimschoots overschrijdt. Schatting van het risico op kanker als gevolg van blootstelling aan PAK via het voedsel wordt belemmerd door gebrekkige dierexperimentele en epidemiologische gegevens. De noodzaak om dit risico te kwantificeren wordt geïllustreerd door het feit dat de dagelijkse blootstelling via deze route in grootte een orde hoger geschat wordt dan die via inhalatoire blootstelling voor een aantal belangrijke carcinogene PAK, zoals benzo[a]pyreen (B[a]P). Omdat niet verwacht wordt dat epidemiologische studies hier op termijn uitkomst kunnen bieden, is grote behoefte aan goed uitgevoerd dierexperimenteel onderzoek.
Om bovengenoemde reden is een carcinogeniteitsstudie uitgevoerd waarbij ratten levenslang oraal zijn blootgesteld aan B[a]P, algemeen beschouwd als een representatieve modelstof voor carcinogene PAK. De in het instituut gekweekte Wistar ratten (52 dieren per dosis en per sexe) zijn per maagsonde vijf dagen per week blootgesteld aan in soja-olie opgeloste B[a]P, in doseringen van 0 (kontrole), 3, 10 en 30 mg/kg lichaamsgewicht. Deze behandeling resulteerde in een dosis-gerelateerde toename in tumorincidentie in diverse organen en weefsels. Veruit de hoogste incidenties tumoren werden gevonden in lever en voormaag, beide organen met een lage spontane tumorincidentie in deze rattenstam. Levertumoren vormden daarnaast de belangrijkste doodsoorzaak in de hoogste dosis-groep in beide sexen. De tumorvorming in dit orgaan in vrouwtjes ratten is vervolgens gebruikt voor het berekenen van de carcinogene risico's volgens een door de Gezondheidsraad aanbevolen methode (HCN, 1994-1996). Dit resulteerde in een "acceptabele dagelijkse dosis" (ADI) van 5 ng B[a]P per kg lichaamsgewicht, d.w.z. overeenkomend met één extra kankergeval per miljoen levenslang blootgestelden. Op basis van de beschikbare gegevens over de carcinogene potentie en het voorkomen van diverse PAK in het voedsel in Nederland wordt voorgesteld een conversie-factor van 10 te gebruiken voor totale PAK-belasting in voedsel, ofwel een

ADI van 0.5 ng B[a]P per kg lichaamsgewicht, met B[a]P als indicator voor in voedsel voorkomende PAK. Dit ‘onverwacht’ lage risico, althans in vergelijking met de bovenvermelde risico’s van PAK bij inhalatoire blootstelling, én de onzekerheden in de database en gebruikte methodiek, worden bediscussieerd.
De vorming van DNA addukten door B[a]P is ook in deze species bestudeerd onder dezelfde blootstellings condities. DNA addukten (bepaald met de ^{32}P-postlabelings-methodiek, die stabiele DNA addukten met grote gevoeligheid kan detecteren) konden in alle onderzochte organen en weefsels worden aangetoond. Omdat tumoren slechts in een beperkt aantal hiervan werden gevonden, kan worden geconcludeerd dat de vorming van stabiele DNA addukten op zichzelf niet voldoende is voor tumorvorming. Ook de totale hoeveelheid DNA addukten (ofwel de dichtheid), of de vorming van specifieke DNA addukten kon niet aan de localisatie van tumorvorming gerelateerd worden. Daarentegen suggereren waarnemingen in de range-finding en sub-chronische studies dat lokale celproliferatie een kritische additionele factor in tumor-vorming zou kunnen zijn. De mogelijke implicaties van deze bevindingen worden bediscussieerd.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) are known widespread environmental contaminants that are formed by (all kinds of) combustion of organic substances from both natural and anthropogenic origin, the latter being by far the greatest contributor. Humans are continuously exposed to this group of compounds by the inhalatory and oral route (food consumption), and occasionally also via the skin (IARC, 1983).

Several experimental studies have demonstrated the mutagenicity and carcinogenicity of PAH in animals, resulting in the conclusion that these compounds should be considered as human carcinogens (IARC, 1983; HCN, 1990). This supposition is strengthened by the observation that exposure of humans to PAH-rich mixtures, e.g. coal tar, coal-gasification, and coke-production, is associated with increased tumour incidences (IARC, 1984, 1985).

On the basis of available epidemiological and experimental data a quantitative cancer risk assessment appears feasible for the inhalatory route of exposure (Slooff *et al.,* 1989; Montizaan *et al.,* 1989). By using AirPEx (Freier *et al.,* 1997), a model developed to account for time- and space-dependent variations in exposure (Van Velze, 1996; Milieubalans, 1998), human cancer risks associated with lifetime exposure to PAH amounting to 20- 25 cases per million were calculated (Freier and Kroese, unpublished results). This figure only represents risks associated with outdoor sources, i.e. excluding indoor PAH sources originating from smoking and fireplaces, for example.

In contrast to the inhalatory route, the estimation of cancer risks associated with oral exposure to PAH is hampered by the absence of adequate epidemiological and experimental data (Slooff *et al.,* 1989). Because of the technical and methodological impossibility of obtaining useful data from epidemiology, the need for experimental oral data is motivated by the fact that daily exposure to PAH by the oral route is about an order of magnitude higher than that via inhalation (Slooff *et al.,* 1989; Heisterkamp and Van Veen, 1997). This latter at least holds for some PAH compounds associated with carcinogenic risks, e.g. benzo[a]pyrene (B[a]P). Consequently, it was decided to conduct a chronic carcinogenicity study in experimental animals (rats) exposed orally to a PAH considered as an adequate representative for the carcinogenic activity of this class of compounds. B[a]P was chosen because of its ubiquitous presence in complex PAH mixtures in the environment as well as in food, and its known relative strong carcinogenic potency compared to that of other PAH compounds (Montizaan *et al.,* 1989; Larsen, 1995; Muller *et al*., 1997).

This report presents the results of the chronic carcinogenicity bioassay and associated studies. It also provides a derived limit value for B[a]P as indicator for PAH, and

further discusses the risks associated with actual exposures to PAH compounds in the Dutch diet.

2. MATERIALS AND METHODS

2.1 Introduction

Details on this section will be described in Appendices A and B. Here only a brief description is provided of the test agent, test animal and experimental designs.

2.2 Animals, test agent and mode of application

Riv:TOX rats of the Wistar strain were used in all experiments performed within the scope of the project 658603. The animals were SPF, bred and housed at the Animal Facility of the National Institute of Public Health and the Environment under standardised conditions, and monitored for microbiological status with additional sentinel animals. Access to the animal room was limited to authorized personnel, that was subjected to strict hygiene measures.

B[a]P was from Serva (Heidelberg, Germany) and had a purity of 98.6 ± 0.4%. The dose-range finding study was performed with B[a]P from Janssen Chimica (Beerse, Belgium), which had a purity of 97.7 %. Claimed purities were verified upon receipt at the Institute.

B[a]P was dissolved in soy-oil and administered by gavage five days a week. Application by gavage was chosen for pragmatic reasons, to avoid the need for preparing homogeneous diets and to prevent hazardous and confounding contamination with B[a]P of the animal rooms. In the range-finding study exposure was for at least 5 weeks, whereas exposure in the carcinogenicity study was for 104 weeks. The resulting additional administration of soy oil was compensated for by equivalent soy oil reductions in the administered standard diet.

Further details are provided in Appendices A and B.

2.3 Experimental design

Based on data from the open literature it was decided that one 5-week dose range-finding study would be sufficient for finding the appropriate dose levels for the chronic study, and that the chronic study would include a scheduled 3-months sacrifice group (90-day study) to have this reassured.

2.3.1 Range-finding study

The prechronic dose-range finding study consisted of groups of 10 animals (per dose, per sex) that were administred B[a]P (dissolved in soy oil) by gavage for at least 5 weeks, 5 days a week at dose levels of 1.5, 5, 15 or 50 mg B[a]P/kg bw. Controls received the vehicle only. Dosing was performed on the basis of mean group weights. Administration of B[a]P started when the animals were 6 weeks of age and after having acclimatized for 10 days. Body weights, food- and water-consumption were recorded weekly. After 5 weeks of exposure the animals were sacrificed and examined for standard haematology and urinalysis parameters. In addition, some clinico-chemical parameters and organ weights were recorded. The animals were also subjected to histopathological investigations, specifically addressing reported and possibly additional target-tissues for toxicity.
Further details are provided in Appendix A.

2.3.2 Carcinogenicity study

In total 74 animals per group and per sex were used (see Scheme 1, next page). Of these, 52 rats (per dose, per sex) were assigned to the carcinogenicity assay and treated for 104 weeks with dose levels of 0, 3, 10 or 30 mg B[a]P/kg bw for 5 days a week.. Administration of B[a]P started at 6 weeks of age, after an acclimatization period of 8 days. Body weights, food and water consumption were recorded at regular intervals. Animals were monitored daily for health status, and intercurrently died or moribund animals were subjected to gross macroscopy and histopathology at earliest convenience. SPF status was monitored with additional sentinel animals. Upon terminal sacrifice all animals were subjected to detailed macroscopic examination and organs and tissues were prepared for further histopathological analysis. Further details are provided in Appendix B.

2.3.2.1 90-day study

In conjunction with the carcinogenicity study, another 10 animals (per dose, per sex) were assigned for a scheduled sacrifice after 3 months (see Scheme 1). Upon sacrifice these animals were subjected to macroscopy, prepared for histopathology and recording of organ weights, especially focussing on target-organs of B[a]P toxicity identified as such in the prechronic range-finding study and clino-chemical analyses. Further details are provided in Appendix B1.

2.3.2.2 DNA adduct study

For the determination of B[a]P-induced DNA adducts a supplementary group of 12 animals (per dose, per sex) was assigned for scheduled sacrifices after 4 and 5 months (6 animals each, see Scheme 1). In addition to the above described treatment, one extra group of 12 animals (per sex) received a dose of 0.1 mg B[a]P/ kg bw with the same regime. Upon sacrifice, blood was collected and the animals were subjected to a total body perfusion before isolation and storage of relevant organs at -70°C. Blood and organs were stored until processed for DNA adduct analysis. Further details are provided in Appendix B2.

Scheme 1. Schematic overview of the various studies conducted: number of rats of either sex used and duration of treatment.

Dose[1]	Carcinogenicity study	90-day study	DNA adduct study	Total
0.0	52[2]	10	12	74
0.1	-	-	12	12
3.0	52	10	12	74
10	52	10	12	74
30	52	10	12	74
Duration[3]	24	3	4 and 5[4]	

[1]) dose in mg/kg bw, 5 days a week; [2]) number of animals allocated to the various groups; [3]) duration of treatment and observation in months; [4]) Two duration periods to confirm the achievement of steady-state levels of DNA adducts.

3. RESULTS

3.1 Introduction

In this chapter only those experimental results are described that are considered of direct, critical significance for the quantitative evaluation of the carcinogenicity of B[a]P. Details on the range-finding study are given in Appendix A. A full histopathology report and other details concerning the carcinogenicity study are described in Appendix B. The results of the 90-day study and DNA adduct analyses are provided in Appendices B1 and B2, respectively.

Based on toxicological effects of B[a]P observed in the range-finding study it was decided to apply as dose-levels for the carcinogenicity assay 3, 10, and 30 mg B[a]P/kg bw (see Appendix A for further argumentation).

3.2 Experimental results

3.2.1 Survival

In the control animals, survival after 104 weeks was about 65% and 50% in males and females, respectively (Figure 1). From health status reports, and macroscopic and histopathological examination upon sacrifice (either intercurrently or at termination) or post-mortem necropsy it was apparent that control animals were generally in good condition. In these animals by far the main cause of death was tumour development in the pituitary, which is consistent with earlier findings in our historical controls (Wester *et al.*, 1985a, 1985b, 1990). In animals exposed to B[a]P treament of both males and females resulted in a dose-related decrease in survival. Most intercurrent mortalitites were from euthanasia due to poor condition, ususally related to tumours (mainly in liver and stomach), while significant non-neoplastic effects were not observed.

3.2.2 Body weight

Only at the highest dose tested (30 mg/kg bw), B[a]P appeared to affect body weight gain in males only, i.e. from week 10 onwards (Figure 2). At the same dose level females appear to be less sensitive for this effect. Accordingly, it was concluded that adequate top-dose levels that were originally chosen were adequate, i.e. around or just below the maximum tolerated dose (MTD).

3.2.3 Food- and water consumption

B[a]P treatment apparently had no significant effect on food consumption (see Figure 1 in Appendix B). For some short periods within the 104 weeks of treatment small (i.e. < 10%) statistically significant differences between the various treatment groups and controls were observed, though without a clear relationship to dose. An exception appears to be the more sustained reduction of food consumption though again of less than 10% of high dosed males starting at about week 36.

B[a]P treatment had no major effect on water consumption in females (see Figure 2 in Appendix B). Water consumption in males appeared to be dose-relatedly increased, starting from week 13 onwards. High-dose males showed an increased consumption of water (more than 50%) briefly before their withdrawal from the study. It was noted that high-dose males also showed a decreased food consumption (see Figure 1 in Appendix B), and a decreasing body weight during this period (Figure 2, below). There is yet no satisfactory explanation for these observations.

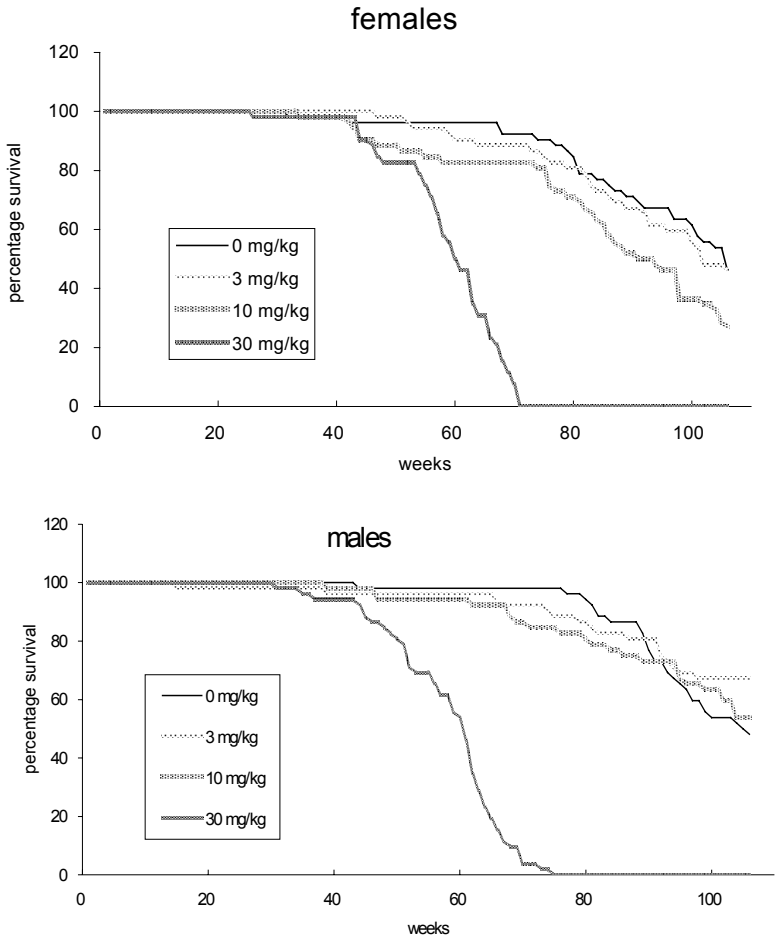

Figure 1: Survival of female and male rats during oral exposure to B[a]P at the indicated dose levels for 104 weeks: Control (——); 3 mg/kg (. . . .), 10 mg/kg bw (·····) and 30 mg/kg bw (–·–·–)

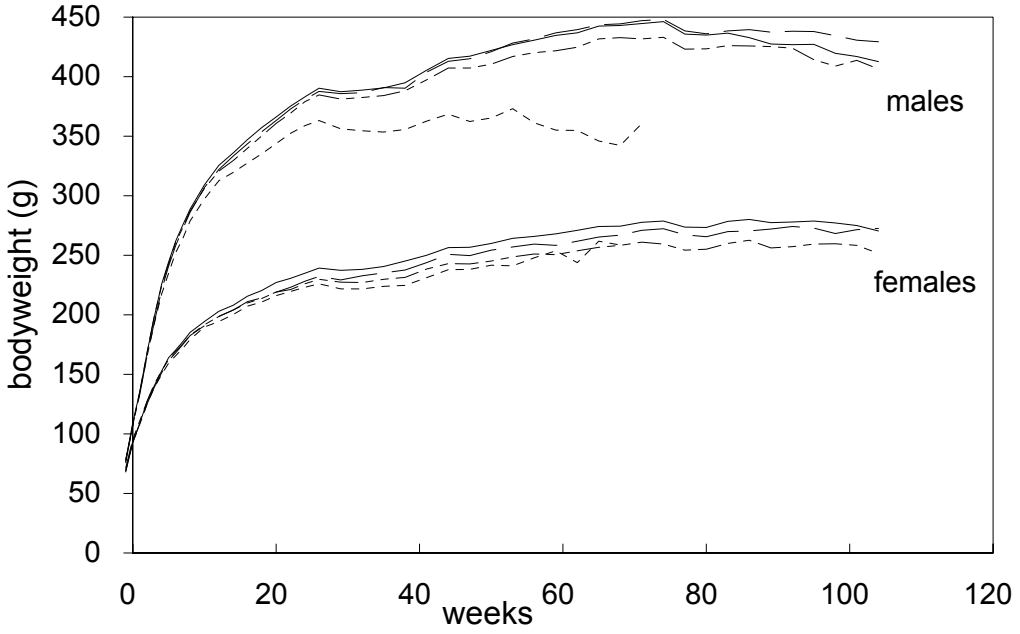

Figure 2. Body weights of female and male rats during oral exposure to B[a]P at the indicated dose levels for 104 weeks. Control (—), 3 mg/kg bw (— —), 10 mg/kg bw (— – –), and 30 mg/kg bw (– – – –).

3.2.4 Non-neoplastic findings

Treatment with B[a]P for 3 months clearly resulted in effects on target organs for toxicity, i.e. increased liver- and decreased thymus weights (see Appendix B1 for 90-day study results and de Jong *et al*., 1999). This was in line with observations in the range-finding study (Appendix A). Histopathological examination showed only minor changes such as slight reactive (proliferative) changes in the mucosa of the forestomach.

3.2.5 Carcinogenicity

B[a]P treatment for 2 years resulted in a dose-dependent increase in tumour incidence in a variety of organs and/or tissues (Tables 1 and 2; see Appendix B for further details). Clearly the most prominent carcinogenic effects of B[a]P were observed in the liver and forestomach, both organs with a low spontaneous tumour incidence in this rat strain (Wester *et al.*, 1985a, 1985b, 1990), as also illustrated by the incidence found in the concurrent controls of the present study. Liver tumour development was also responsible for the high mortality rate in the highest tested doses in both sexes. At the two highest dose levels multiple tumours in the liver was a common finding, together with tumour development in the forestomach. Besides these two target sites, B[a]P treatment also induced soft tissues sarcomas at various sites (oesophagus, skin, mammary), as well as tumours of the auditory

canal, skin, and oral cavity. Tumours were, also, observed in the small intestine (at the top dose; especially males), and in the kidney (the two highest dose levels, males only) (see Appendix B for further details).

Table 1. Incidences of some major treatment-related neoplasms in female rats treated with B[a]P. The most advanced stage of lesions is scored.

Site	Dose[1] mg/kg bw	0	3	10	30[2]
forestomach	*Examined*	*52*	*51*	*51*	*52*
	squamous cell papilloma	1	3	20***	25***
	squamous cell carcinoma	0	3	10**	25***
liver	*Examined*	*52*	*52*	*52*	*52*
	hepatocellular adenoma	0	2	7*	1
	hepatocellular carcinoma	0	0	32***	50***
auditory canal	*Examined*[3]	*0*	*1*	*0*	*20*
	squamous cell papilloma	0	0	0	1
	carcinoma[4]	0	0	0	13**

[1]) 5 days a week; [2]) note that this group had a significantly shorter lifetime (see Figure 1);
[3]): these tissues were examined only when abnormalities were observed upon macroscopic examination; [4]) composite tumours of squamous and sebaceous cells apparently arisen from the pilosebaceous units / "Zymbal glands".

*p<0.01, **p<0.001 ***p<0.00001, Fisher's exact test; analyses of tumour incidence of the auditory canal was based on n=52

Table 2. Incidences of some major treatment-related neoplasms in male rats treated with B[a]P. The most advanced stage of lesions is scored.

Site	dose[1] mg/kg bw	0	3	10	30[2]
forestomach	*examined*	*52*	*52*	*52*	*52*
	squamous cell papilloma	0	7*	18***	17***
	squamous cell carcinoma	0	1	25***	35***
liver	*examined*	*52*	*52*	*52*	*52*
	hepatocellular adenoma	0	3	15***	4
	hepatocellular carcinoma	0	1	23***	45***
auditory canal	*examined*[3]	*1*	*0*	*7*	*33*
	squamous cell papilloma	0	0	0	4
	carcinoma[4]	0	0	2	19***

[1])5 days a week; [2]) note that this group had a significantly shorter lifetime (see Figure 1);

[3]): these tissues were examined only when abnormalities were observed upon macroscopic examination; [4]) composite tumours of squamous and sebaceous cells apparently arisen from the pilosebeceous units / "Zymbal glands".

*p<0.01, **p<0.001 ***p<0.00001, Fisher's exact test; analyses of tumour incidence of the auditory canal was based on n=52

4. EVALUATION

4.1 Introduction

In this chapter we will first focus on the carcinogenic effects induced by B[a]P in the Riv:TOX rat under the present treatment conditions. Subsequently, from these data a limit value for B[a]P will be derived using the carcinogenic risk assessment procedure adopted by the Dutch National Health Council (HCN, 1994, 1995, 1996). In addtion, the role of B[a]P as a representative of the group of PAH compounds will be discussed and a proposal for an appropriate limit value for B[a]P as PAH indicator will be presented. Finally, using the above derived limit values, we will estimate the cancer risks associated with exposure to PAH via the diet as the main source of oral PAH. A critical discussion of the extrapolation methods employed in the various phases of the above mentioned risk assessment will be the subject of the next chapter.

4.2 Derivation of a limit value for B[a]P

4.2.1 B[a]P-induced carcinogenic effects

The present results clearly showed B[a]P to be a potent carcinogen upon chronic oral administration. Tumours were induced at multiple sites in both sexes of rats, i.e. liver, forestomach, auditory canal, oral cavity, skin, and intestines, and additionally the kidney in males, and the mammary and oesophagus in females. The most potent carcinogenic effects of B[a]P under the present conditions were observed in the liver and forestomach, both organs with a low spontaneous incidence in this rat strain.

The induction of liver tumours by B[a]P is a somewhat unexpected finding. Though this site is the most frequently observed target site for mutagenic rat carcinogens, i.e. in 35% of the cases (Gold *et al.*, 1993), data sofar had identified B[a]P as a “typical” non-hepatocarcinogen in rodents (Hesse *et al.*, 1982; Hunt, 1988). Probably, the absence of a properly conducted study in rats has precluded the identification of this site as a target for carcinogenesis by B[a]P.

Full malignant forestomach tumours, also, have not been observed before in this species upon B[a]P treatment. On the other hand, they have been observed in most previously performed oral carcinogenicity studies with mice and hamsters; The advisory limit value for oral exposure to PAH derived by the RIVM in 1989 actually was based upon B[a]P-induced tumours at this site and the upper digestive tract in mice (Slooff *et al.,*1989). Of the other tumour sites found in the present study auditory canal (“Zymbal gland”) tumours have not been reported previously with B[a]P (Soderman, 1985; Muller *et al.*, 1997). Interestingly, the

strict association between tumours of the auditory canal and potent mutagenic (i.e. Salmonella positive) carcinogens in rodents, e.g. 2-acetylaminofluorene, azoxymethane, and vinyl chloride (Ashby and Paton, 1993; Gold and Zeiger, 1997), is reinforced by the present observations.

Also remarkable is the observation of tumours in the small intestine and the kidney, which has not been reported previously for B[a]P treated rats (Soderman, 1985; Muller *et al.*, 1997).

An important aspect in interpreting rodent carcinogenicity results is the notion of a possible exceedance of the maximum tolerated dose (MTD), i.e. the highest tested dose that induces minimal signs of toxicity (OECD, 1987). In case tumours are only induced at dose levels that exceed the MTD, it has been argued that the meaning of this phenomenon for human risk assessment should be considered questionable at least (Hoel *et al.*, 1988; Ames and Gold, 1990; Haseman and Lockhart, 1994). The present results show that only the top dose in males may possibly be regarded as exceeding the MTD, and therefore, the carcinogenic effects observed in the other dose groups are not subject to the above mentioned restrictions with regard to extrapolation to humans.

4.2.2 Methodology adopted by the Dutch Health Council for derivation of a virtually safe dose (VSD)

In the Netherlands a procedure for derivation of health-based limit values for the general population for genotoxic carcinogens like B[a]P has been recommended by the Dutch Health Council (HCN, 1994, 1995, 1996). It implies that exposure levels without any risk for carcinogenic effects cannot be identified for stochastically acting agents. Consequently, an exposure level representing a negligible (acceptable) risk level to humans is established, the so-called virtually safe dose (VSD): an exposure level associated with an additional risk for cancer of no more than one per million lifetime exposed people.

For the derivation of this VSD preferably data from epidemiological studies should be used. However, in the absence of adequate human data, as is the present case, results from animal studies have to be applied. Briefly the procedure is as follows. Within the animal study the lowest dose level associated with a significantly increased tumour response is identified. This dose level is used as a point of departure for linear extrapolation to zero dose, representing the no-effect level for genotoxic compounds. After correction for background tumour incidence, the VSD is calculated from this linear response curve. In its motivation the Health Council recognizes the multistep nature of the carcinogenic process, but assumes linearity at (very) low exposures as the compound is suspected under these conditions to account only for one rate limiting, relevant mutagenic step in this process (i.e. in addition to

background mutations). It should be noted, however, that linear extrapolation from the observable effect range to zero dose is recognized as being a conservative approach for risk estimation, in the sense that it is likely to overestimate actual carcinogenic risks. This kind of extrapolation also assumes that humans and animals are equally sensitive towards genotoxic carcinogenic compounds (HCN, 1994, 1995, 1996).

4.3 Derivation of a VSD for B[a]P

From the present data several points of departure for linear extrapolation and derivation of a VSD are possible. The most susceptible organs for B[a]P-induced tumourigenesis are the liver and forestomach. Tumours were found at the lowest tested dose of 3 mg/kg bw, though at a (borderline) non-significant incidence. Several approaches are possible here. One is to take liver tumours as point of departure, as these are the most prominent of all. This choice is further supported by the argument that forestomach tumours may be less relevant as humans do not possess a clearcut counterpart to this rat organ, and the mode of application in the animal experiment (gavage) resulted in unrealistically high local concentrations of B[a]P that most probably have seriously promoted the development of tumours at this site (Wester and Kroes, 1988). On the other hand, there are also arguments in favour of taking forestomach tumours as a starting point. These tumours have also been observed in mice exposed to diets supplemented with B[a]P (Culp *et al.*, 1998) implying that they are not strictly related to 'bolus-injection' by the gavage-application technique. The direct use of forestomach tumours observed in experimental animals for quantitative cancer risk assessment purposes for humans has been discussed by Wester and Kroes (1988). Their conclusion is that, although humans do not possess a homologue organ, the forestomach may be regarded as a sensitive model for organs having equivalent epithelial lining cells, e.g. the eosophagus. Indeed, B[a]P also induced tumours at this site in mice, be it at a clearly much lower efficiency. Furthermore, dermal and inhalation studies have shown B[a]P to be quite effective in inducing carcinogenic effects at the site of application (Slooff *et al.*, 1989; Montizaan *et al.*, 1989). Indeed a number of oesophagus- associated tumours were observed in the present study, but these were sarcomas instead of carcinomas, and attributed to accidental local tissue deposition of gavaged material (injection site sarcomas). Finally, one could take both tumour sites combined as a point of departure, i.e. the number of animals either bearing liver or forestomach tumours, or include other tumours, when regarded treatment-related, as well (TBA, no. of tumour bearing animals). This latter approach is based on the notion that from a human health point of view, cancer risks count as such, irrespective of the tumour type or site as long as these are considered relevant to humans. As tumour incidences in liver and forestomach do not differ much between males and females, one could

combine the two sexes for this purpose. However, preferably dose levels as far as possible below those inducing toxicity should be selected, and, therefore, females are taken here as species for low dose extrapolation (since the top dose in males elicited obvious toxicity, as noted above). Another issue relevant to the choice of the point of departure concerns the nature of the tumour considered: one could either start from malignant tumours only or from malignant ánd benign tumours combined: both approaches are taken into consideration in this report (see Table 3).

Before calculating and comparing VSDs for these approaches some corrections have to be made to deduce an acceptable lifetime daily dose from the present experimental design. First of all, the 5-days-a-week application regimen has to be corrected into a 7-days-a-week exposure: this is achieved by simply multiplying the applied doses with a factor of 5/7 (HCN, 1995). The next step is a correction for observation-period in relation to the lifespan of the species, which by default is 1000 days for rats (HCN, 1995). Exposure was for 104 weeks, whereas the mean observation period was 106 weeks. Therefore, the formula to estimate the additional risk for cancer of one per million lifetime exposed is:

$VSD = (10^{-6}/ I_{sign}) \times TD_{sign} \times 5/7 \times 728/1000 \times 742/1000$

where: I_{sign} = observed incidence at the TD_{sign} minus background incidence;
TD_{sign} = the lowest dose level associated with a significantly increased tumour response.

The VSDs that result from the approaches outlined above are summarized in Table 3. It is shown that whatever approach is taken, the range of VSDs calculated from the present rat study ranges from 5 to 19 ng B[a]P/kg bw. For the derivation of the VSD the most sensitive species/site combination for tumour development is applied, and as long as there is no reason to question this on the basis of human relevance for these types of tumours (HCN, 1996). Thus, from the present data a VSD of 5 ng B[a]/kg bw is derived.

Table 3. VSDs based on the results of the oral carcinogenicity study with B[a]P in the rat. The incidences depicted are those from female rats (for explanation see text).

Site	TD_{sign} [1]	I_{sign} [2]	VSD [3]
Liver hepatocellular adenomas & carcinomas combined hepatocellular carcinomas	10	0.75 0.62	5 6
Forestomach squamous cell papillomas & carcinomas combined squamous cell carcinomas	10	0.57 0.20	7 19
TBA[4], liver & forestomach benign and malignant tumours malignant tumours	10	0.73 0.65	5 6
TBA, All treatment-related tumours[5] benign and malignant tumours malignant tumours	10	0.83 0.67	5 6

[1]) TD_{sign}= the lowest dose level (mg/kg bw; 5 times a week) associated with a significantly increased tumour response; [2]) I_{sign}= observed incidence at the TD_{sign} minus background incidence;[3]) calculated daily dose in ng B[a]P/kg bw (numbers rounded); [4]) TBA: tumour bearing animals; [5]) also skin and oesophagus tumours included.

4.4 Derivation of a VSD for B[a]P as indicator for PAH compounds

The above calculated VSDs are derived for B[a]P as a single chemical. In practice, however, humans will not be exposed to this single PAH, but to mixtures of hundreds of PAH compounds instead, including B[a]P. At the time of preparation of the Integrated Criteria Document PAH in 1989 studies relating to cancer risks and oral exposure to PAHs were not available (Slooff *et al.,* 1989; Montizaan *et al.,* 1989), and the authors, therefore, had to estimate the relative contribution of these other PAH to the cancer risk by extrapolation. Based on the available poor data it was estimated that the carcinogenic risks associated with PAH mixtures might amount to 10 (Slooff *et al.,* 1989; Montizaan *et al.,* 1989) or even 25 times that of B[a]P alone (Kramers and van der Heyden, 1988). Thus, if B[a]P is taken as PAH-indicator, it is proposed to divide the VSD calculated above for B[a]P by a factor of 10 to account for cancer risks induced by all PAHs together. Daily exposure to PAH via food, therefore, should not exceed 0.5 ng /kg bw, equivalent to 35 ng/person (assuming an average body weight of 70 kg).

4.5 PAH exposure via the diet: estimation of associated cancer risks

Exposure to PAH via the diet has been investigated by Vaessen *et al.* (1988) and de Vos *et al.* (1990). Vaessen *et al* analysed duplicate diets of 50 individuals in Dutch home settings for 15 PAH in the period 1976-1985, and found a mean daily intake for B[a]P of 80 ng per person (see Table 1, Appendix D). De Vos *et al.,* analysed 17 PAH in 10 'market baskets' of eighteen year-old Dutch males sampled over a 2.5 year period (1984-1986). They estimated a daily intake of B[a]P between 120 and 290 ng, when taking food groups with 'not detectable' B[a]P as either zero or equal to the detection limit. (see Table 1, Appendix D). This latter method clearly is a "worst case" approach.

From analytical data on food groups (de Vos *et al.,* 1990) and information on daily food intake for the Dutch population (VCP, 1990), Heisterkamp and Van Veen (1998) calculated the distribution of the daily B[a]P intake by the Dutch population. For this purpose they applied a statistical model, STEM, to estimate the interindividual variation in life-time intake (Slob, 1993). Their results are shown in Table 1 of Appendix D and graphically depicted in Figure 3. The calculated mean daily B[a]P intake is 205 ng when taking mean detected B[a]P levels, and additionally all food groups with "not detectable" B[a]P as half the detection limit (Heisterkamp and van Veen, 1998). It appears that this distribution is lognormal with a 95%-distribution factor of 1.85, which is rather small. However, as PAH content has only been determined for a few food items, this 95%-distribution factor is expected to be an underestimate.

From the above data on B[a]P exposure via the diet one can conclude that estimates of the mean intake range from 80 to 205 ng per person a day. When taking the above derived VSD for B[a]P as PAH indicator into account (that is based solely on the rat data), i.e. daily 35 ng B[a]P per person, this exposure amounts to an additional lifetime cancer risk for the general population of 2 to about 5 per million exposed. Based upon the estimated distribution of individual intakes, even those individuals that represent the 99th percentile only have an approximate 3-fold higher risk as compared to that calculated for the average individual. As stated above, however, the estimated distribution is expected to underestimate the true variation in individual B[a]P intake, but it is unknown to what extent.

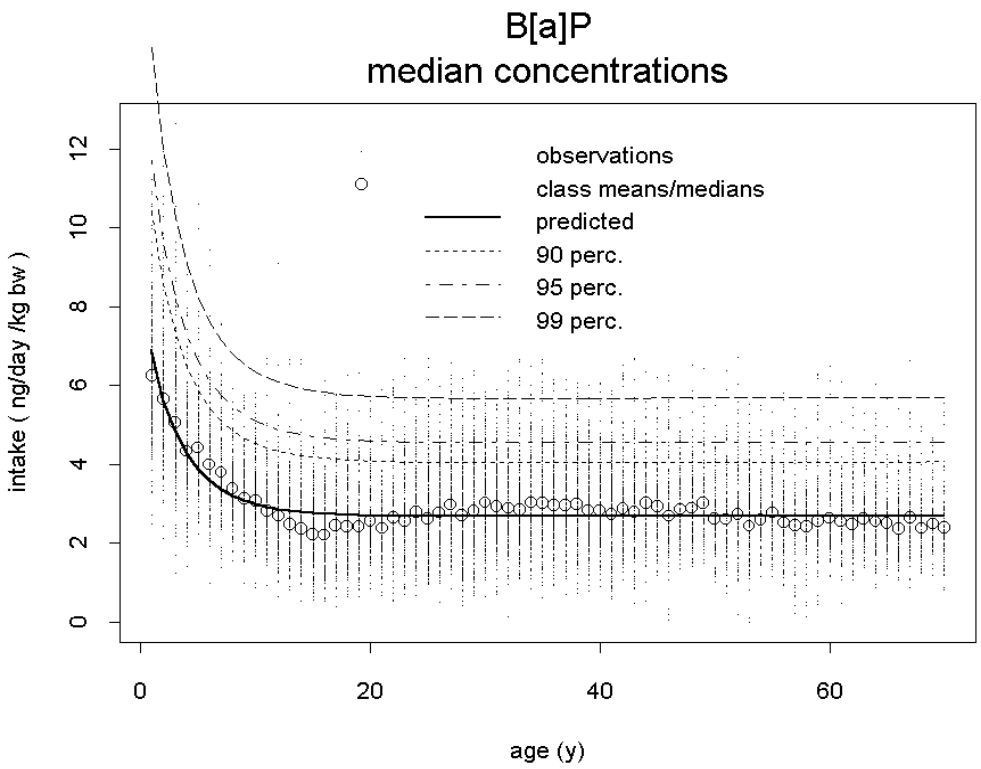

Figure 3. Distribution of dietary intake of benz[a]pyrene in the general Dutch population. The values are derived by using median measured concentrations in food items with detectable levels of PAH, and half the detection-limit in food items with no detectable levels of PAH (from Heisterkamp and Van Veen, 1997).

5. DISCUSSION

5.1 Introduction

This chapter will briefly discuss the VSD for B[a]P as PAH indicator, that was derived in the previous chapter based on rat data: i.e. the uncertainties in the applied methodology to derive at this VSD, as well as the adequacy of the presently available database[1]. For further details, reference will be made to the corresponding Appendices.

5.2 VSD estimates for B[a]P derived from animal studies

First of all, the above derived VSDs from the carcinogenicity study in the rat should be compared to those presented by the RIVM in the Integrated Criteria Document PAH in 1989 (Slooff *et al.,* 1989; Montizaan *et al.*, 1989). At that time the available bioassay data were all judged to be of insufficient quality. Among these, the studies of Horie *et al*. (1965) and Chouroulinkov *et al.* (1967), in which mice were exposed to B[a]P via the diet and drinking water, respectively, were selected for low dose extrapolation. The VSD calculated from these studies was based upon forestomach and other upper digestive tract tumours and amounted to 20 - 40 ng/kg bw when only malignant tumours were counted. If, on the other hand, also related benign tumours were included, a VSD of 4 - 6 ng B[a]P/kg bw was calculated (Montizaan *et al.,* 1989). Thus, the range of VSDs from these 'old' and 'inadequate' mice studies was from 4 to 40 ngB[a]P/kg bw, and remarkably similar to the presently calculated VSDs for the rat.

Concomitantly with the present study with rats, a 2-year carcinogenicity study was conducted at the NCTR with female B6C3F1 mice exposed to B[a]P via the diet (Culp *et al.*, 1998; see Appendix C). Target tissues for carcinogenicity found in this latter species were the forestomach, oesophagus and tongue, confirming earlier observations (Horie *et al.*, 1965; Chouroulinkov *et al.*, 1967; Rigdon and Neal, 1966; Neal and Rigdon, 1967). No clear treatment-related induction of liver tumours was found in the mouse study, despite the recognized predisposition of that species for liver tumours (Drinkwater *et al.,* 1989, Haseman *et al.,* 1998), though this may not have been noticed because of a focus on (the (early) occurrence of) tumours in the forestomach. A VSD calculated from the mouse bioassay in a similar way as previously described for the rat data, would amount to approximately 5 ng B[a]P/kg bw (Table 2, Appendix C). A lifetime correction was not performed here as the

[1] This discussion is not to detract from the provided risk estimates, but rather to position them in a context that provides a better understanding and to justify the conclusions drawn in the next chapter.

default value of 750 days for this species (HCN, 1994) was more or less matched by the experimental period of 735 days.

Conclusion

Both the earlier discussed animal studies, as well as the recent NCTR study did lead to VSD values comparable to that derived from the rat study reported here. Values range from 5 to 40 ng B[a]P per unit body weight for the "old" mice studies, and from 5 to 19 for the recent studies: i.e. around 5 ng B[a]P when based on the recent mouse and rat data, respectively. Thus based on all these data a VSD of 5 ng B[a]P per kg bw seems a justified value, for the following reasons.

5.3 Uncertainties of the derived VSD for B[a]P

Despite this apparently convenient concordance in animal data, uncertainties exist with regard to interpreting the above VSD value as derived via methodology adopted by the Dutch Health Council. The estimation of a VSD for humans from data obtained in animal studies basically implies two extrapolations, from animal to man, and from high to low dose[2], that will both be briefly commented.

5.3.1 Extrapolation from animal to man

In its calculation of cancer risks for genotoxic agents after oral exposures the Health Council assumes (in the absence of further data) humans to be as sensitive as animals (HCN, 1996). The default approach chosen for interspecies scaling is on the basis of the daily dose per unit body weight. Some support in favour of this approach was provided by Crump *et a*l., (1989), and Goodman and Wilson (1991) in their empirical analysis of the potency of carcinogens in humans and animals. However, others have provided evidence for using either surface area scaling (i.e. per unit body weight$^{0.67}$) or scaling according to caloric demand (i.e. per unit body weight$^{0.75}$) (Crouch and Wilson, 1979; Davidson *et al.,* 1986), or even on the basis of the cumulative dose (Dedrick and Morrison, 1992; Lijinski, 1993). In 1991 U.S.Governmental Agencies (EPA, FDA and CPSC) have agreed to extrapolate on the basis of caloric demands (U.S.EPA, 1991). As a reasoning for this the Agencies put forward both pragmatic and empirical grounds, as well as a "biological rationale", i.e. taking into account the principles of interspecies allometric variation in anatomy, physiology and pharmacokinetics (U.S.EPA, 1991).

[2] i.e. from experimentally applied doses to exposures actually experienced by humans, or: from the observable effect range to exposures at which effects cannot be observed.

Despite considerable study and debate none, of the scaling methods has emerged as clearly preferable over the others, either on empirical or theoretical grounds. One should recognize, however, that VSDs calculated via different methods may result in substantially different values; e.g. scaling on caloric demand results in a VSD that is 4 or 7 times lower, when based on rat or mouse data, respectively, as compared to scaling on unit body weight.

In the context of interspecies extrapolation it is worth mentioning that scaling on caloric demand is also applied in the Netherlands when dealing with cancer risks associated with inhalatory exposure to genotoxic carcinogens; i.e. these are based on air concentrations which can be considered equivalent to scaling in caloric demand (HCN, 1995, 1996). This needs to be considered when comparing cancer risks associated with exposure to PAH via other routes.

5.3.2 Extrapolation from high to low dose

For the extrapolation from high to low dose, the Health Council assumes a "conservative" linear dose-response (as outlined in paragraph 4.3.1). As with interspecies scaling, there is no international harmonization on this point either. Based on the same theoretical grounds as put forward by the Dutch Health Council, other extrapolation models (with or without low-dose linear behaviour) have been used, e.g. the linearized Multistage model (LMS) by, among others, U.S. Agencies (US.EPA, 1986; CPSC,1992). This LMS and most of the other models make use of the entire dose-response curve in the observable region. In many instances all these models give a good fit to the observed response-curve, but result in VSD estimates that may vary by orders of magnitude (Cothern, 1986). Recognizing this uncertainty (and the inability to verify low-dose risks), and because of intrinsic difficulties of the LMS (Lovell and Thomas, 1996; ECETOC, 1996), U.S.EPA has recently revised its cancer risk guidelines in a way more resembling the Dutch approach (U.S.EPA, 1996): an LED_{10} (95% Lower confidence value of the effective dose inducing a 10% extra tumour incidence) is first deduced by curve fitting of the observed dose-response, which is subsequently taken as a point of departure for linear extrapolation to zero dose.

Apart from this international harmonisation towards linear extrapolation, the question remains whether in the specific case of B[a]P there are grounds to deviate from the conservative default methodology. In other words: are there any signs or indications that the observed tumour development in the rat bioassay is induced (or promoted) by high-dose effects of B[a]P, that are not supposed to exist, and therefore donot contribute, at actual low dose levels encountered by humans? To address this question we have, among others, examined DNA adduct formation in various organs and tissues of B[a]P exposed rats in the present study. Two main conclusions can be drawn from the results obtained (for further

details see Appendix B2). First, the dose-response relationship between administered dose and DNA adduct formation in target sites for carcinogenesis is fairly linear within the investigated 300-fold dose range of 0.1 to 30 mg B[a]P/kg bw, i.e. down to levels 30-fold below the lowest dose showing tumourigenic effects (3 mg/kg bw). Secondly, DNA adduct formation *per se* is not sufficient for (observable) tumour-induction: B[a]P-DNA adducts were observed in all examined tissues, of which only a few turned out to be target for carcinogenesis. Thus, additional physiological events appear to be needed in these tissues before B[a]P tumourigenicity becomes manifest. One candidate factor is immunosuppression as tumor promoter: B[a]P induces thymusatrophy and reduced NK cell activity, a cell population critical in immunesurveillance of cancer (de Jong *et al.,* 1999). Another candidate factor is B[a]P-induced organ-specific mitogenicity. Interestingly, cells of the two major target-organs for carcinogenesis, namely the liver and forestomach, show increased mitogenic responses upon B[a]P treatment (as outlined in the Appendices A and B1, describing the dose-range finding, and 90-day study, respectively). These are conditions which have been shown previously to induce and promote carcinogenesis (Ames *et al*., 1990, 1996). Additionally, the dose response curves for both tumour types appear to show some sublinearity. Indeed, induction of preneoplastic lesions as well as tumours in the liver of B[a]P treated rats have been observed by others, but only after additional application of potent mitogenic stimuli, i.e. partial hepatectomy and prolonged exposure to phenobarbital, a well-known non-genotoxic liver mitogen (Pereira *et al.,*1988; Glauert and Pitot, 1988).

If mitogenesis is of relevance for carcinogenesis by B[a]P, the next question will be how to address the contribution of this mitogenic factor over the entire extrapolation range in a quantitative sense, and to see whether a deviation from the linear default is justified. There is, as yet, no validated solution for this. One possibility may be to apply dose-response models that have incorporated part of the biology of carcinogenesis, including chemically-induced mitogenesis, i.e. so-called biological-based models. One example of these, often referred to, is the MVK model (for further details see Moolgavkar and Luebeck, 1990; and Appendix F). When this model is used to describe the liver tumour-incidence data of the present rat study it fits better the data when mitogenic activity - instead of mutation induction - was taken as directly proportional to dose. Low-dose extrapolation with this best fitting model resulted, however, in a VSD of 18 ng B[a]P/kg bw, i.e. about 3 times the 5 ng B[a]P/kg bw that was derived for this tumour-site via linear extrapolation (see Table 4; see also Appendix F).

Conclusion

Clearly, some basic uncertainties are inherent to the approach adopted by the HCN, as well as to several other approaches applied internationally. These uncertainties may lead to

substantially different VSD values in some cases, as with benzo[a]pyrene[3]. Unfortunately, however, we are not able to verify the behaviour of the dose-response curve at very low doses, and the risks associated with it.

5.4 Use of PBPK modeling

A different approach to both interspecies and low dose extrapolation for B[a]P, has recently been explored by Zeilmaker *et al.* (1999a,b). Based upon the various kinetic studies performed within this project on B[a]P in the rat (Lusthof *et al.*, 1993, 1994, 1996; Olling *et al.*, 1995; Klaassen *et al.*, 1996), they developed a PBPK model to describe the distribution of B[a]P, its biotransformation to genotoxic metabolites, including the formation of DNA adducts in the liver (a major target site for carcinogenesis) under chronic oral exposure conditions; a model for P450 enzyme-induction by B[a]P was also incorporated (Zeilmaker *et al.*, 1999a). DNA adducts were incorporated because they were regarded as most critical for the tumour-development, and consequently, represent the most representative internal dose-surrogate for carcinogenesis in the liver. Moreover, by using this approach the identification and detection of the ultimate carcinogenic metabolite(s) could be circumvented. In order to describe the kinetics of B[a]P and the associated DNA adduct formation accurately, evidently some simplifying assumptions had to be made (Zeilmaker *et al.*, 1999a).

This rat PBPK model was subsequently scaled to a human PBPK model, by replacing rat model parameters by their human equivalents. When unknown, human parameters were assumed to be identical as their "rat equivalents". Examples of such parameters are those which determine the rate and repair of DNA adducts in the liver. Thus, in this approach humans were assumed to be as sensitive for DNA adducts in the liver as animals. Under these conditions the PBPK model results in a VSD that is substantially lower than the VSD as derived above, based on the administered dose. The reason is that, at the same administered dose, the PBPK model predicts a higher accumulation of DNA adducts in the human liver than in the rat liver. This result, however, has to be interpreted with caution. Sensitivity analysis showed that the PBPK calculation of the VSD is quite sensitive for the assumptions made on DNA adduct formation in the human liver. It is therefore concluded that, until the uncertainty which is inherent to the model assumptions is not substantially

[3] To illustrate this: US.EPA (1997) has derived at a so-called oral slope factor for B[a]P of 7.3 $(mg/kg\ bw,day)^{-1}$ based on animal studies of Neal and Rigdon (1967), and Brune (1983). From this a VSD can be calculated via linear extrapolation to be 0.14 ng/kg bw, quite different from the values presented here (or in the ICD on PAH in 1989 by Slooff *et al.*); this is due to differences in: i) animal data selected for extrapolation, ii) dose scaling, iii) other correction factors, and iv) using a 95% confidence limit, instead of a mean estimate.

reduced, PBPK modeling will not form an alternative for the more classical linear default of deriving a VSD.

Conclusion

The currently available database on B[a]P that can be usefully applied for PBPK modelling is too small to provide a serious alternative to the current approach of deriving a VSD for B[a]P.

5.5 VSD for dietary PAH

One of the ultimate goals of the rat bioassay described in this report was to provide a VSD for dietary exposure of humans to PAH. In the absence of effect-data for this route in humans, a VSD is derived for B[a]P alone, and, subsequently, a correction factor is applied to compensate for the contribution to the carcinogenicity by other PAH present in the human diet (like the approach taken by Sloof *et al.* (1989) in their ICD on PAH). This paragraph will shortly discuss this approach. In parallel to this, the results of the first animal studies examining the carcinogenic effects of dietary exposure to specific PAH mixtures, i.e. diets mixed with coal tars will be discussed (Culp *et al*., 1998).

As an alternative to the above mentioned approach this paragraph will also briefly discuss the use of existing human cancer risk data on PAH for a different routeof exposure (inhalation), to derive a VSD for oral exposure to PAH by combining the associated VSD and a route-to-route extrapolation.

5.5.1 Use of animal data

In order to derive a VSD for PAHs in the human diet based on the data on B[a]P several steps have to be made. First of all, data on the composition of PAHs in the Dutch diet should be listed, focussing on those PAHs considered relevant with respect to the carcinogenicity of this mixture. Subsequently, the relative contribution of B[a]P to the carcinogenicity of these PAH should be determined to define the size of the correction factor. This will be outlined below. More information is found in Appendices C-E.

5.5.1.1 Concentration and carcinogenicity of PAH in Dutch diet

Table 4 shows that the current state of knowledge on the presence of carcinogenic PAH in the diet of the Dutch population is rather poor. The available data are those published by Vaessen *et al.* (1988), and de Vos *et al.* (1990) (i.e. the first two columns of Table 4), and stem from the period 1976-1986. The PAH considered in the ICD by Slooff *et*

al. (1989) are all estimated to be of a relatively low carcinogenic potency as compared to B[a]P. Those PAH considered to be relatively potent and of which the levels were determined (e.g. DB[ah]A, DB[ah]Py, and 3-MC), all appear to have levels not exceeding those of B[a]P. This probably also holds for the assumed potent carcinogen DB[al]Py, if the other two examined dibenzopyrenes, i.e. DB[ah]Py and DB[ai]Py, may be taken as concentration-indicators for these compounds. Thus, B[a]P may still be considered one of the more relevant PAH with respect to its contribution to the overall carcinogenicity of dietary PAH. One should realize, though, that the available data on relative carcinogenic potencies of the various PAH are all derived from quite diverse experimental designs, and notably, mainly concern non-oral exposure routes.

5.5.1.2 The size of the correction factor

The data on the dietary composition of carcinogenic PAH demonstrates that it is not possible to apply a Toxic Equivalency Factor (TEF) approach here: i.e. to calculate the overall carcinogenic potency of dietary PAH by summing their individual contributions (and assuming these to be additive; US.EPA, 1986b; Nisbet and Lagoy, 1992). Instead, one could take a conservative correction factor to account for the contribution of other PAH in the diet (as did Sloof *et al.*, 1989). The proposed factor of 10 (by Slooff *et al.*, 1989) still seems a reasonable first guess if one makes the following assumptions:

- the contribution of equipotent or more potent PAH will sum up to about 5 times that of B[a]P, and,
- that of PAH compounds of intermediate potency (between 0.1 and 1.0 times the potency of B[a]P) will together contribute to about 2 times that of B[a]P.

It would be desirable, though, if this factor could be validated; e.g. by comparing the carcinogenic responses in mice exposed to B[a]P and coal tars, respectively, as recently published by Culp *et al.* (1998; see next paragraph).

5.5.1.3 Use of coal tar studies in mice

It would be ideal to expose animals to an average dietary PAH profile to derive a VSD for PAH in the human diet. Such an approach is impracticable. However, recently an animal study was published examining the carcinogenic effects of orally applied PAH mixtures, namely coal tars (Culp *et al.*, 1998). Based on this study, verification of the oral carcinogenic potency of PAH mixtures relative to B[a]P is possible, to a certain extent. Culp *et al.* (1998) exposed female B6C3F1 mice via the diet to either B[a]P or one of two coal tar mixtures for 2 years (see also Appendix C). It appears that the cancer potency of the coal tar mixtures investigated in these mice is about 2 - 5 times that expected from B[a]P alone:

VSDs for B[a]P are around 5 ng/kg bw, whereas those for the coal tar mixtures range from 1 – 3 ng B[a]P per kg bw (see Appendix C). Thus, these estimates are smaller than the above suggested value of 10 for the correction factor. However, a number of comments need to be made. First of all, one would like to have comparable profiles of (potent) carcinogenic PAH in coal tar and human diet for this purpose. As shown in Table 4, a judgement about this cannot be made yet, due to a shortage of information on levels of relatively potent PAH in both (last two columns).

Secondly, an additional complicating factor is the difference in target-site profile in this mouse strain between coal tars and B[a]P: B[a]P induces tumours in the forestomach, oesophagus and tongue, whereas exposure to the coal tar mixtures leads to tumours in lung, liver, forestomach, and small intestine, but also to sarcomas in multiple organs, e.g. hemangiosarcomas and histiocytic sarcomas. Thus, coal tar mixtures cannot simply be regarded as a sum of "B[a]P- equivalents" . One could state inversely that B[a]P apparently is not an ideal reference chemical for coal tars because constituents other than B[a]P (non-PAH?) might contribute in a critical way.

Finally, the factor expressing the indicator role of B[a]P for coal tars, here estimated from the VSDs to be 2 to 5, intrinsically contains a lot of uncertainty if the shape of the dose-response curves for the relevant tumour differ greatly (i.e. for B[a]P alone, or for coal tar). This is because the points of departure for linear extrapolation, i.e. the TD_{sign} (to arrive at a VSD), that is critical for the determination of this factor, are arbitrarily chosen during the design of the study. One may circumvent this approach by comparing B[a]P doses of exposures that result in comparable tumour incidences. This leads to estimates for the correction factor in the order of 3 to 4.Those values do not exceed the suggested factor of 10 (see paragraph 5.5.2).

Table 4. Relative concentration and relative estimated carcinogenic potency of dietary PAH as compared to B[a]P (B[a]P set at ≡ 1.0). The relative potency was ordened into 4 groups based on the results of various carcinogenicity tests as published in the literature (see Appendix G). Residual PAH not shown here (but listed in Appendix G) are of an estimated potency of at least two orders lower than that of B[a]P.

PAH[1]	Vaessen *et al.*, 1988[2]	De Vos *et al.*, 1990[3]	others[4-7]	Culp *et al., 1998*[8] CT 1	CT 2
group 1: potency (probably) > B[a]P[9]					
B[j]AA					
DB[ah]A	0.5		0.08- 0.5[5-7]	0.1	0.1
DB[ah]Py	<0.38[15]				
DB[al]Py					
3-MC		0.8			
5-MCH					
group 2: 1/10 B[a]P < potency ≤ B[a]P[9]					
An					
B[e]AA					
B[l]AA					
B[b]F	'5.3'[10]	2.6		1.1	1
B[rst]pp (?)[11]					
CPP (?)					
7H-DB[cg]C (?)					
6-NCH[12]					
DNF's (?)					
DNPy's[13] (?)					
group 3: potency ≤ 1/10 B[a]P[9]					
IP	2	0.7		0.7	0.7
B[a]A	2	1.7		1.3	1.2
B[k]F	'0.1'[10]	0.8		0.4	0.4
CH	15	7.2		1.3	1.1
B[ghi]Pe	2	1.7		0.8	0.8
B[j]F	'2.1'[10]				
DB[aj]A		4.5		0.18[14]	
DB[ah]Ac					
DB[aj]Ac					
DB[ae]F					
DB[ae]Py		0.08			
DB[ai]Py	<0.38[15]				
DB[el]Py (?)					
2-MCH					
1-NP					
2-NF					
DNPy 1,3-					
group 4: potency ≤ 1/100 B[a]P[9]					
F	34	8.3	6.8[4]	2.7	2.3

Legend to Table 4 (previous page):
[1]) PAH shown in **bold** are those selected by Slooff *et al.* in their Integrated Criteria Document (1989); For abbreviations see Appendix G; If not depicted in the Table PAH is considered hardly if at all carcinogenic (e.g. **Ph**, **A**, and **N**); [2]) Analysed 50 duplicate diets in Dutch home settings for 15 PAH; these 50 were selected from 311 samples because of their expected "elevated" PAH-levels; [3]) Analysed 10 market baskets (23 food commodity groups) of 18 year old youngsters for 17 PAH: 'not detected' was taken as zero; [4]) Analysed diet exposure of general population via ad 3. and VCP (1990): 'not detected' was taken as half detection limit; between brackets: figure represents 5-percentiles of population with low and high PAH exposures; [5]) Modified from results by Dennis MJ, *et al.* (1983); [6]) Modified from results by Lodovici M, *et al.* (1995); [7]) Turrio-Baldassarri *et al.* (1996); [8]) Modified from analytical data by Culp *et al.* (1998), CT 1:coal tar mixture, CT 2: coal tar mixture 2; [9]) The potency ordening is based on the results of various carcinogenicity tests as published in the literature (see Appendix E). [10]) figure calculated from sum of {B[k]F, B[b]F, and B[j]F} (see Table 2 Appendix D) based on their ratio within environmental compartments which appears to be fairly constant: 1, 70 & 29%, respectively (van Velze, 1996); [11]) ?: belonging to this potency group?: based on only one test or mean of various tests showing wide variation in results; [12]) *italics*: indicates heterocyclic PAH; [13]) i.e. 1,6- and 1,8-DNPy's; [14]) Modified from results presented by Muller *et al* (1997): Table C311221, page C-7; [15]) actually not detectable; detection limit was 30 ng per kg;

Conclusion

In order to obtain a VSD for dietary PAH the currently available data do not offer an alternative to the approach outlined by Slooff *et al.* in 1989. So the most appropiate solution is to take the oral VSD for B[a]P of the most sensitive site/species combination, and use a correction factor to compensate for the contribution of the other carcinogenic PAH compounds. A factor of 10 appears to be justified as an educated first guess. The VSD for B[a]P as indicator for dietary PAH, therefore, is suggested to amount to 0.5 ng/ kg body weight, or 35 ng B[a]P per person (taking a mean body weight of 70 kg).

5.5.2 Use of human data on cancer risks associated with inhalatory exposure to PAH

As an alternative to the above mentioned approaches based on animal data, one could apply available human cancer risk data for the inhalatory route to derive a VSD for dietary PAH, namely by combining the VSD for inhalatory exposure to PAH with a route-to-route extrapolation. The obvious advantage of this is the absence of a need for an interspecies extrapolation step with its implicit uncertainties. The VSD for inhalatory exposure to PAH is determined to be 0.01 ng B[a]P/m^3, in which B[a]P is used as indicator for all PAH present (Slooff *et al*., 1989). This figure is a mean of estimates from three epidemiological studies in which PAH exposures were found in association with increased incidences of lung tumours. Using the average daily volume inhaled, i.e. 18 m^3 (HCN, 1995), this amounts to a daily dose

of 0.18 ng B[a]P (as PAH indicator). If one assumes that all PAH inhaled this way are absorbed (which is the default position in the absence of further data on this: HCN, 1995), a VSD of 0.18 ng B[a]P (as PAH indicator) is obtained, which is clearly two orders of magnitude below the VSD for the oral route derived above from animal data: 35 ng B[a]P (as PAH indicator). From this, one might conclude that the animal data result in a clear underestimation of human cancer risks from exposure to dietary PAH. However, in interpreting these estimates the following critical points should be recognized.

First of all, the 0.01 ng B[a]P/m^3 estimate provided by Slooff *et al*. (1989) is a mean of risk estimates obtained from three different epidemiological studies, two of which are 95% upper confidence limits (UCL) on the mean risk estimate; one of these UCL values amounts to even 15.6 times the mean estimate (US.EPA, 1984). Note that the VSD for oral exposure derived from the above presented animal data is a mean estimate. Next, implicit in the derivation of risk estimates for inhalatory exposure is the scaling according to caloric demands: by taking air concentrations as dose-measure, body burdens are dependent upon inhalation rates. Note that for oral exposure in the above outlined VSD from animal data, the dose-measure is amount per unit body weight. When adjusting for this (extrapolating from rats to man) an additional scaling factor of 4, i.e. resulting in a 4-fold smaller VSD value, would be obtained. Finally, there is some suspicion that factors in addition to PAH, e.g. particulates and irritative gases, may have contributed to the carcinogenic effects observed in these epidemiological studies, as is clearly shown in experimental animals (Gallagher *et al*., 1994; Nikula *et al*., 1995; Morrow *et al*., 1996; Dasenbrock *et al*., 1996). Thus, when correcting for these factors, the difference in VSD values for exposure to dietary PAH as derived above when starting from either animal or human data may not be that big anymore.

In the end, though, there still may be a difference in carcinogenic potency for PAH when entering the body via different routes. When comparing the cancer potency of B[a]P (and other PAH compounds) in experimental animals, quite different values are obtained for the different routes. PAH compounds appear the least potent upon oral exposure, i.e. on average about two orders of magnitude lower when compared to inhalatory exposure (Slooff *et al*., 1989; Muller *et al*., 1997). Such a route-specific difference in carcinogenic potency apparently also applies to PAH mixtures tested, i.e. to coal tars (Heinrich *et al*., 1994; Culp *et al*., 1998).

Conclusion

When starting from human cancer risk data on inhalatory exposure to PAH to derive a VSD for oral exposure (and thus avoiding an interspecies extrapolation step), a lower VSD estimate is obtained (i.e. up to around one order of magnitude, after correction for some factors) as when starting from animal data on oral exposure to B[a]P (or PAH). A

qualitatively similar route-dependent difference in cancer potency by PAH is found in animal experiments. Therefore, these data are taken to support the proposed VSD based on animal data.

5.6 Cancer risks associated with PAH exposure via the diet in The Netherlands

Another goal of the rat bioassay described in this report was to provide an estimation of the cancer risks associated with the actually experienced daily exposure to dietary PAH in the Netherlands. In this paragraph we will briefly discuss the risk estimates associated with this exposure as well as those exposure situations that appear not acceptable from a health perspective, i.e. situations that ask for intervening action by the Inspectorate.

5.6.1 Distribution of cancer risks due to exposure to dietary PAH

In chapter 4 cancer risks associated with exposure to dietary PAH are estimated at 2 to 5 per million people, i.e. in the order of the so-called negligible risk level of one per million. In this calculation the VSD for B[a]P as PAH indicator (38 ng B[a]P per person per day), and the measured/calculated average daily B[a]P intakes in the Dutch population (from 80 to 205 ng B[a]P) were combined. This risk estimate, as well as the calculated distribution of cancer risks for the Dutch population, deserve some comments (see also Appendix D for further comments).

The average B[a]P intake values basically originate from only two different and independently performed small-scale studies by Vaessen *et al.* (1988), and de Vos *et al.* (1990). These values nicely compare to the outcome of a different approach by Heisterkamp and van Veen (1997), i.e. by applying results of a national food survey: VCP (1990). As these intake values are also in rather good agreement with internationally reported measurements, at least some support can be given to their outcome. It should be noticed, though, that these Dutch studies were performed in the period 1976-1986. It may well be that both food items and consumptions patterns have changed since then.

The highest estimated risk of 5 per million relates to the calculated intake of 205 ng B[a]P per day (i.e. by using VCP, 1990). Because all food items were considered here to contain PAH (those with undetectable levels were included with levels half the detection-limit) this approach may be regarded as a "worst case approach".

The risk estimates given hold for the population as a whole, i.e. represent the mean risk for the Dutch population. Individual risks due to exposure to dietary PAH may be substantially higher (or lower), depending on the personal food patterns. The calculated

distribution of individual long-term PAH consumptions is rather small, i.e. having the 5th and 95th percentiles within a factor of 2 from the mean exposure, but this will most probably be an underestimate of the unknown, true dispersion[4] (as already noted in chapter 4). Individual risks also critically depend on an unknown variability in sensitivity, that may be quite substantial.

5.6.2 Exposure situations that may require intervention by the Inspectorate

From the preceeding paragraphs it is clear that cancer risks from ingested PAH in the Netherlands are estimated to average slightly above the so-called negligible risk level of one per million. Next, some guidance is required for situations in which B[a]P (or PAH) levels clearly exceed these negligible risk levels, i.e exposure situations that may ask for action (e.g. intervention) by the Inspectorate. We will briefly comment on this.

Two points should be discerned when an exposure situation has to be judged as unacceptable from a health point of view. First of all, risk levels should be established that are considered unacceptable. This clearly is a policy decision. For example, within environmental health policy a risk level of 10^{-6} per year is considered maximally permissible (reduction to a negligible risk level of 10^{-8} per year is pursued, if possible; VROM, 1997). Secondly, the risk associated with the exposure that exceeds the VSD should be calculated. The methodology adopted by the Health Council (HCN, 1995, 1996) also gives guidance on how to estimate cancer risks under conditions of non-chronic exposures that exceed the VSD. It is implicitly assumed that the risk associated with a high dose of a carcinogen received over a shorter period of time is equivalent to the risk associated with a corresponding low dose spread over a lifetime, or in other words: the cumulative dose received over a lifetime is assumed to be an appropriate default measure of exposure to a carcinogen and the associated risks (HCN, 1996). Thus, one may estimate this extra cumulative dose people will be exposed to during the period the VSD is exceeded and compare it to the cumulative dose resulting from a lifetime exposure to the "regular" VSD of 0.5 ng B[a]P per kg body weight daily (as PAH indicator), to arrive at the extra cancer risk. Taking this into account short-term exposures in excess of the VSD will in most cases not result in substancial increases in risk: e.g. an exposure of 100 times the VSD (i.e. daily dietary exposure to 50 ng B[a]P per kg body weight

[4] It should be noted that individuals may not be always at the top of the distribution (i.e. representing those highly exposed): some may be during their teenages, but not as adult, or vice versa. The available database, unfortunately, does not include information of the same subjects through the years. Also important to recognize is that the applied software is not yet capable of identifying products that have a major contribution to the average daily PAH burden via diet.

as PAH indicator) for half a year not even results in a doubling of the negligible lifetime cancer risk for this chemical[5]. In the case of short exposures to very large excesses of the VSD (so-called “peak” exposures), one may - in the absence of additional relevant information on this chemical - consider the use of an extra safety factor of 10 to the estimated 'extra' lifetime risk, as proposed by a HCN report (HCN, 1994a). The application of this factor in case of 'peak' exposures to PAH, should be based on expert judgement of the specific exposure situation.

Another way of estimating risks associated with exposures exceeding the VSD might be approached as follows. The accurateness of a risk estimation in this situation can be increased by reassessing the data on exposure and dose response. With respect to this latter point it is recognized that the adopted methodology for deriving a VSD may overestimate risks at low doses in certain cases (HCN, 1995, 1996). As outlined in paragraph 5.3.2, some data support a sublinear behaviour of the dose response curve for B[a]P. Evidence for this also directly stems from an analysis of the observed dose-response relationship for liver tumours. If the next lower dose to the TD_{sign}, i.e. 3 mg/kg bw, is chosen for linear extrapolation (by the way as outlined in Appendix F), a VSD of 9 ng B[a]P is derived.Thus, neither this alternative approach, nor the MVK approach (that resulted in a VSD of 17.5 ng/kg bw: see Appendix F) result in a substantially different VSD value.

Conclusion

When based on the VSD obtained by methodology adopted by the HCN, estimation of the cancer risks associated with daily exposures to dietary PAH in the Netherlands are slightly above one per million incidence level, and thus can be considered negligible. It should be noted, however, that the exposure data might not be applicable for current exposures, as they stem from the period 1976-1986.

For the situation that exposure levels substantially exceed the VSD, it has been shown that short-term exposures in most cases are not expected to result in substancial increases in lifetime cancer risk.

[5] To illustrate this: an exposure of 100 times the VSD for half a year is (100 x 0.5 / 1 x 75) = 0.67 times the negligible risk, or these people experience a lifetime risk of $1{,}67\ 10^{-6}$, when lifetime is taken as 75 years.

6. CONCLUSIONS

Based on the available animal data it is concluded that the oral VSD for B[a]P is 5 ng/kg body weight. It should, however, be emphasized that the approach applied to estimate the VSD is not without uncertainties. However, these uncertainties are not specifically associated with B[a]P, but adhere to all VSDs for genotoxic carcinogens derived from animal experiments as the only data source. With B[a]P the animal results among themselves, in fact, are remarkably consistent.

It is concluded that when B[a]P is used as an indicator for other dietary PAH, a VSD of 0.5 ng/kg bw is justified, by applying a factor of 10 to the VSD for B[a]P. The reason for applying a (correction) factor is because the database on dietary PAH considered relevant with respect to carcinogenicity still is rather poor, both with respect to dietary concentrations as to potential carcinogenicity. A VSD, therefore, cannot be calculated directly from this specific PAH profile.

In the Netherlands exposure to dietary PAH has not been determined since the period 1976-1986. The average daily B[a]P intake in this period was estimated/calculated to range from 80 to 205 ng. By applying the presently derived VSD for B[a]P (as PAH indicator) an average population cancer risk of 2 to 5 per million was estimated to be associated with exposure to dietary PAH.

Finally, it is shown that the presently adopted methodology for estimating cancer risks implies that increases in cancer risks above the negligible level of one per million lifetime exposed persons can be achieved only by prolonged periods of substantial excesses of the VSD.

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APPENDICES

The following Appendices provide details concerning the range-finding study (A), the carcinogenicity study (B), and other background information considered not directly relevant to risk managers primarily involved in application of the outcome of this project (these details were for that reason not included in the main text). Still, it will be confined to those data judged by the authors to be relevant to interested readers; more information and raw data are available within the files of this project that will be archived for **7** years at the Institute.

APPENDICES

Study related

A Range-finding study

B Carcinogenicity study

- B1 90-day study
- B2 DNA adduct analysis study

Miscellaneous

C NCTR carcinogenicity study in mice (Culp *et al.,* 1998)

D Exposure of the general Dutch population to PAH in the diet

E Methods for calculating cancer risks associated with PAH in Dutch diet

F Alternative VSD calculations

G Carcinogenic potency of PAH relative to B[a]P (Tables)

APPENDIX A Range-finding study

Preface

1. Experimental design
2. Results & comments
3. Conclusion on dose-levels for the carcinogenicity study

Range-finding study

Preface

This Appendix will provide details concerning the experimental design of the range-finding study and the safety measures taken to ensure minimization of potential risks for personnel and environment. Also, the results will be presented together with some brief comments. More information and raw data are available within the files of this project.

1. Experimental design

1.1 *Animal housing, treatment, monitoring and necropsy*

SPF Riv:TOX rats of the Wistar strain were used. They were bred at and derived from the Animal Facility at the Institute. At the age of 4-5 weeks they were shipped to the animal rooms for acclimatization one week before the start of treatments. They were *ad random* assigned to the various dose groups by standardized procedures. The animals were housed in macrolon cages with a wire floor, two per cage. Animals were identifiable by tail staining and cagenumber. Aimed housing temperature and relative humidity were 19 - 25 °C and 40 - 72%, respectively. Food (SSP-Tox, Hope Farms BV, Woerden) and tapwater (public drinkingwater, WMN, Utrecht) were supplied *ad libitum*. From the start of treatment the applied food contained a reduced amount of soy oil to compensate for the soy oil used to administer the test chemical (see below). Analyses of food and water for contaminants were performed according to standard procedures. Additionally, sentinel animals were used for monitoring the animal's microbial health status.
Groups of 10 animals (per dose, per sex) were administered B[a]P (dissolved in soy oil) by gavage 5 days a week at dose levels of 1.5, 5, 15 or 50 mg B[a]P/kg bw. Concurrent controls only received the vehicle. The dosing of animals was stopped one day preceeding their sacrifice, and was on the basis of mean group weights (per dose, per sex). The animals were examined daily for behaviour and clinical symptoms. Body weights, food- and water-consumption were recorded weekly. During the sixth week of exposure the animals were killed by exsanguination under ether anesthesia and blood, urine and tissues were taken for investigations on standard toxicological parameters, i.e. for haematology (Hb, Ht, RBC, MCV, MHC, MCHC, WBC, PLT), urinalysis (pH, protein, glucose, ketone, bilirubin, blood, nitrate, and urobilinogen; all by reagent strips, N-Multistix SG, Ames Division, Miles Nederland), clinico-chemical analysis (GGT, ASAT, ALAT, LDH, and kreatinine), and for organ weights (liver, lung, thymus, spleen, kidneys, adrenals, and ovaria). Also, liver enzyme induction was monitored by EROD (ethoxyresurfin-O-deethylase) activity in plasma. All animals were subjected to macroscopical examination and tissue samples were processed for further histopathological investigations.

1.2 *Testagent*

B[a]P was from Janssen Chimica (Beerse, Belgium), which had a purity of 97.7 %. The claimed purity was verified upon receipt at the Institute by the Laboratory of Organic Chemistry (Analytical Residue Research section). B[a]P was dissolved in soy oil (De Oliehoorn BV, Zwaag) under heating (< 50°C) and ultrasonic vibration. Fresh solutions were prepared weekly. The stability of B[a]P under conditions of dissolution and storage (once

dissolved) was verified. Due to several corrections (e.g. purity) actually achieved dose levels were a few percent below those targeted.

1.3 *Mode of application*

B[a]P was dissolved in soy oil in volumes of 0.4 and 0.5 ml per female and male rat, respectively. B[a]P was dissolvable in soy oil up to ≈ 30 g/l. The length of the gavage needle used assured exposure of (at least the caudal part of) the oesophagus (a known target-site for carcinogenesis). Application was always in the morning for all groups and took about 0.5 -1 hour.

1.4 *Safety measures*

B[a]P is a recognized animal carcinogen, and presumed to be a human carcinogen, i.e. classified by IARC into group 2A (IARC, 1983). In order to avoid contamination of the animal rooms with B[a]P and its metabolites, to protect personnel, and to avoid exposure of the animals via other routes, several measures were taken. First, all personnel allowed to enter the animal rooms were commissioned to wear special clothing (including haircap), gloves, and a compressed-air-mask. Animal rooms were at underpressure relative to the entrance-room to avoid leakage of B[a]P (and metabolites) to the outside. B[a]P was applied by gavage and all animal excreta were collected on moistened paper that was removed the first thing in the morning. By the end of the day the animal rooms were cleaned using a water-hoover. Air-samples were taken in the animal rooms during several days in the first and second week of exposure, and the (airdust-)filters were analysed for B[a]P content. As these analyses revealed B[a]P concentrations within the animal rooms to be only slightly above outdoor air concentrations, it was decided that the compressed-air masks could be replaced by (more comfortable) standard mouth caps. Clothing prescriptions also remained in force during autopsy; these animals were not dosed the preceeding day. Taking a shower was an obligatory step in the procedure for leaving the animal room area.

2. Results & comments

2.1 *Survival*

None of the animals died within the 5-week treatment period.

2.2 *Body weight*

There were no differences in body weight development between controls and B[a]P-treated animals within the 5-week period (data not shown).

2.3 *Food- and water consumption*

The results on daily food- and waterconsumption are given in Table 1 (note this is per cage, i.e. per two animals). There are statistically significant, though small differences (i.e. < 10%) between the various treatment groups and controls. An increase in food consumption with treatment was observed for females, whereas for males a decrease was found. Whereas treatment did not affect water-consumption by females, it did reduce consumption by males (except for the highest dose), which was about 12% in excess over control value.

There are no explanations for the observed changes in food- and water consumption.

Table 1. Food- and waterconsumption of animals treated with B[a]P. Data represent mean ± SEM for 10 animals over the 5-week period. Note: all values are per cage, i.e. for two animals together.

	females		males	
(g/day) dose (g/kgbw)	food	water	Food	water
0	23.3 ± 0.2	35.1 ± 0.5	32.8 ± 0.3	41.6 ± 0.4
1.5	23.3 ± 0.2	34.4 ± 0.3	30.4 ± 0.3*	39.1 ± 0.2*
5	24.3 ± 0.2*	34.6 ±0.3	29.7 ± 0.4*	37.2 ± 0.2*
15	24.2 ± 0.2*	34.9 ±0.3	30.1 ± 0.3*	38.8 ± 0.2*
50	25.5 ± 0.3*	35.5 ±0.4	31.2 ± 0.4*	48.1 ± 0.5*

Statistical significant differences (at <0.05 level) compared to control values: *) <0.01

2.4 *Organ weights*

B[a]P treatment did not have any significant effects on organ-weights of lung, spleen, kidneys, adrenals, ovaria, and testis (data not shown). A reduction in thymus weight and an increase in liver weight was observed (see Table 2).

Table 2. Effect B[a]P treatment on organ-weights of thymus and liver. Data represent mean ± SEM in milligrams (thymus) or grams (liver) for 10 animals.

	females		males	
dose (g/kgbw)	thymus	liver	Thymus	liver
0	326 ± 12	4.28 ± 0.11	471 ± 19	6.10 ± 0.26
1.5	367 ± 23	4.40 ± 0.73	434 ± 20	6.19 ± 0.19
5	351 ± 25	4.37 ± 0.11	418 ± 26	6.13 ± 0.10
15	317 ± 30	4.67 ± 0.17	342 ± 20*	6.30 ± 0.14
50	271 ± 16*	5.03 ± 0.15*	317 ± 21*	7.20 ± 0.18*

Statistical significant differences (at <0.05 level) compared to control values: *) <0.01

2.5 *Haematology*

Apart from some non-statistical, small, dose-related decreases in Hb (both sexes), and RBC counts (males), B[a]P treatment did not have any significant effects on investigated haematological parameters.

2.6 *Clinical chemistry*

B[a]P treatment did not have any significant effects on GGT, ASAT, and ALAT values. The observation of slight haemolysis within a substantial number of samples paralleled the variably increased LDH values. A small increase with treatment was observed on creatinine levels in males only (see Table 3).

Table 3. Effect B[a]P treatment on creatinine levels in blood. Data represent mean ± SEM (in U/l) for 10 animals.

dose (g/kgbw)	females	males
0	53 ± 6	45 ± 0.3
1.5	49 ± 0.3	47 ± 0.6*
5	51 ± 0.4	49 ± 0.3*
15	47 ± 0.4	48 ± 0.4*
50	51 ± 1	51 ± 0.5*

Statistical significant differences (at <0.05 level) compared to control values: *) P<0.01

2.7 *Urine analysis*

Of the urinalysis parameters investigated, B[a]P treatment only induced some increase in nitrite concentration in females.

2.8 *Liver enzyme induction (EROD)*

B[a]P treatment had a very pronounced effect on liver microsomal EROD activity (as determined by Burke and Mayer; see Figure 1). The induction level at a dose of 50 mg/kg bw appears to be the maximal achievable in male rats (under the present treatment conditions); a level which is about 36 times control values. Induction was already apparent (i.e. about 5 times increase) at the lowest tested dose of 1.5 mg/kg bw. In females the highest induction level appeared not to be reached at 50 mg/kg bw. In the absence of B[a]P treatment (during the weekends) EROD activity appeared to rapidly decline to almost control values, indicating the dynamics of the induction mechanism (see Table 4).

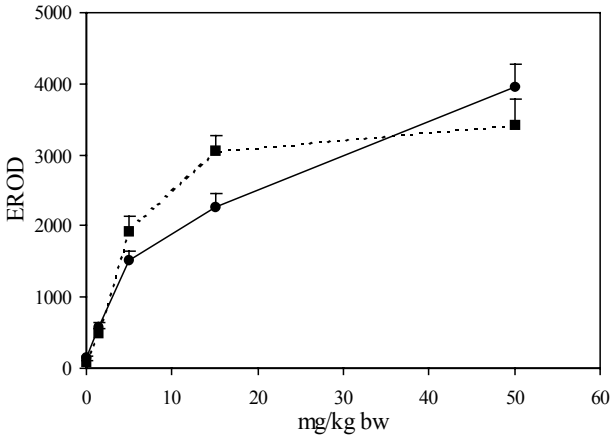

Figure 1. Effect of B[a]P treatment on liver EROD activity (as nmol/min,gr protein); males (■), females (●). Values are mean ± SEM of 8 animals, sacrificed two by two a day during the last week of B[a]P treatment; animals sacrificed on monday (which had their last B[a]P dose on the preceding friday) were excluded.

Table 4. Effect of B[a]P treatment on EROD activity. Data represent mean for 2 animals. Values shown are from the 6th and last week of treatment: Monday ('mon'), Tuesday ('tue'), and Friday ("fri"), respectively[1]. EROD activity is expressed as (nmol/min,gr protein). Note:values shown are from different animals.

	females				males			
dose[2]	control	Fri	mon	tue	control	fri	mon	tue
0	150				94			
1.5		552	154	605		455	155	580
5		1421	375	1991		2482	254	1471
15		2963	318	2211		2702	422	3366
50		3254	429	3338		2504	516	4192

[1]) note that "mon" represents the activity after 2 days without treatment, "tue"one day after the first treatment day of the week, and 'fri' after 4 days of treatment, respectively; [2]) dose in mg/kg bw, 5 days a week.

2.9 *Pathological findings*

In life observations

No effects were observed during this 5-week treatment period, either on behaviour or upon handling.

Necropsy

No treatment-related effects could be discerned after the 5-week B[a]P exposure period. Occasionally, some common background changes were obeserved; single cases of petechiae at the thymus, hydronephrosis, and a dilated uterus

Histopathology

All animals of the highest dose groups and controls were histopathologically examined for their oesophagus, stomach, duodenum, liver, kidneys, spleen, thymus, lung and mammary gland (females only). In case of abnormalities the intermediate dose-groups were examined additionally. The observed changes are described below.

Forestomach. Attention was paid to the forestomach since it is a known target-organ for carcinogenesis by B[a]P, especially after gavage administration. Small hyperplastic responses were observed in the basal layer of the epithelium, without any pattern and to a variable extent. This response was characterized by an increased number of cells with an oval rather than rounded nucleus and oriented perpendiculary to the basementmembrane, or cells clearly showing hyperthrophy. Also, the regular occurrence of mitotic figures in the basal as well as suprabasal layer, vacuolization within and around the nucleus, nuclear polymorphy, atypia, and eosinophilic nucleoli reflected this mitotic response. Furthermore, the border with the lamina propria sometimes was irregular and not distinct due to nests of proliferating epithelial cells and/or influx of predominantly mononucleated inflammatory cells. If two of these criteria were met, the changes were considered 'slight', and they were regarded as significant within the context of the relatively short exposure period (as compared to the commonly used 90-day subchronic study). Table 5 shows the dose-response for these changes, with 15 mg/kg bw as a marginal effect dose (but statistically significant).

Liver. No clear treatment-related changes were observed in this organ. Therefore, intermediate dose-groups were not examined.

Table 5. Effect B[a]P treatment on basal cell hyperplasia in forestomach. Data represent number of animals showing some degree of hyperplastic response: '0', no; '±', 'very slight'; and '+', 'slight'. Groups consisted of 10 animals each.

dose (mg/kgbw)	females			males		
	0	±	+	0	±	+
0	8	2	0	5	4	1
1.5	5	4	1	5	4	1
5	8	1	1	2	4	4
15	3	4	3*	3	4	3
50	1	2	+7**	2	1	7

Statistically significant differences (at <0.05 level) compared to control values: *) <0.05 **) <0.01

Oesophagus. Inflammatory lesions, muscle regeneration, and atrophy were observed in a number of cases. As these are unusual, and show no relation with B[a]P treatment, it is assumed that they are secondary to trauma by gavage application. Local mucosa appeared normal, probably due to a higher regerative capability.

Thymus. An increased incidence of brown pigmentation of red pulp (hemosiderin) was observed in treated animals of both sexes. Because of the very small difference (as compared to controls), and the absence of any further support for this (e.g. hematological changes) intermediate groups were not examined.

3. Conclusion on dose-levels for the carcinogenicity study

The dose levels applied in this range-finding study did not induce notable toxicity within the period of treatment. With regard to the effects induced, i.e. basal cell hyperplasia in the forestomach as well as the effects upon liver- and thymus-weight, a dose of 50 (mg/kg bw,day) seemed to be somewhat too high for a chronic treatment. Also, it is important to recognize that sustained proliferation in the forestomach should be excluded, as this may lead to tumours at this site, and consequently undesired premature loss of animals, and thereby possibly masking other tumour-sites considered of higher human relevance. As this range-finding study was of relatively short duration (i.e. compared to the normally used 3-months subchronic study period) we decided to verify this forestomach proliferation using the BrdU-incorporation technique: this study essentially showed similar results[6].

[6] Female rats (8 per dose) were treated as described (at 5, 15, and 50 mg/kg bw). One hour prior to scheduled kill the animals were administered BrdU i.p.(in saline, 100 mg/kg bw), and forestomachs were isolated as described, but subsequently injected with 70% alcohol to allow a better quantification of BrdU-incorporation by immunohistochemical techniques. Comparable results as those described in Table 6 were observed for normal histopathology, slightly increased mitotic activity at 15 and 50 mg/kg bw, respectively; BrdU incorporation was also significantly increased at 50 mg/kg bw only, as measured by the number of staining cells per stretching meter (more details in files).

As the next lower dose of 15 mg/kg bw day, on the other hand, hardly induced toxic effects, it was decided to take as the highest dose in the 104 week lasting carcinogenicity study 30 mg/kg bw day. It was anticipated that at least some effects on forestomach, liver and thymus could be expected at this dose level. The next lower dose levels were taken at reasonable intervals of this dose, i.e. at 10 and 3 mg/kg bw, day, respectively. In order to verify this expectation an additional 90-day group was incorporated and scheduled for sacrifice after 3 months.

Literature

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APPENDIX B Carcinogenicity study

Preface

1. Experimental design
2. Results
3. Discussion & Conclusion

Carcinogenicity study

Preface

This Appendix will provide further details concerning the experimental design of the carcinogenicity study and address the safety measures taken to ensure minimization of potential risks for personnel and environment. It includes the results not presented in the main text together with some brief comments. Animals used for 90-day sacrifice (Appendix B1) and DNA adduct analysis (Appendix B2) were housed, treated similarly and simultaneously. More information and raw data are available within the files of this project that will be archived for at least 8 years at the Institute.

1 Experimental design

1.1 *Animal housing, treatment, monitoring and necropsy*

SPF Riv:TOX rats of the Wistar strain were used. They were bred at and derived from the Animal Facility at the Institute. At the age of 4-5 weeks they were shipped to the animal rooms for acclimatization before the start of treatments. They were *ad random* assigned to the various dose groups by standardized procedures. The animals were housed in macrolon cages with a wire floor, two per cage. Animals were individually marked by tattooing. Housing temperature and relative humidity were 19 - 25 °C and 40 - 72%, respectively. Food (SSP-Tox, Hope Farms BV, Woerden) and tapwater (municipal drinkingwater, WMN, Utrecht) were supplied *ad libitum*. From the start of treatment the applied food had a reduced amount of soy oil to compensate for the soy oil used to administer the test chemical (standard SSP-Tox food contains 4.55% soy oil on a weight basis; see paragraph 1.3).

Groups of 74 animals (per dose, per sex) were administered B[a]P (dissolved in soy oil) by gavage, 5 days a week at dose levels of 3, 10, or 30 mg B[a]P/kg bw. Controls received the vehicle only. Of these, 52 animals (per dose, per sex) were subjected to this regime for 104 weeks, i.e. for carcinogenicity testing. The remaining animals were either subjected to an 90-day interim sacrifice (see Appendix B1) or used to study DNA adduct formation by B[a]P under these conditions (see Appendix B2) (see also scheme 1 in the main text). Dosing was on the basis of extrapolated mean group weights (per dose, per sex). Exposure started when the animals were 6 weeks of age after having acclimatized for 10 days to the housing conditions. Analyses of food and water for contaminants were performed according to standard procedures. Sentinel animals were used for monitoring the animals' microbial health status.

The animals were examined daily for behaviour and clinical symptoms and by palpation. Food and water consumption were recorded twice weekly during the first 18 weeks, and once every two weeks for the remaining period. Body weights were registered every week (week 1 - 8), every 2 weeks (week 9-18), or every 3 weeks (week 19-104), respectively.

All animals were subjected to complete necropsy after death, or intercurrent or terminal sacrifice. The rats were killed by exsanguination from the abdominal caval vein under ether narcosis. From week 32 onwards exsanguination was performed under inhalation anaesthesia with CO_2 / O_2. After macroscopic inspection the following organs were collected for further microscopic analysis: brain, pituitary, heart, thyroid, submandibular salivary glands, lungs (including trachea and infused with fixative), stomach (mounted on paraffin

wax disks after opening along curvature maior and flushing), oesophagus, duodenum, jejunum, ileum, caecum, colon, and rectum (swiss rolls), thymus, kidneys, urinary bladder (after instillation with fixative), spleen, mesenteric, axillary and mandibular lymph nodes, liver, pancreas, adrenals, sciatic nerve, biceps femoris muscle, femur (diaphysis), vertebral column and head (after flushing the nasal cavity), skin including mammary tissue (ventral abdominal strip adjacent to midline, swiss rolled), ovaries/uterus or testis/accessory sex glands. In addition, all gross abnormalities, in particular masses and lesions suspected of tumourous nature were sampled. After fixation (and for vertebral column, femur and skull: after subsequent decalcification in formic acid 20% as adequate) samples were trimmed, processed, and paraffin wax embedded. From the head, transverse sections (5) of the nose were taken, as well as a transverse section at the level of the auditory canal. Sections (5μm) were cut and stained routinely with haematoxylin and eosin (H&E). Fixation, paraffin wax embedding, sectioning, staining, and all other histotechnical procedures were performed according to the Standard Operating Procedures of the Laboratory of Pathology of the Institute.

1.2 *Testagent*

B[a]P was from Serva (14800, lotnr.24022; Heidelberg, Germany) and had a purity of 98.6 ± 0.4%. The claimed purity was verified upon receipt within the Institute at the Laboratory of Organic Chemistry (Analytical Residue Research section). B[a]P was dissolved in soy oil (De Oliehoorn BV, Zwaag) under heating (up to 60°C) and ultrasonic vibration. Fresh solutions were prepared every week (week 1 - 8), every 2 weeks (week 9-18), and every 3 weeks (19-104), respectively. B[a]P appeared to be stable under conditions of dissolution and storage (once dissolved). B[a]P solutions were prepared as follows. First, mean group weights were determined (per sex, per dose). Then, the mean group weight was estimated at half the dosing-period by simple extrapolation on the group's growth curve (this 'extrapolated' mean group weight was used to prepare the solutions; this resulted in some slight initial overdosing). Due to several corrections (e.g. purity) actually achieved dose levels were somewhat lower, i.e. 2.9 ± 0.3, 9.6 ± 1.0, and 29 ± 3 mg/kg bw per application, respectively. Testsubstance solutions in use were regularly verified for their B[a]P content.

1.3 *Mode of application*

B[a]P was dissolved in soy oil. At the start of treatment B[a]P was applied in 0.4 and 0.5 ml soy oil per female and male rat, respectively. Upon body weight gain and herewith amount of B[a]P per rat these volumes were adjusted to 0.6 and 0.75 ml, respectively. Solubility of B[a]P in soy oil was ≈ 30 g/l. The length of the gavage needle used assured exposure of the distal half of the oesophagus (a known target-site for carcinogenesis). Application was always in the morning for all groups and took about 1.5 - 2.5 hours. Rats were not dosed at some national or christian celebration days, i.e.12 days in total.

1.4 *Safety measures*

B[a]P is a recognized animal carcinogen and a presumed human carcinogen, i.e. classified by IARC into group 2A (IARC, 1983). In order to avoid contamination of the animal room with B[a]P and its metabolites, to protect personnel in the animal room, and to avoid exposure of the animals via other routes, several measures were taken. First, all personnel allowed to enter the animal rooms were commissioned to wear special clothing (including haircap), gloves, and a compressed-air-mask. Animal rooms were at underpressure

relative to a dress-sluice, which was itself at underpressure to the inlet-room to avoid leakage of B[a]P (and metabolites) to the outside. B[a]P was applied by gavage and all animal excreta were collected on special moistened filter paper and removed the first thing in the morning. By the end of the day the floor of the animal room was cleaned using a water-hoover. Air-samples were taken in the animal room during several days in the first and second week of exposure, and the (airdust-)filters were analysed for B[a]P content. As these analyses revealed B[a]P concentrations within the animal room to be slightly above outdoor air concentrations, it was decided that the compressed-air masks could be replaced by (more comfortable) standard mouth caps. Application of the testsubstance was by two biotechnicians: one (re)filled and offered the gavage syringe, the other fixed the animal and applied the testsubstance. Clothing prescriptions also remained in force during autopsy, starting at week 106; these animals were not dosed for at least nine days to reduce B[a]P content of animal tissues and waste. Taking a shower was an obligatory step in the procedure for leaving the animal room area.

2 Results

2.1 *Survival*

The survival among controls and B[a]P-treated animals is described and depicted in the main text and Figure 1 therein. The mortality in the highest dosed groups was 100% after about 70 weeks. This mortality was mainly due to sacrifice for humane reasons when the rats became emaciated, with distended abdomen in which frequently one ore more palpable masses were present in the cranial area (liver). Despite the presence of palpable masses, the condition and behaviour, with particular emphasis on animal welfare aspects, remained reasonably well.

Liver tumours were the most predominant tumour type encountered in this study in terms of morbidity and mortality (see 2.5 *Carcinogenicity* below).

2.2 *Body weight*

Body weight development during exposure to B[a]P is described and depicted (Figure 3) in the main text.

2.3 *Food- and water consumption*

Food- and water consumption are depicted below in Figures 1 and 2, and described in the main text. There is no explanation for the reduced water consumption during week 30 to 40 yet.

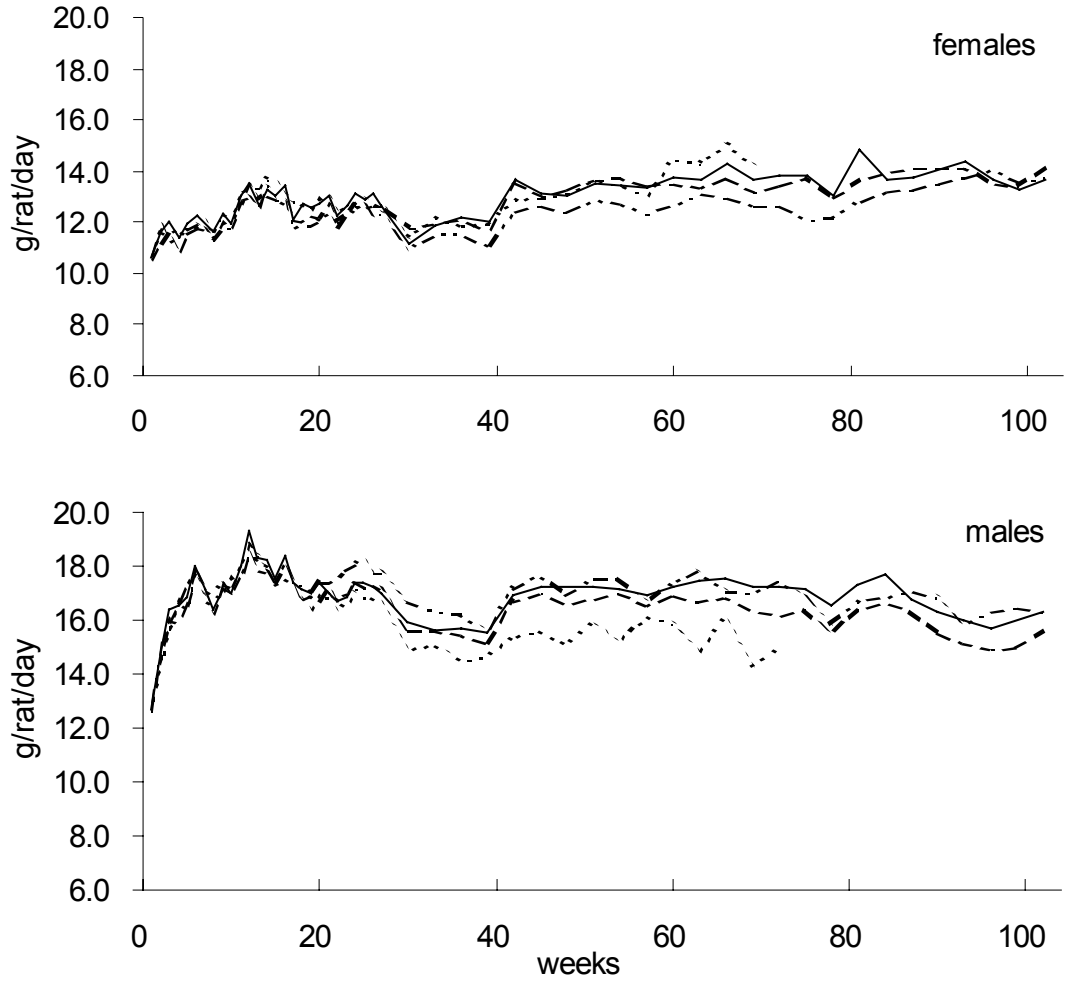

Figure 1. Food consumption of female and male rats during oral exposure to B[a]P at the indicated dose levels for 104 weeks. Control (——), 3 mg/kg bw (— —), 10 mg/kg bw (— -- —), and 30 mg/kg bw (- - - -).

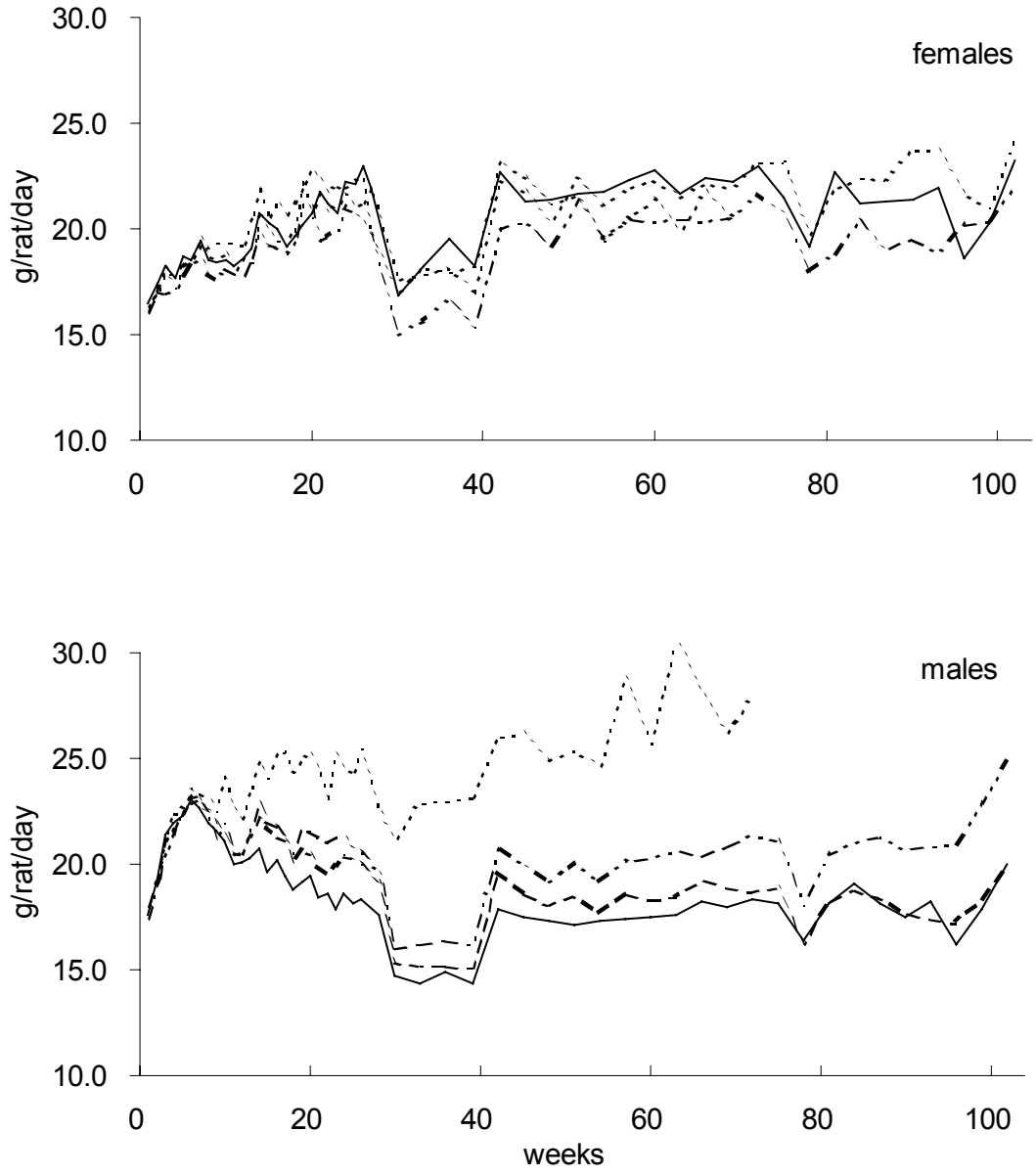

Figure 2. Water consumption of female and male rats during oral exposure to B[a]P at the indicated dose levels for 104 weeks. Control (——), 3 mg/kg bw (— —), 10 mg/kg bw (— - - —), and 30 mg/kg bw (- - - -).

2.4 *Non-neoplastic findings*

Since this study was principally intended to identify and quantitate the tumour occurrence for risk assessment purposes, attention was focused on tumourous and suspected or related lesions, and exceptional changes, especially those leading to death. Common background lesions were not scored unless excessive. Background lesions not taken into consideration are listed in Table 1.

Table 1. Common background lesions that were not entered in histopathology:

brain:	slightly dilated ventricles - corpora amylacea
heart:	cardiomyopathy - myxoid valvular change
lung:	perivascular cuffs - clusters of foamy alveolar macrophages
liver:	microgranulomas - portal lymphoid infiltrates - cholangiofibrosis / hyperplasia - fatty change
spleen:	moderate extramedullary hemopoiesis - moderate hemosiderosis -capsular cysts - moderate lymphoid hypertrophy / atrophy
kidney:	corticomedullary mineralisation - chronic progressive nephropathy - basophilic tubules
adrenal:	focal degenerative changes; dilated sinusoids (haemocysts)
sciatic nerve & ganglia:	demyelinisation
thymus:	involution
lymph nodes:	activation / atrophy
gland.stomach:	senile atrophy
thigh muscle:	atrophy
leg:	pododermatitis
ovaries / testes:	senile atrophy
prostate etc.:	inflammation, concrements

For the description of 'early' non-neoplastic and possible pre-neoplastic effects induced by B[a]P, i.e. after 3 months of exposure, the reader is referred to paragraph 2.8 of Appendix B1, which reports the results of the histological examination of the 90-day sacrifice on the following tissues: liver, stomach, esophagus, thymus, lung, spleen and mesenteric lymph node.

Non-neoplastic changes observed at necropsy during the two-year study are shown in Table 2. Again a selection is made, i.e. only those organs are shown that also appeared as target-organs for carcinogenesis (see below).

Table 2. Incidences of non-neoplastic changes in female and male rats treated with B[a]P. Only those organs and sites are shown in which treatment-related tumour-incidences were observed.

Site & lesion[1] dose[2] sex	0 f	3 f	10 f	30[3] f		0 m	3 m	10 m	30[3] m
Oral cavity *examined* [4]	*19*	*21*	*9*	*31*		*24*	*24*	*37*	*38*
squamous cyst	2	2	0	4		0	0	4	1
abscess	9	14	2	7		14	14	12	3
(pilo)sebaceous hyperplasia	10	10	5	11		10	13	19	13
Oesophagus *examined*	*52*	*52*	*52*	*52*		*50*	*52*	*52*	*52*
inflammation	0	2	0	2		0	0	2	0
abscess	0	1	0	0		1	0	0	0
phlegmona	0	0	2	0		0	0	0	0

(Table continued on next page)

Table 2 (continued-1)

Site & lesion[1] dose[2] sex	0 f	3 f	10 f	30[3] f	0 m	3 m	10 m	30[3] m
Forestomach *examined*	*52*	*51*	*51*	*52*	*50*	*52*	*52*	*52*
squamous cyst	2	1	0	0	2	0	0	0
inflammation	0	0	0	0	2	0	0	0
ulcer	1	0	0	0	2	0	0	0
hyperplastic limiting ridge	0	1	0	0	0	1	0	0
basal cell hyperplasia	1	8	13	2	2	8	8	0
Duodenum *examined*	*49*	*48*	*50*	*51*	*51*	*50*	*51*	*49*
ulcer	0	0	0	1	0	0	0	0
enteritis	0	0	0	0	0	1	0	0
Jejunum *xamined*	*50*	*48*	*50*	*51*	*51*	*50*	*51*	*49*
peritonitis	1	0	0	0	0	0	0	0
enteritis	0	0	0	0	0	1	0	0
tubular cell hyperplasia	0	0	0	0	0	0	1	0
Liver *examined*	*52*	*52*	*52*	*52*	*52*	*52*	*52*	*52*
necrosis	1	0	1	2	0	1	0	0
FCA[5], basophilic	3	5	1	0	0	2	1	0
FCA, eosinophilic	1	7	3	1	3	2	0	0
FCA, tigroid	0	2	0	0	1	0	0	0
FCA, clear cell	22	33	4	2	8	22	1	1
FCA, amphophilic	0	4	0	0	0	5	0	0
FCA, mixed	0	1	0	0	0	0	0	0
lobular hyperplasia	0	0	0	0	1	0	0	0
vacuolated focus	4	7	1	1	3	5	2	0
Kidney *examined*	*52*	*52*	*51*	*50*	*52*	*52*	*52*	*52*
papillary necrosis	0	0	0	0	1	0	0	0
pelvic mineralisation	0	0	0	0	1	0	0	0
pyelonephritis	0	0	0	0	0	1	0	0
urothelial hyperplasia	2	1	1	0	1	1	0	3
tubular proliferation	0	1	0	0	0	0	2	0
Urinary bladder *examined*	*50*	*52*	*51*	*50*	*52*	*52*	*52*	*49*
metastatic mineralisation	0	0	0	0	2	0	0	0
urothelial hyperplasia	0	1	0	0	1	3	4	8
Auditory canal *examined*[4]	*0*	*1*	*0*	*20*	*1*	*0*	*7*	*33*
squamous cyst	-	1	-	0	1	-	3	4
(pilo)sebaceous hyperplasia	-	0	-	5	0	-	1	2
hyperplastic plaque	-	0	-	0	0	-	0	4

(Table continued on next page)

Table 2 (continued-2)

Site & lesion[1] dose[2] sex	0 f	3 f	10 f	30[3] f		0 m	3 m	10 m	30[3] m
Skin & mammary *examined*[4]	*52*	*52*	*51*	*52*		*52*	*52*	*52*	*51*
squamous cyst	0	0	0	0		0	0	3	1
galactoceles	30	29	10	1		19	9	8	2
atrophy	1	0	0	0		0	0	0	0
necrosis	1	0	0	0		0	0	0	0
granuloma	0	1	0	0		0	0	0	0
ulcer	0	0	0	0		1	0	0	0
dermatitis	1	1	0	0		0	0	0	0
mammary stimulation	4	2	0	0		3	0	0	2
dermoid cyst	0	0	0	0		3	0	1	0
(pilo)sebaceous hyperplasia	0	0	0	0		0	0	0	1
hyperplastic mammary tissue	3	4	3	2		1	3	2	2
phlegmona	0	0	1	0		0	0	0	0

[1]) tissues listed in Table 1 but not in this tabel did not reveal any remarkable changes; [2]) dose in mg/kg bw, 5 days a week; [3]) this group had a significantly shorter lifetime (see Figure 1, main text);
[4]) some tissues were examined only when abnormalities were observed upon macroscopic examination;
[5]) FCA: foci of cellular alteration

2.5 *Carcinogenicity*

In life observations

Next to the palpable liver tumours (see *Survival* section), frequently tumours were seen on the base of the ear which eventually could result in ulceration or eruption through the auditory canal. Often these occurred bilateral. In the intermediate groups, subcutaneous tumours were often found in the neck area between pharynx and axillary region. When these tumours compromised feeding or mobility, or became ulcerated, the animal was necropsied and the tumour was qualified as fatal.

Necropsy

Gross changes in the highest dose group included masses in the liver (99/104), papilliferous thickening or masses in the forestomach (89/104) and nodules in the lungs suspect for metastatic spread. Also, frequently masses in the skin (back / head / neck / lips) or at the basis of the auditory canal were found. In a number of cases (predominantly in groups 3 and 4) typically masses were found in the neck / prescapular or mediastinal area. Ususally these tumours were closely associated with the esophagus and / or neck musculature. Occasionally masses were observed on other organs and tissues, such as kidney, small intestine, etc (see Tables 1 and 2, below). Other gross findings occurred occasionally or in accordance with common age and strain related background pathology. Necropsy and histopathological observations for some major tumours will be briefly presented below.

Liver. Masses in the liver were found in the intercurrently died rats, the first case observed in week 35 (male, highest dose). These were most often found in the middle and right lateral lobes, but in advanced cases multiple tumours were found in multiple lobes. Tumours were often pleomorphic: solid, cystic, necrotic areas, haemorrhagic areas. In

advanced cases, adhesions were formed with surrounding organs and omentum, and intra-abdominal metastatic spread and ascites could be present. Liver tumours were the most frequent cause of death (i.e. indication for euthanasia).

In the lower dose group, a much lower incidence and manifestation at a later time point was observed.

Forestomach. Masses in the forestomach were seen in most animals (86%) from the high dose group, and in 65 and 14% of the lower dose groups. These appeared as isolated small protrusions with a papilliferous surface. The common site of origin was the apex of the forestomach, and in advanced cases multiple masses became confluent. In some cases (pre)perforative local peritonitis had occurred, occasionally with evidence of intra-abdominal spread. In a few cases, the forestomach tumours were considered to be fatal, as decided upon presence of peritonitis and / or metastatic spread.

Histopathology

Since this study was principally intended to identify and quantitate the tumour occurrence for risk assessment purposes, attention was focused on tumourous and suspected or related lesions, and exceptional changes, especially those leading to death. Common background lesions were not scored unless excessive. Some organs were 'missing' and apparantly were lost during processing (these are described in the files of this project; it is concluded that these will not interfere with the conclusions of this study.

Liver. Tumours of the liver were usually complex, multiple and malignant in nature. A large proportion had metastasised to the lungs (59 of 150) and some to the abdominal cavity. The growth pattern and cytology were highly variable, while strikingly metastases were cytomorphologically often well differentiated. Therefore, cytomorphology was not a powerful criterion in discriminating between benign and malignant tumours. Malignant liver tumours were often associated with cystic structures that could be either of hepatocellar or cholangiocellular origin. Since these cholangiocellular lesions usually did not occur separately, but within or associated with malignant liver tumours, they have not been monitored as a separate entity with a few exemptions where they were clearly separated.

Histologically only the most progressed lesion was scored, thereby encompassing overdiagnosing. Thus, since tumours were usually multiple, if carcinoma was scored, this implies the concomitant presence of adenomas and foci of cellular alteration. Scoring was quantified as one, few or multiple.

Foci of cellular alteration (mainly clear cell type) were scored in tumour free livers (see Table 2), and quantified as one, few or multiple[7].

Forestomach. Lesions in the forestomach consisted of (multi)focal hyperplasia of basal cells, followed in advanced cases by papillomas, which ultimately could have resulted in invasive squamous cell carcinomas. In a few instances metastatic spread into the abdominal cavity and organs or lungs had occurred.

[7] Foci of cellular alteration (mainly clear cell type) were scored in tumour free livers (see Table 2), and quantified as one, few or multiple in H&E stained slides. However, this approach is less pertinent since separate sections were stained with GST-P as enzyme marker for "putative preneoplastic foci of cellular alteration"; using this staining technique more quantitative morphometry was performed which is more suitable for the purpose of the study.

Forestomach lesions could be monitored in a time-sequence fashion swing to the intercurrent sacrifices. Small and /or early lesions were characterised by focal or confluent multiple hyperplasia of basal cells. In more advanced lesions hyperplasia of the squamous component, including squamous cell papilloma, became apparent. Invasive growth usually occurred by the squamous component (squamous cell carcinoma), in some cases resulting in perforation, contact metastasis in the abdomen, and lung and liver metastases. Consequently, the diagnosis of squamous cell carcinoma implicitly signifies the presence of both papilloma and basal cell hyperplasia, and the diagnosis papilloma implicitly includes the presence of basal cell hyperplasia.

The glandular stomach or oesophageal mucosa were generally unaffected.

Soft tissue sarcomas. Most sarcomas were found in the subcutis of neck, prescapular or axillary area, often involving the oesophagus, thyroid or neck muscles by invasion. For this reason most have been entered under "oesophagus" or "skin and mammary". Occasionally this type of tumour was found at other locations (chest wall, groin, abdominal cavity (3x), stomach wall (2x), uterus / ovary (2x) and nasal cavity. These sarcomas typically revealed a variety of characteristics within the same mass, such as rhabdo-myosarcoma, fibrosarcoma, malignant fibrous histiocytoma, undifferentiated sarcoma, etc. When one feature predominated, this served as the basis of classification. In a proportion of these tumours transparant spaces were observed with material suggestive of oil. These spaces were covered by thin elongated fibroblast or macrophage like cells, but without florid inflammatory response.

Auditory canal. The tumours were histologically composed of squamous or sebaceous cells with the range of intermediate types, although in the larger tumours the squamous component predominated. These lesions apparently arose from the pilo-sebaceous units (sebaceous glands, including the Zymbal gland) in the auditory canal. In some cases a normal Zymbal gland existed in association with a tumour. This lesion was usually not preceded by (diffuse) hyperplasia, but focal dysplasia, hyperplasia or cystic change occurred instead. Malignant tumours of the auditory canal tended to infiltrate the masticatory musculature, and occasionally the skull and brain. Metastasis to the lung was found once.

Oral cavity. Tumours at this site usually were derived from pilosebaceous tissue, normally present as clusters in the lip commissure. In many cases, including control animals, hyperplasia of the units was found, often with abscesses, dilation of hair shafts / glandular outlets. The incidence of these hyperplastic and inflammatory lesions appeared not to be treatment related. Sizeable tumours usually were of the squamous or mixed squamous / sebaceous cell type, and were often multiple / bilateral, and dose-related increased.

Skin. Skin tumours were basically characterised as arising from epidermis (squamous cell papilloma, carcinoma or keratoacanthoma) or appendages (basal, hair follicle or sebaceous cell types and mixtures thereof). For that reason the diagnostic differentiation was not always unequivocal and was based upon the most prominent or most malignant component. In fact it may be realistic to pool these varieties of skin / appendage. These tumours were often multiple and variable, therefore the total number of tumours may seem overestimated when related to number of tumour bearing animals.

Kidney. Tumours of the kidney mainly included cortical adenoma and occurred in the two highest dose group males. These benign tumours were usually small and could only be diagnosed incidentally by careful histopathology. A clear distinction with hyperplasia is not always feasible, even with the commonly accepted criteria (IARC / SNTP), since there is a

gradual continuum between hyperplasia and adenoma. Therefore the lesions might be merged. Since the observation of these lesions is particularly chance-dependent, this lesion is under this protocol (one section of either kidney) most probably underscored. Occasionally adenocarcinoma, and tumours of pelvis or ureter were found.

Small intestine. Tumours of the small intestine were observed mainly in the top dose males and were sometimes multiple. Malignant tumours occasionally metastasised to the liver and were often associated with obstruction and / or peritonitis.
Tables 3 and 4 depict the incidences of the most remarkable sites of tumour development associated with B[a]P treatment. For comments (additional to the above) one is referred to the main text.

Table 3. Incidences of treatment-related neoplasms in female rats treated with B[a]P. Only those organs and sites are shown in which incidences were either significantly increased with treatment,or with remarkable observations per se.

Site		dose[1]	0	3	10	30[2]
Oral cavity		*examined* [3]	*19*	*21*	*9*	*31*
	papilloma		0	0	0	9
	squamous cell carcinoma		1	0	0	9
	basal cell adenoma		0	0	1	4
	sebaceous cell carcinoma		0	0	0	1
Oesophagus		*examined*	*52*	*52*	*52*	*52*
	sarcoma undifferentiated		0	0	2	0
	rhabdomyosarcoma		0	1	4	0
	fibrosarcoma		0	0	3	0
Forestomach		*examined*	*52*	*51*	*51*	*52*
	squamous cell papilloma		1	3	20	25
	squamous cell carcinoma		0	3	10	25
Duodenum		*examined*	*49*	*48*	*50*	*51*
	adenocarcinoma		0	0	0	2
Jejunum		*examined*	*50*	*48*	*50*	*51*
	adenocarcinoma		0	0	0	2
Liver		*examined*	*52*	*52*	*52*	*52*
	hepatocellular adenoma		0	2	7	1
	hepatocellular carcinoma		0	0	32	50
	cholangiocarcinoma		0	0	1	0
	anaplastic carcinoma		0	0	1	0
Auditory canal		*examined* [3]	*0*	*1*	*0*	*20*
	benign tumour		0	0	0	1
	squamous cell papilloma		0	0	0	1
	carcinoma [4]		0	0	0	13
Skin &		*examined*	*52*	*52*	*51*	*52*
mammary	fibroadenoma		3	13	5	1
	fibrosarcoma		0	0	1	1
	mammary adenoma		5	2	1	0
	fibrous histiocytoma (M) [5]		0	0	4	1
Pituitary		*examined*	*46*	*52*	*47*	*52*
	adenoma		22	17	11	8
	carcinoma		8	7	1	0

[1]) dose in mg/kg bw, 5 days a week; [2]) this group had a significantly shorter lifetime (see Figure 1, main text); [3]) some tissues were examined only when abnormalities were observed upon macroscopic examination; [4]) of mixed tumours of squamous and sebaceous cells apparently arisen from the pilosebeceous units; [5]) M: Malignant

Table 4. Incidences of treatment-related neoplasms in male rats treated with B[a]P. Only those organs and sites are shown in which incidences were either significantly increased with treatment, or with remarkable observations per se.

Site	dose[1]	0	3	10	30[2]
Oral cavity	*examined*[3]	*24*	*24*	*37*	*38*
	papilloma	0	0	2	10
	squamous cell carcinoma	1	0	5	11
	keratoacanthoma	0	0	0	2
	sebaceous cell carcinoma	0	0	0	2
Forestomach	*examined*	*52*	*52*	*52*	*52*
	squamous cell papilloma	0	7	18	17
	squamous cell carcinoma	0	1	25	35
Duodenum	*examined*	*51*	*50*	*51*	*49*
	adenocarcinoma	0	0	0	1
Jejunum	*examined*	*51*	*50*	*51*	*49*
	adenocarcinoma	0	0	1	8
Liver	*examined*	*52*	*52*	*52*	*52*
	hepatocellular adenoma	0	3	15	4
	hepatocellular carcinoma	0	1	23	45
	cholangioma	0	0	1	0
	cholangiocarcinoma	0	0	0	1
Kidney	*examined*	*52*	*52*	*52*	*52*
	cortical adenoma	0	0	7	8
	adenocarcinoma	0	0	2	0
	urothelial carcinoma	0	0	0	3
Auditory canal	*examined* [3]	*1*	*0*	*7*	*33*
	benign tumour	0	0	1	0
	squamous cell papilloma	0	0	0	4
	sebaceous cell adenoma	0	0	0	1
	carcinoma [4]	0	0	2	19
Skin &	*examined*	*52*	*52*	*52*	*51*
mammary	basal cell adenoma	2	0	1	10
	basal cell carcinoma	1	1	0	4
	squamous cell carcinoma	0	1	1	5
	keratoacanthoma	1	0	1	4
	trichoepithelioma	0	1	2	8
	fibrosarcoma	0	3	5	0
	fibrous histiocytoma (*M*) [5]	0	0	1	1
Urinary bladder	*examined*	*52*	*52*	*52*	*49*
	squamous cell papilloma	0	0	1	0
	transitional cell carcinoma	0	0	1	0
Pituitary	*examined*	*51*	*52*	*51*	*50*
	adenoma	29	29	20	1
	carcinoma	3	2	3	0

[1]) dose in mg/kg bw, 5 days a week; [2]) this group had a significantly shorter lifetime (see Figure 1, main text); [3]) tissues were examined only when abnormalities were observed upon macroscopic examination; [4]) of mixed tumours of squamous and sebaceous cells apparently arisen from the pilosebeceous units; 5) M: malignant.

3 Discussion & Conclusion

The most predominant site for tumour development by B[a]P encountered in this study in terms of morbidity and mortality was the liver, a site not previously reported upon B[a]P exposure. The first tumours appeared in week 35 (highest dose group males). In mild cases tumours mostly occurred in one lobe, but in advanced cases, and in particular fatal ones, more lobes or even the total liver was affected. Tumours were usually complex, showing various gross and histomorphological features which were not further histologically differentiated. Counting the number of individual tumours was not possible due to advanced character of the disease. A considerable proportion had metastasised to the lungs (59 / 150) and some to the abdominal cavity.

B[a]P-induced malignant tumours in the forestomach also have not been reported previously in this species. Only "multiple hyperplasia" and papillomas were reported previously at this site by Gibel (1964), and Brune *et al.* (1981) after lifetime oral exposure to B[a]P. Based on the basal cell proliferation observed in the range-finding and 90-day study in the present rat strain (see appendices A and B1), tumour-development at this site was anticipated. It also is in line with the full malignant tumours observed at this site in mice after feeding B[a]P (Horie *et al.*, 1965; Neal and Rigdon,1967; Chouroulinkov *et al.*, 1967). Since the proliferation at this site in the 90-day study in the present rats was not associated with diffuse hyperplasia, it was considered not a result of a mitogenic effect, but as the consequence of replacement of damaged cells, i.e. by cytotoxicity or genotoxicity. Occasionally only focal hyperplasia of basal cells was observed (in the 90-day study), which in a later stage may have progressed to papilloma and invasive carcinoma.

The distal oesophagus, being exposed to B[a]P as well (as concluded by the length of the gavage needle), and covered with similar epithelium, showed none of these features. For this reason, the absence of a forestomach analogue in humans, and the artificial (pulse) exposure, forestomach tumours were considered of less relevance for human risk assessment. In fact, some tumours were found in close association with the oesophagus, but these were composed of a different tissue than the mucosa, i.e. it concerned soft tissue sarcomas. Most of these were fatal and were predominantly found in the cervical part of the oesophagus. As they occurred in B[a]P treated animals without a clear dose response, and occasionally had transparant spaces with material suggestive of oil, it was concluded that these represented deposits of soy bean oil containing B[a]P by erroneous application, thus serving as an (unintended) sustaining delivery site of locally acting carcinogen[8]. From the literature, induction of (rhabdomyo-) sarcomas by subcutaneous application of B[a]P is well known (Buening *et al.,* 1983). Therefore, it was considered that indeed (micro)trauma at the level of the pharynx could have resulted in submucosal deposition of benzo[a]pyrene, also in view of the absence of epithelial changes or other evidence of trauma in the oesophagus. With regard to this pathogenic mechanism, these tumours were also considered inappropriate endpoints for human risk assessment.

[8] The exact site of origin was usually not to be determined because of the widespread invasion in oesophagus, thyroid, cervical vertebrae / skull, etc. In occasional specimen the oesophageal origin could indeed be confirmed. These sarcomas were usually poorly differentiated, and frequently showed a variety of aspects (MN cells, fibroblasts, smooth and striated muscle cells, etc.). Careful examination revealed empty spaces in a significant proportion of these sarcomas. These were usually lined by flat elongated mesenchymal cells (fibroblasts or histiocytes); often these spaces showed some evidence of oil-like material, despite the histochemical procedures. Surprisingly no significant inflammatory response with eventual elimination of this oil was seen, indicating a high biocompatibility of the vehicle and a persistent presence of B[a]P.

The mechanism of induction of epidermal tumours is unclear. Auditory canal tumours are commonly seen after application of mutagenic carcinogens in this species (Ashby and Paton, 1993; Gold and Zeiger, 1997), possibly as a result of accumulation or excretion of carcinogens. The other epithelial tumours may have the same etiology, at least their histogenesis is common as far as the basic cell type is concerned. Whether appendage tumours, often located at potential contact sites such as lip and anterior body (through grooming behaviour?) may also have a systemic component, as is most probably the case with the auditory canal tumours, remains unclear. Lip and auditory canal tumours occurred almost exclusively in the top dose group, and more in males, while other epidermal tumours occurred in males only, with an increase in the top dose group. Contact site carcinogenesis associated with PAH is well known in humans (scrotal cancer in chimney sweeps, lip cancer in pipe smokers) and animals, albeit mainly squamous in nature. On the other hand, several PAHs have acnegenic potential probably through Ah receptor binding, and this may support the theory of a systemic mechanism.

Tumours of the small intestine are extremely unusual in our strain of rats, and thus the number of 13 adenocarcinomas in 104 animals that had died within 1-1.5 years (top dose) is significant. Probably this is due to local activity of B[a]P before or during intestinal absorption and may be of human relevance. However, for risk assessment the high dose group should be neglected and the number of liver tumours significantly exceeds all other tumour types.

Surprisingly, no (increase in) lung tumours were found despite the high level of DNA adducts compared to the liver (see Appendix B2), and even though often microdroplets of oily material were present within alveoli, probably from inhalation of microdroplets (data not shown). These oil droplets were not associated with an inflammatory / phagocytic response and thus the material was apparently biocompatible and not rapidly taken up.

Most of the common background tumour lesions are age associated, and in view of the high mortality, consequently these occurred in a lower incidence in the top dose group, such as those encountered in the pituitary.

In conclusion, this study demonstrated the carcinogenic potential of benzo[a]pyrene in a variety of organs when administered by gavage to rats. Major target organs were liver, forestomach and epidermal structures, of which the liver is considered the most relevant for human risk assessment in terms of pathogenesis and sensitivity.

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APPENDIX B1 90-Day study

Preface

1. Experimental design
2. Results & comments
3. Verification of dose-levels for the carcinogenicity study

90-Day study

Preface

Within the groups of 74 animals (per dose, per sex) subjected to the chronic treatment regimen with B[a]P 10 animals were assigned for a scheduled sacrifice after 3 month (see scheme 1, main text). The purpose of this 90-day kill was to reassure the appropriateness of the dose levels chosen for the chronic carcinogenicity study. Special attention is paid to differences with the carcinogenicity protocol. Also, the results of necropsy and investigated toxicity parameters will be presented together with some brief comments. Focus was on target-organs of B[a]P-toxicity identified as such in the prechronic range-finding study (Appendix A). More information is available within the files of this project.

1 Experimental design

1.1 *Animal housing, treatment, monitoring and necropsy*

10 animals (per dose, per sex) were housed, treated, and monitored as described for animals subjected to the 104 week testing protocol (see Appendix B). This, of course, also applies to the test agent used, the mode of application, and the safety measures applied for personnel and environment.

The 90-day kill after 3 months of exposure to B[a]P was designed as follows. On the day before sacrifice the animals received their last B[a]P dose, and were allocated in metabolism cages for collection of urine. On the day of sacrifice blood was taken under ether narcosis by orbita puncture. Blood and urine were for clinico-chemical examinations (i.e. ALAT, ASAT, γ-GT, LD, creatinine), urinalysis (volume, creatinine and protein), and haematological investigations (WBC, RBC, Hb, Ht, MCV, MCHC, PLT). Prior (1h ± 15') to euthanasia the rats received an i.p. injection of 2 ml buffered saline containing 20 mg BrdU/ml. The animals were sacrificed by exsanguination from the abdominal aorta. After general gross inspection the following organs were sampled: brain; pituitary; thyroid (attached to larynx); thymus; heart; lungs (after formalin infusion); liver (two lobes); spleen; pancreas; kidneys; adrenals; ovaries / testes; uterus / prostate with seminal vesicles; sciatic nerve; quadriceps muscle; gastro-intestinal tract (stomach opened and spread on paraffin wax disks; swiss rolls from oesophagus, duodenum; jejunum; ileum; caecum; colon and rectum); lymph nodes (axillary, mesenteric and mandibular); ventral abdominal skin (including subcutaneous mammary tissue) and femur. Liver, thymus and spleen were weighted. All tissues were fixed in 4% neutral buffered formaldehyde. For initial histological examination the following tissues were designated as protocol organs: liver, stomach, esophagus, thymus, lung, spleen and mesenteric lymph node. Other organs were archived separately for backup purposes (possible unexpected tumour yields) and possible other future research, in view of the unique character of the material.

Histological specimens of protocol organs were processed routinely (paraffin embedding and H&E staining of 5 μm sections). From the stomach, a putative target organ and selected for morphometric counting of BrdU labelling, two samples were taken perpendicular to the limiting ridge, containing both fore- and glandular stomach. Other samples were processed according to Standard Operation Procedures of the Laboratory of Pathology.

Initially only control and high dose groups animals were examined. Organs displaying changes that could be dose-related, were further analysed by "blind reading", and if a difference was apparent, also the intermediate dose groups were studied.

Morphometrical analysis of the forestomach was carried out for the prevalence of S-phase epithelial cells displaying BrdU incorporation after immunohistochemical staining (modified from Schutte *et al*., 1987). From each section of the forestomach five representative samples (if possible), each over the length of the monitor were counted, and the total per stomach was expressed in relation to the surface of the corresponding muscularis mucosae. The latter was done to compensate for variation in stretching of the stomach wall. Morphometry was performed using the IBAS 2000 image analysis system (Kontron Bildanalyse GmbH, Eching, Germany); the data were analysed statistically by ANOVA for treatment and by Student-t test for group wise-comparison.

2. Results & comments

2.1 *Survival*

There were no differences in survival between controls and B[a]P-treated animals within the 3-month period (see Figure 1, main text).

2.2 *Body weight*

Body weight development during the first three months of exposure for these animals is as described and depicted (Figure 3) for the whole group in the main text.

2.3 *Food and water consumption*

Food and water consumption during the first three months of exposure for these animals is a as described in the main text and Appendix B.

2.4 *Organ weights*

As indicated in the Preface this 90-day kill focused on those parameters known to be affected by B[a]P treatment. Clear target-organs of toxicity were the liver and thymus, as was already diagnosed in the range-finding study (Appendix A). Effects of B[a]P treatment on weights of liver and thymus are shown in Tabel 1. Spleen weights were only just significantly increased ($P<0.05$) at the top dose in both sexes (data not shown).

Table 1. Effect B[a]P treatment on organ-weights of liver and thymus. Data represent mean ± SEM in grams (liver) or milligrams (thymus) for 10 animals.

	liver		thymus	
dose[1]	females	males	females	males
0	5.54 ± 0.70	7.49 ± 0.97	320 ± 60	380 ± 60
3	5.42 ± 0.76	8.00 ± 0.85	310 ± 50	380 ± 110
10	5.76 ± 0.71	8.62 ± 1.30*	300 ± 40	330 ± 60
30	6.48 ± 0.78*	9.67 ± 1.17**	230 ± 30**	270 ± 40**

[1]) dose in mg/kg bw, 5 days a week; Student t-test (unpaired, two-tailed): *)P<0.05; **) P<0.001.

From the range-finding is was concluded that changes in weight of liver and thymus would preferentially be confined to the highest dose level. As can be seen from Table 1 this is achieved, except for the liver of males, in which also 10 mg/kg bw induced a substantial (15%) weight increase.

2.5 *Haematology*

Apart from some non-statistical, small, dose-related decreases in RBC count and Hb (both sexes), B[a]P treatment did not have any significant effects on the investigated haematological parameters[9].

2.6 *Clinical chemistry*

No treatment-related group-differences, nor dose-response relationships could be discerned for ALAT, ASAT, LD, or creatinine. For γ-GT a small dose-related decrease in activity was found in males. The treatment-related small increase in creatinine levels observed in the range-finding study with males (paragraph 2.6, Appendix A) could not be reproduced under the present conditions.

2.7 *Urine analysis*

Urinary volume was increased in males at the highest B[a]P-dose tested, though in the absence of a clear dose-response relationship. Urinary creatinine levels appeared increased at this treatment level in males and females. Both male and female rats showed a dose-related increase in urinary protein levels. In the absence of further investigations, underlying mechanisms are yet unresolved.

2.8 *Non-neoplastic findings*

In life observations

No remarkable effects were observed during this 3-months treatment period, either on behaviour or upon handling.

[9] In a later study in this strain on B[a]P-induced immunotoxicity with an almost identical experimental design, significant decreases were observed after 5 weeks 10 and 30 mg/kg bw for RBC, Hb, and Ht (de Jong *et al*., 1999)

Necropsy

A consistent finding in most animals was a discolouration of the mandibular lymph nodes, and occasionally other regional lymph nodes (axillary). This discolouration varied from reddish to brown / grey, although in the table this discolouration was named brown / red. This phenomenon was attributed to minute local haemorrhages caused by manipulation (gavage), which was conveyed to the regional lymph node. Other changes occurred at a low incidence and were consistent with common background pathology. The gross findings are summarised in Table 2.

Table 2. Incidences of gross findings in rats treated with B[a]P for 3 months. Only those organs and sites are shown in which changes were observed.

Site & lesion[1] dose[2] sex *examined*[3]	0 f *10*	3 f *10*	10 f *10*	30 f *10*		0 m *10*	3 m *10*	10 m *10*	30 m *10*
Axillary lymph node									
brown/red discolouration	0	0	0	3		0	0	0	0
Caecum									
petechiation	0	1	0	0		0	0	0	0
Lung									
haemorrhage	0	0	0	0		2	0	0	1
Nasal cavity									
Haemorrhage/trauma	0	0	0	0		0	1	0	0
Mandibular lymph node									
Brown/red discolouration	5	8	7	10		8	9	8	10
Mesenteric lymph node									
Brown/red discolouration	0	1	0	1		0	0	0	0
Spleen									
Serous cyst	0	1	0	0		0	0	0	0
Testis									
Haemorrhagic lesion	-	-	-	-		0	0	0	1
Uterus									
Dilatation	2	1	4	2		-	-	-	-
Hypertrophy	0	0	0	1		-	-	-	-

[1]) note: liver and thymus did not reveal any remarkable changes; [2]) dose in mg/kg bw, 5 days a week; [3]) no.of animals examined.

Histopathology

The histopathological findings are summarised in Table 3.

Forestomach. The epithelium of the forestomach of the 30 mg/kg group in particular demonstrated minimal changes that could be summarized as "basal cell disturbance". The diagnosis basal cell disturbance was based on the combination of various features that occurred either focal or diffuse. These features were: increase in number of basal cells, mitotic figures

and remnants of necrotic (apoptotic?) cells; occasionally early nodule development; infiltration by inflammatory cells (mainly histiocytes); capillary hyperaemia, often in combination with the previous changes. Typical changes are shown in **Fig. 1-3** and are summarised in Table 3 under the diagnostic term “basal cell hyperplasia”.

Intermediate dose groups were also studied, and the changes were scored by "blind reading". Although difficult to determine by this way and based on data from only a limited number of animals, the lowest dose without effect could be estimated as 3 mg/kg for males and 10 mg/kg for females, or 3 mg/kg for sexes combined. The nodular hyperplasia, probably an indication of early neoplasia, was found in one animal of the 30 mg/kg group in either sex (**Fig. 4**).

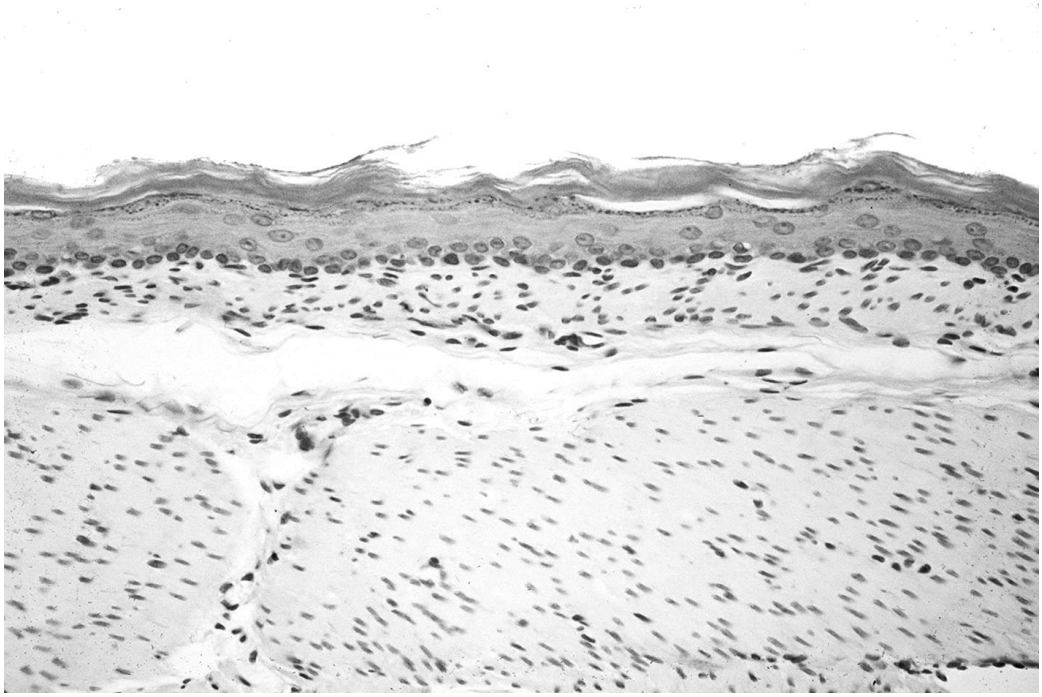

Figure 1. Histology of a rat forestomach after 3 months control treatment (H&E stained).

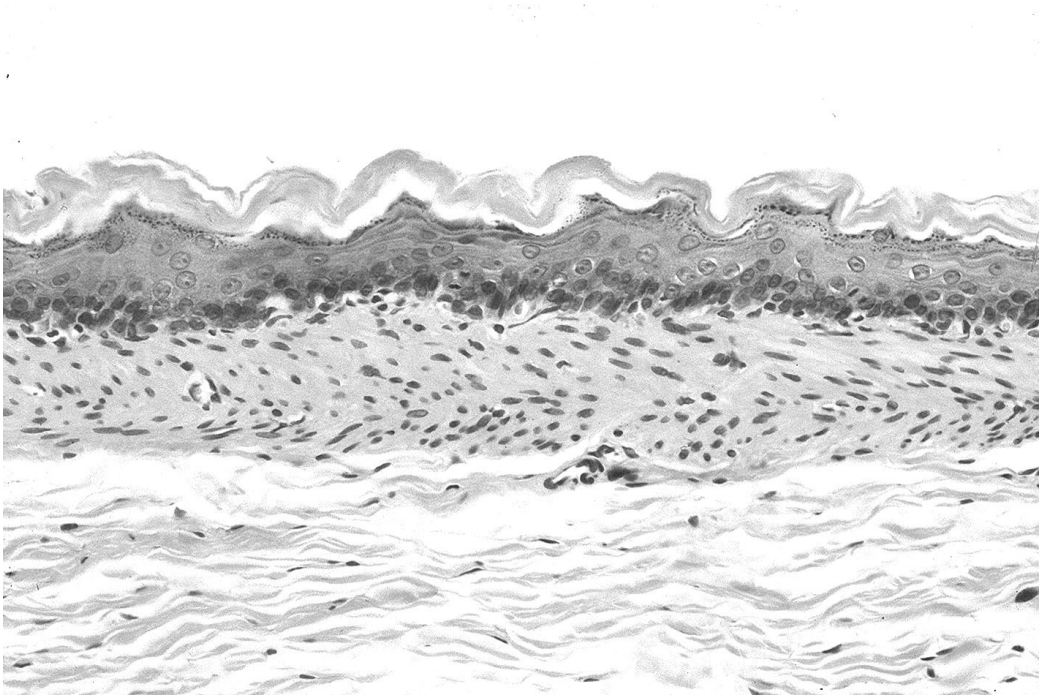

Figure 2. Histology of the forestomach of a rat treated with 30 mg B[a]P /kg bw for 3 months. Note the diffuse increase in cellularity (including mitotic figures) of the basal layer of the mucosa compared to the control in fig 1. (H&E stained).

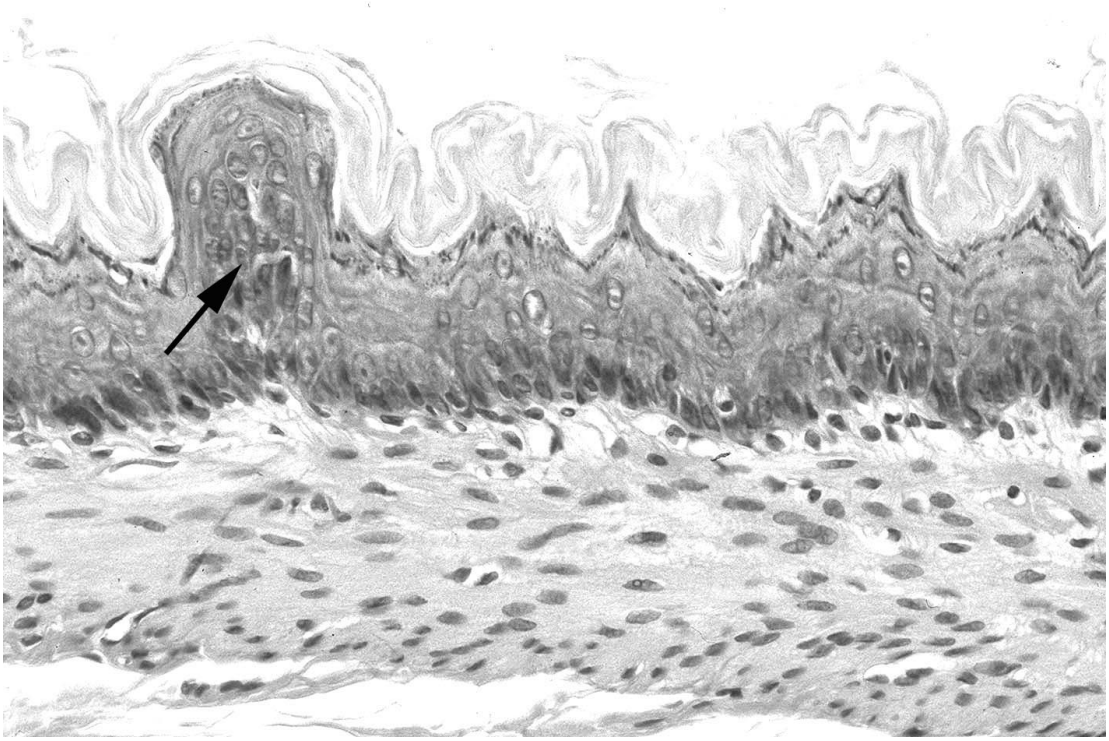

Figure 3. Histology of the forestomach of a rat treated with 30 mg B[a]P/kg bw for 3 months. Note the diffuse increase in cellularity of the basal layer of the mucosa and the focal early nodule (arrow) (H&E stained).

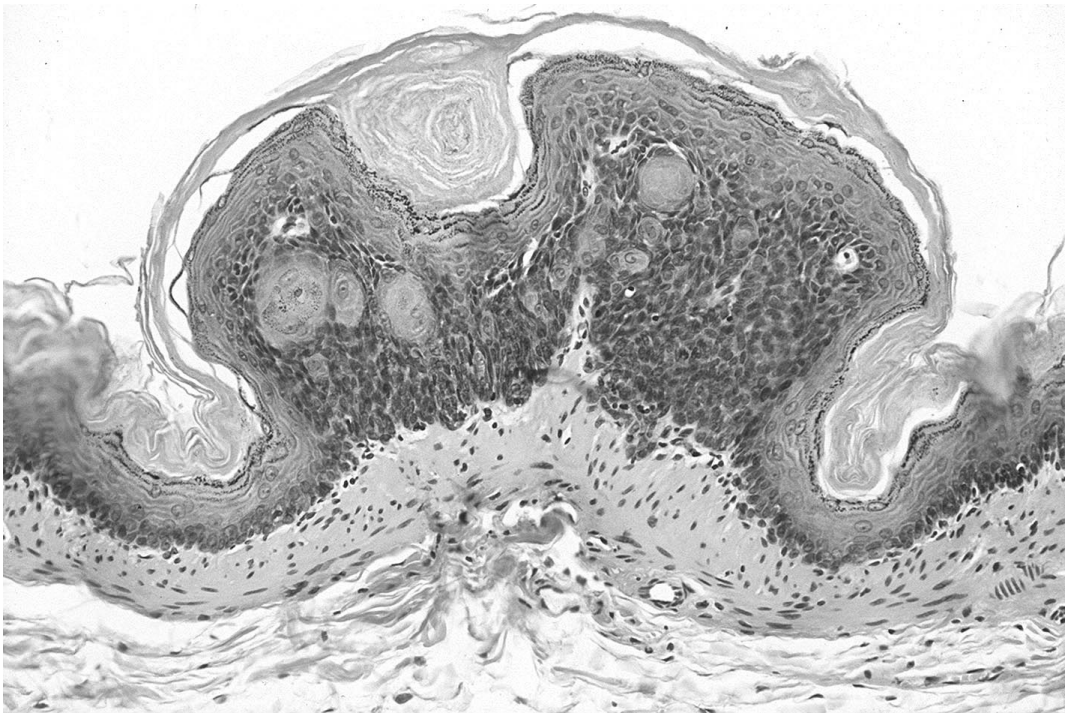

Figure 4. Histology of the forestomach of a rat treated with 30 mg B[a]P/kg bw for 3 months. Note the nodular hyperplasia of basal cells with dysplasia and focal differentiation, and the sharp demarkation from normal mucosa: this lesion presumably represents early tumour development (H&E stained).

Morphometry was carried out for mitotic cells as a discrete parameter for the changes listed above. Representative examples of BrdU staining results are shown in **Fig. 5**. The group data are presented graphically in **Fig. 6**. It can be concluded that a steep increase in mean number of mitotic cells was found with increasing dose, although the vatiation was considerable. ANOVA demonstrated a significant relation with dose, and Student-t revealed statistical significance (two-tailed) for groups treated with 3 and 10 mg/kg bw, respectively, when compared to controls (see **legend Figure 6**).

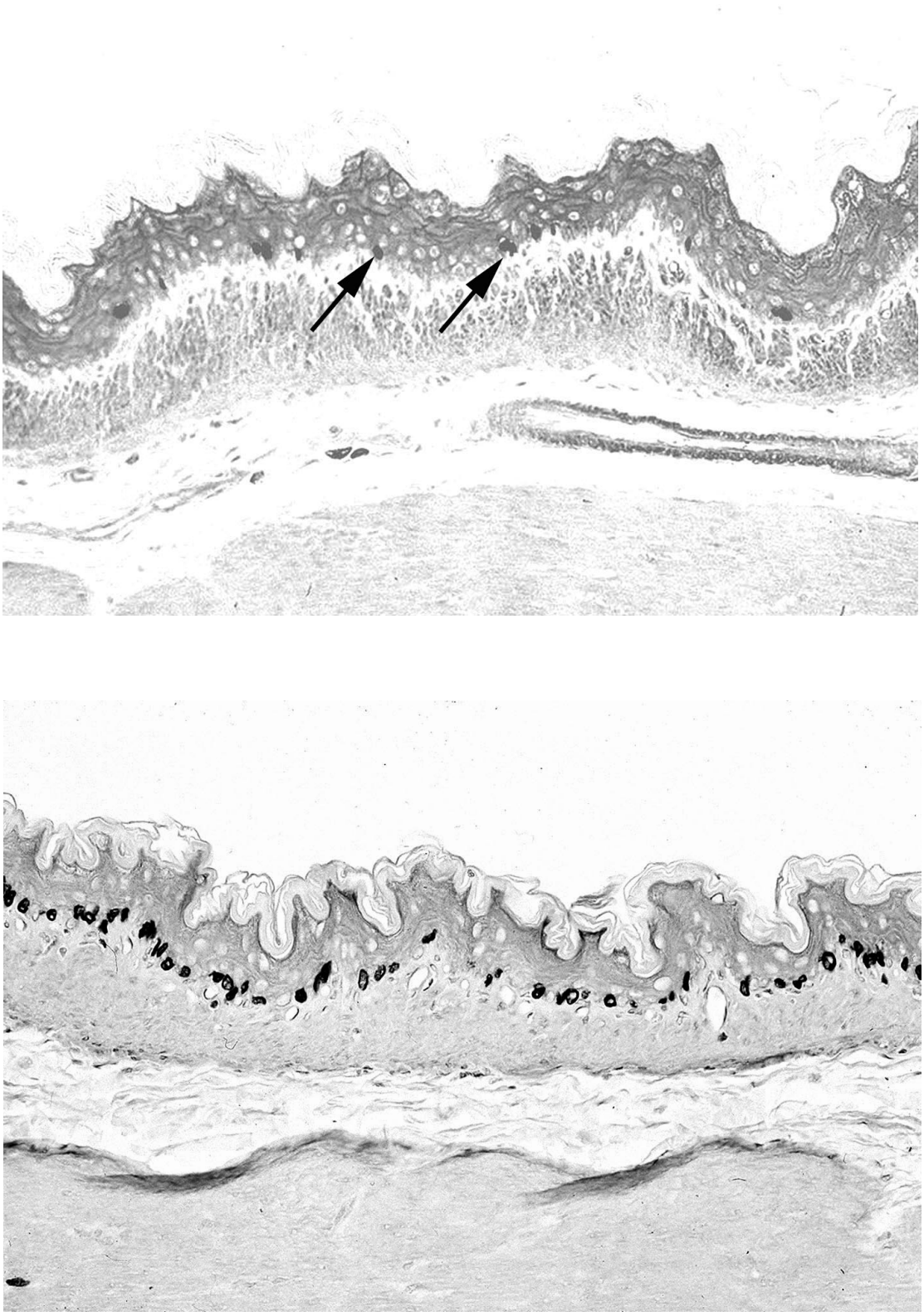

Figure 5. Photograph of anti-BrdU staining of the forestomach of control (top) and B[a]P (30 mg/kg bw for 3 months) treated rat (bottom). BrdU reactive nuclei in the basal layer (arrows) are clearly visible by their dark labeling. Note the clear increase in treated animals (anti-BrdU stained).

Table 3. Incidences of relevant non-neoplastic changes in female and male rats treated with B[a]P for 3 months. The following tissues were examined: liver, stomach, esophagus, thymus, lung, spleen and mesenteric lymph node[1].

Site & lesion[2] dose[3]	0	3	10	30		0	3	10	30
sex	f	f	f	f		m	m	m	m
No.examined	*10*	*10*	*10*	*10*		*10*	*10*	*10*	*10*
Forestomach									
not remarkable	3	3	4	0		4	3	2	0
nodular hyperplasia,	0	0	0	1		0	0	0	1
basal cell hyperplasia	7	7	6	10		6	7	8	10
minimal	7	5	3	3		4	7	2	3
slight	0	2	3	7**		2	0	6	7#
Glandular stomach									
not remarkable	10	-	-	10		10	-	-	10
Liver									
focal vacuolation	1	-	-	2		0	-	-	0
(fatty) vacuolation	4	-	-	3		3	-	-	7
necrosis	0	-	-	0		1	-	-	1
inflammatory cell foci	10	-	-	10		10	-	-	10
atypical nuclei	0	-	-	0		1	-	-	0
Lung									
not remarkable	6	-	-	4		7	-	-	7
inflammatory cell foci	1	-	-	5		2	-	-	2
histiocytosis	0	-	-	1		0	-	-	0
arterial mineralisation	2	-	-	0		1	-	-	3
increased septal cellularity	1	-	-	1		0	-	-	1
Mesenteric lymph node									
not remarkable	10	-	-	10		10	-	-	10
Oesophagus									
not remarkable	9	-	-	10		7	-	-	9
degeneration	0	-	-	0		0	-	-	1
regeneration	1	-	-	0		1	-	-	0
inflammatory cell infiltration	0	-	-	0		2	-	-	0
Spleen									
not remarkable	10	-	-	8		10	-	-	7
haemosidirin pigment	0	-	-	1		0	-	-	0
hyperaemia	0	-	-	0		0	-	-	1
lymphoid depletion	0	-	-	1		0	-	-	1
reticulum cell hyperplasia	0	-	-	0		0	-	-	1
Thymus									
not remarkable	10	10	10	7		10	8	9	4
atrophy, slight	0	0	0	3		0	2	1	6**

[1]) only highest dose and controls were examined, except for forestomach and thymus; [2]) tissues listed in Table 1 but not in this tabel did not reveal any remarkable changes; [3]) dose in mg/kg bw, 5 days a week; Statistical testing by Fisher‘s exact test: **: P<0.01 (2-tailed), #: P,0.05 (1-tailed).

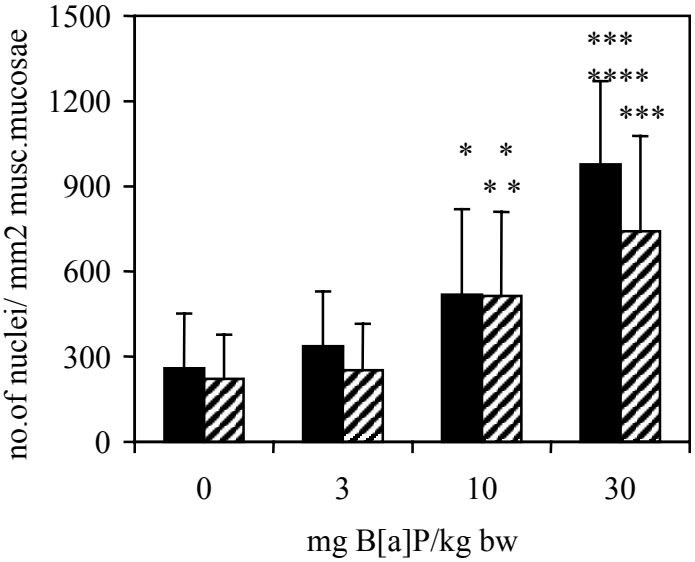

*Figure 6. BrdU incorporation in forestomach epithelium of rats treated with 0, 3, 10 or 30 mg/kg bw for 3 months (filled, females; shaded, males). Depicted is the morphometric determina-tion of the number of BrdU-staining nuclei per unit surface area of the underlying lamina muscularis mucosa; histograms depict mean ± SD for 8 to 10 animals. Levels of significance, Student-t test, two-tailed: * P< 0.05 as compared to '0' (= 0 mg/kg bw); **) P< 0.05 as compared to '3'; ***) P<0.01 as compared to '0' and '3'; ****) P < 0.01 as compared to '10'.*

Thymus. Thymus atrophy was diagnosed in relation to the structure of the thymic lobes, i.e. the relative quantity, shape and distribution of cortical and medullary areas. In animals displaying thymus atrophy, the proportion of medullary areas was increased with irregular boundaries. The incidence of this slight thymus atrophy in the 30 mg/kg groups appeared increased compared to the controls. For this reason also the intermediate groups were evaluated; this finding was recorded by blind reading. The incidence in these groups were nil or insignificantly low. Therefore the lowest dose without effect can be established at 10 mg/kg.

Liver. All animals contained a few to many small inflammatory cell foci consisting mainly of macrophages, occasionally surrounding a necrotic cell (hepatocyte?). When this lesion was quantified (categories 0-5; 5-10 and >10 foci per 2 liver sections), no difference between control and top dose group was apparent. In one male of both control and 30 mg/kg group a necrotic area was seen in the liver.

Slight fatty vacuolation was seen in a significant proportion of both males and females, with a slightly higher incidence in high dose males (when "vacuolation" and "fatty vacuolation" are combined). The distribution was more or less zonal, and occurred mainly in the periportal (zone I) areas. In a few females (1 in control, 2 in the top dose group) the vacuolation occurred more focally and was more of the glycogen type. This change was suggestive for early clear-cell foci.

Lung. Minimal inflammatory changes (inflammatory cell foci, histiocytosis, increased septal cellularity) occurred at a low incidence, although the number of animals affected was higher in the 30 mg/kg females (6/10 vs 2/10). This, as well as the arterial mineralisation observed is considered as a common background lesion.

Oesophagus. Changes in the oesophagus consisted of degeneration and regeneration of muscle fibers, and focal inflammation of the muscular wall. Although the covering mucosa appeared intact, these changes are considered as a result of blunt traumatic damage due to gavage application of the test compound. These lesions occurred occasionally and were not related to benzo[a]pyrene exposure.

Spleen. Occasional changes such as increased haemosiderin pigment, hyperaemia, lymphoid depletion or reticulum cell hyperplasia, were observed. Although these changes were found exclusively in treated animals, the incidence was too low to be considered significant.
No changes were observed in mesenteric lymph nodes and glandular stomach.

3. Verification of dose levels for the carcinogenicity study

As outlined in paragraph 3 from Appendix A, the aim of this 90-day sacrifice was to justify the dose regimen, and eventually, to adapt it if deemed necessary. This would mean that no frank toxicity would be tolerated.

In particular the forestomach was considered a target organ, in which ultimately the development of tumours was expected. A possible persistent irritation with frank hyperplasia induced by the compound would reduce the value of the tumour induction for extrapolation purposes (Wester and Kroes, 1988), and possibly reduce the life span of the rats. Fortunately, the hyperplasia was only minimal and could be characterised as "basal cell disturbance", and only in two out of 20 animals from the high dose group a small hyperplastic nodule was found, one of which showing dysplasia indicative of early neoplasia. However, due to the small size (no grossly observable changes!) and the random sampling, this figure is probably underestimated. Further analysis of the basal cell hyperplasia was carried out by morphometric counting of BrdU incorporation. This confirmed the minimal changes observed by histopathology, and in addition provided data that could be analysed statistically by which a significant increase was found from 10 mg/kg onwards**.**

Another phenomenon was the atropy of the thymus, a confirmation of the reduction of thymus weight observed at necropsy, which occurred only at the top-dose.

Despite the clear increase in liverweight by B[a]P (see Table 1), examination of histo-pathology at the microscopic level, as well as of clinico-chemical parameters (ALAT, γGT, LDH) did not reveal any signs of hepatocellular toxicity.

In conclusion, this study identified the forestomach and thymus as target organs for toxicity with minimal effects at the higher dose levels. Consequently, there was no indication for a need to change the dose regimen.

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APPENDIX B2 Study on DNA adduct formation

Preface

1. Experimental design
2. Results & comments
3. Conclusions

Study on DNA adduct formation

Preface

Within the groups of 74 animals subjected to the chronic treatment regimen with B[a]P 6 animals (per dose, per sex) were assigned for scheduled sacrifices after 4 and 5 months for examining DNA adduct formation by B[a]P. Only differences with this carcinogenicity protocol will be outlined here. The relevant results of these investigations will be described and will be supplied with some brief comments. Also, results obtained in preliminary investigations on DNA adduct formation by B[a]P will be shortly described and commented. More information is available within the files of this project.

1 Experimental design

1.1 *Animal housing, treatment, monitoring, necropsy and method of DNA adduct analysis*

Groups of 12 animals (per dose, per sex) were housed, treated, and monitored as described for animals subjected to the 104 week testing protocol (see Appendix B). Additionally, for purpose of DNA adduct analysis only, 12 animals (per sex) were treated with a dose level of 0.1 mg B[a]P/kg bw (the actually achieved dose level was 0.1 ± 0.02 mg/kg bw per application). The testagent used, the mode of application, and the safety measures applied for personnel and environment are those described for the carcinogenicity study (see Appendix B).

Groups of 6 animals (per dose, per sex) were sacrificed after 4 and 5 months of exposure to B[a]P, respectively, and the design was as follows. Upon induction of ether anesthesia blood was withdrawn by abdominal aortic puncture, immediately followed by total body perfusion via the abdominal caval vein with phosphate-buffered saline (PBS) of 37°C untill exsanguination was apparently complete. Finally, the total body perfusion was finished with ice-cold PBS for about 2 minutes. The following organs were then isolated, carefully cleaned (for further DNA adduct analysis), and stored at -70°C untill DNA isolation: brain, pituitary, heart, thyroid, lungs (stripped from trachea and bronchi), forestomach, stomach, esophagus, duodenum, jejunum, ileum, caecum, colon, and rectum, thymus, kidneys, urinary bladder, spleen, liver, pancreas, adrenals, sciatic nerve, quadriceps muscle, femur, skin, mammary, uterus or testis, ovary, prostate, and adipose tissue. DNA isolation and purification were according to standard procedures. DNA adduct analysis was by ^{32}P-postlabeling using the Nuclease P1 method essentially as described by Reddy and Randerath (1986), and quantitation of DNA adduct formation was via the use of an internal standard (i.e. adducted DNA derived by *in vitro* reaction of DNA with BPDE). DNA adducts and nucleotides on the TLC plates were visualised and quantified using a Storm phosphorimager (Molcular Dynamics) and imagequant software. The necessary validation experiments will not be described here, but are well documented in the files of this study-dossier.

2. Results & comments .

2.1 *Survival, body weight, and food- and water consumption*

Survival and body weight development for these animals was as described and depicted (see Figures 1 and 3, respectively) for the whole group in the main text. As can be concluded from this, no differences in survival or weight-gain between controls and B[a]P-treated animals could be observed within this four- or five-month period. Also, food- and waterconsumption of these animals seems not influenced by B[a]P treament within these periods, as is described in Appendix B and in the main text**.**

2.2 *DNA adducts*

Preliminary studies

In experiments with this animal strain conducted in preparation of the analysis of DNA adduct formation by B[a]P under conditions of the carcinogenicity assay several observations were made. First of all, DNA adducts by B[a]P were demonstrated in all organs examined, i.e. liver, forestomach, and white blood cells. Secondly, in these organs in total four different major adduct types of B[a]P could be identified (i.e. based upon their chromatographic behaviour on tlc plates in the postlabeling assay; see Figure 2 for illustration); these four adducts contributed quantitatively most to the total DNA adduct formation by B[a]P (and determined via this methodology), and were designated 1, 2, 3 and 4 based on their chromatographic localization on tlc plates. Thirdly, based on co-chromatographic behaviour with the synthetic analogue, adduct 2 could be identified as the well-known BPDE-N2-dG adduct (Weinstein *et al.,* 1976). Finally, the adducts appeared to have different half live times in different tissues, as determined by stop-experiments (Table 1).

Table 1. Half-life values (in days) of the four identified DNA adducts of B[a]P induced in liver, forestomach, and in white blood cells (WBC). Numbers are mean ± SD for the indicated number of animals.

adductnumber[1]	liver	forestomach (n=16)	WBC (n=14)
1		11 ± 3	
2 (BPDE-N2-dG)	28 ± 14 (n=12)	12 ± 3	13 ± 2
3		12 ± 3	19 ± 5
4	22 ± 2 (n= 6)	15 ± 8	

[1]) based upon chromatographic behaviour (see Figure 2).

Steady state levels of DNA adducts were considered critical for the 2-years study, as these were regarded to represent most appropriately the internal adduct dose to which tumour-development could be related. From the half-life values of the various adducts it was estimated that it would take about 4 months of continuous B[a]P exposure (i.e. by the applied gavage regime) to obtain steady state levels for all types of DNA adducts. Therefore, it was decided that DNA adduct formation had to be examined at the 4th and 5th month during the carcinogenicity test for establishing this steady state.

Carcinogenicity assay

Figure 1 depicts the results for the adducts having the longest half-lifes after 4 and 5 months of B[a]P-treatment. It shows that steady state levels are indeed achieved by 4 months of B[a]P exposure.

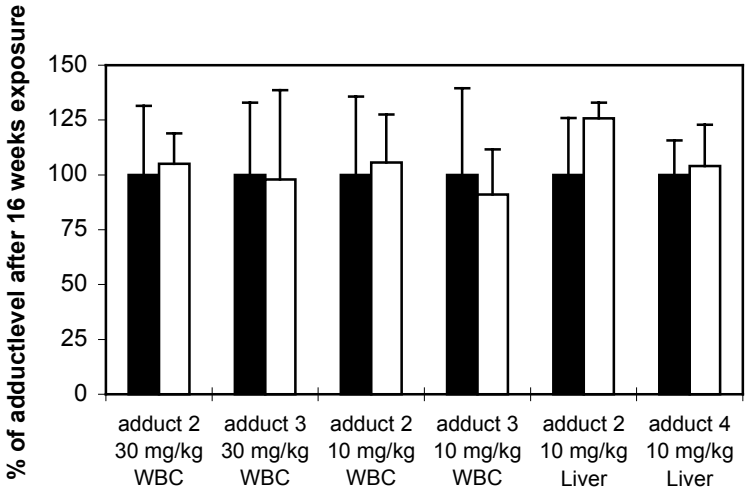

Figure 1. Relative DNA adduct levels (mean ± SD) in liver (n=4) and WBC (n=6) of male rats treated with 10 or 30mg B[a]P/kg bw for 4 months (filled columns; set to 100%) or 5 months (open columns).

Steady state DNA adducts levels in various organs and tissues

Figure 2 shows phospor-images of DNA adducts in a number of organs and tissues in the female rat after 4 months of treatment with 10 mg B[a]P/kg bw. From these images it is concluded that B[a]P induced DNA adducts in all investigated organs and tissues, though with qualitative differences in types and amount of adducts per site. Table 2 provides these data in a quantitative way.

Tabel 2 Steady state levels of B[a]P adducts at nine different sites in a female rat treated with 10 mg B[a]P/kg bw for 4 months. Values are individual numbers from the same animal.

	absolute number of adducts per 10^{10} nucleotides per adduct type						% of total per adduct type				
adduct type / organ	1	2	3	4	11[1]	total	1	2	3	4	11[1]
liver	636	1920	118	24305	232	27211	2	7	0	89	1
kidney	1331	1842	168	9855	900	14096	9	13	1	70	6
heart	4543	7388	1160	372	1907	15370	30	48	8	2	12
lung	2099	3283	510	369	944	7205	29	46	7	5	13
skin	476	3158	233	820	0	4687	10	67	5	17	0
WBC	165	2662	521	51	0	3399	5	78	15	2	0
forestomach	557	2316	204	153	219	3449	16	67	6	4	6
gl. stomach	313	777	77	243	0	1410	22	55	5	17	0
brains	111	523	70	0	136	840	13	62	8	0	16

[1]) unknown DNA adduct of B[a]P encountered in some tissues.

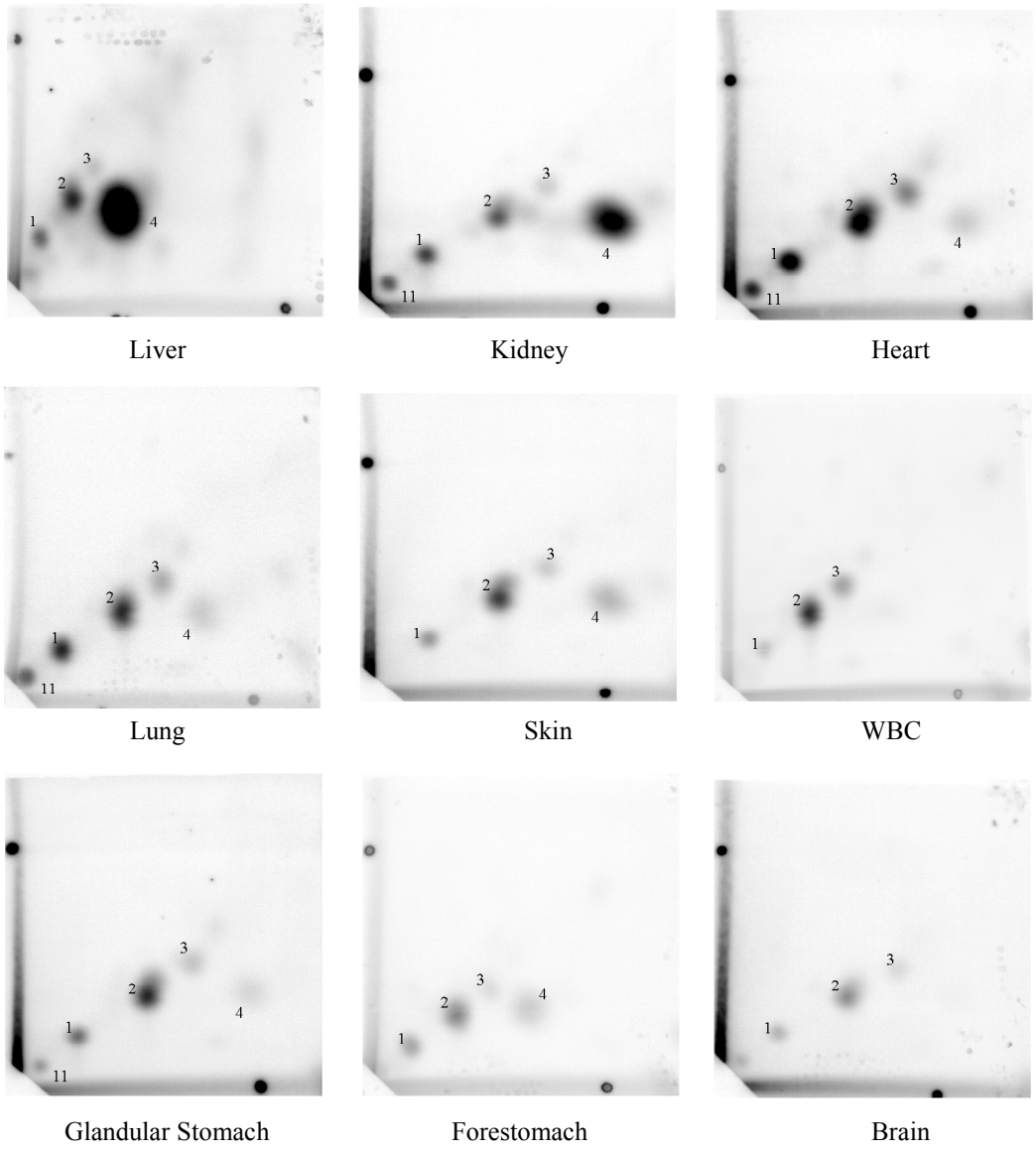

Figure 2. Phorphorimages of DNA adducts induced by B[a]P in nine organs and tissues of a female rat treated with 10 mg B[a]P/kg bw for 4 months. These images are the results of a two-dimensional tlc run of ^{32}P-labeled adducts; the intensity of the spot relates to the amount of adducts formed: these images only provide a qualitative picture of the types and amounts of DNA adduct found in the various tissues, and need several corrections (e.g. background signal, label specificity, recovery of the standard etc) for quantification (resulting in data provided in Table 2.)

Although B[a]P most probably induces DNA adducts in all exposed organs and tissues of the rat, at this dose level of 10 mg/kg bw only in a few of these sites tumours ultimately develop: i.e. liver, forestomach, oesophagus, and skin & mammary. This implies that the presence of DNA adducts *per se* is not a pivotal factor in tumour development, and other possible events should be involved.

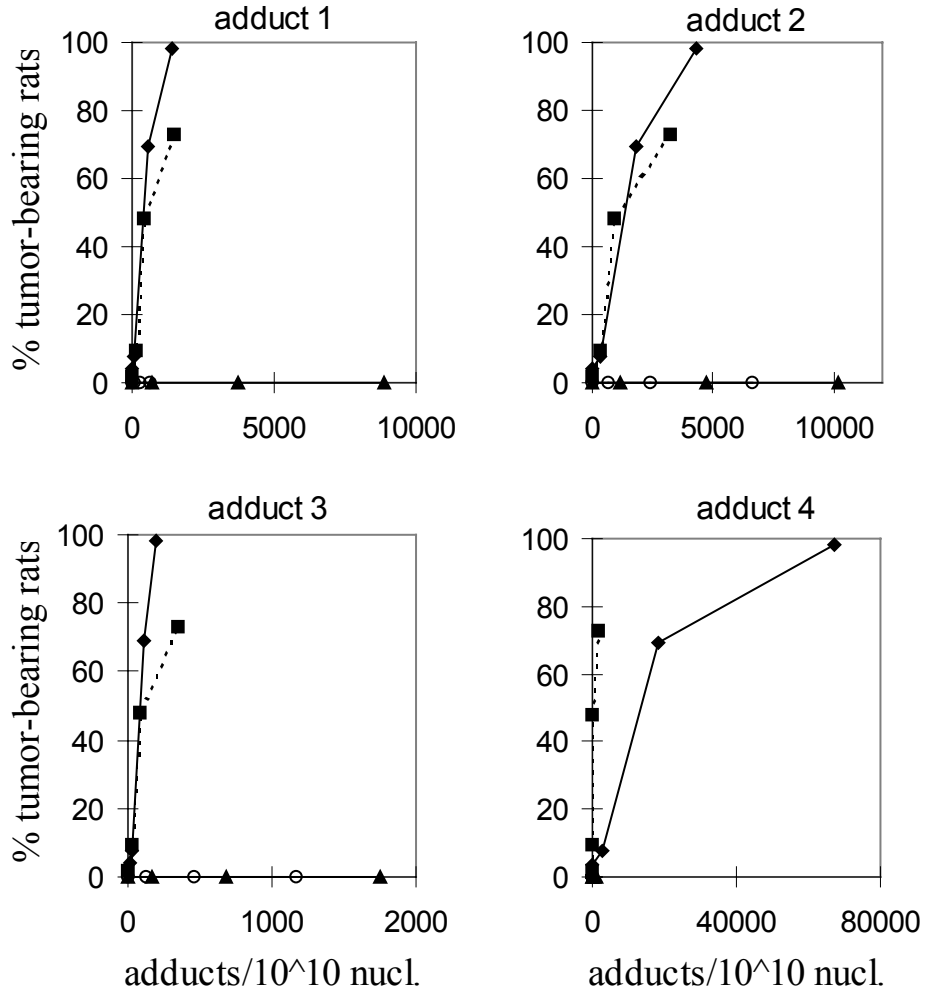

Figure 3. Relationship between steady state levels of specific DNA adducts after 4 months of treatment and tumour-induction by B[a]P after 2 years in the female rat. Depicted DNA adduct values are mean ± SD for 6 animals (liver, ◆; forestomach, ■) or for 4 animals (lung, ▲; WBC, ○).

This conclusion for DNA adducts of B[a]P was recently also reported by Culp *et al.* (1998). For example, specific DNA adducts, or the total number of DNA adducts may be considered next as critical factors here. However, as shown by the data presented in Table 2 and Figure 3, both these explanations apparantly do not hold as well[10]. Remarkably, the highest levels of DNA adduct 2 are found in the lung, an organ devoid of any tumour development or even indications of preneoplastic changes in the present set-up, but a clear target-organ after inhalation exposure to B[a]P (Laskin *et al.,* 1970). Therefore, factors additional to DNA adduct formation apparently are critical to tumour development by B[a]P. A serious candidate factor for this is increased local cell proliferation. For both major target organs for

[10] Some caution is warranted here as: [1st]) the presented DNA adduct figures are from the tissue as a whole, and clear regional or zonal differences in DNA adduct formation, as well as differences between cell-types within the tissue may still exist; Yet, as the presence of adducts at all sites (apart from adduct '4') may be (partly) explained by migration of albumin-bound precursor species (Ginsberg and Atherhold, 1989; Wall *et al.*, 1991; own unpublished data), such a possibility is not really supported; [2nd]) B[a]P DNA adducts may not be able to bring about mutations in tissue-specific genes essential for tumor-formation.

carcinogenesis, liver and forestomach, increased mitogenic activity upon B[a]P- treatment has been shown by an increase in weight (liver) and proliferation markers e.g. certain histopathological characteristics and BrdU-incorporation (forestomach) (see Dose range-finding study, Appendix A). Liver weight increase was due to hyperplasia, i.e. an increase in cell-number, and not to hypertrophy (as demonstrated by morphometric analysis; data not shown). The critical dependence of a parallel increased mitogenic activity yet has not been fully demonstrated, as its absence was not verified in non-target-organs. It should be recognized, though, that this is rather difficult to prove: minimal increases in these organs at specific time-intervals, if at levels detectable at all, may be easily missed.

If one assumes local cell proliferation as the critical additional factor needed for tumour-induction (strongly supported by other studies: see main text), the underlying mechanism also is a critical issue, as it will have clear implications for the dose-response for tumour induction and by this the risk assessment as well. At present, it is unclear which mechanism underlies this proliferation in liver and forestomach. One possible mechanism for the liver, i.e. regeneration in response to cytotoxicity (as shown for various hepatotoxicants) seems to be ruled out here: histopathological examination and clinical chemistry did not show evidence for hepatotoxicity (see Appendices A and B1; one should realize, however, that this has not been monitored over the first few weeks).

For those organs developing tumours upon B[a]P treatment a dose-response relationship could be observed indeed between the number of DNA adducts and tumour incidence (see Figures 4 and 5).

High to low dose extrapolation

One of the various reasons for studying DNA adduct formation by B[a]P in this project was their presumed critical role in tumourigenesis and their potential detectability at dose levels where treatment-related increases in tumour incidence is not observable anymore (i.e. within the number of animals normally used in bioasssays). The reason for this being relevant is that in the estimation of human cancer risks associated with exposure to genotoxic carcinogens, experienced exposure levels are usually several orders of magnitude lower than those used in animal studies. Also, the risk levels accepted in environmental and public health policy are in the order of one per at least 10.000 exposed or one per million exposed, respectively, i.e. far below incidences observable in experimental studies. For these risk calculations it is assumed that the exposure-effect relationship outside the observable domain is linear. A basic element in this is the assumed linearity between applied dose and DNA adduct formation. As one cannot extrapolate dose-tumour responses to low doses, dose-DNA adduct responses may somehow function as a surrogate for this.

Figure 4 shows the relationship between applied dose and DNA adduct levels in four organs, two of which are target for carcinogenesis. DNA adducts are still detectable at the lowest tested dose of 0.1 mg B[a]P/kg bw, which is roughly 30 times below the dose level showing small and marginal carcinogenic responses in two of these organs, i.e. liver and forestomach. Over the investigated 300-fold dose-range, i.e. from 0.1 to 30 mg/kg bw, there is a fairly linear relationship between the applied dose of B[a]P and DNA adduct formation (Figure 4). This is also suggested by the presentation of the data in Figure 5.

A more subtle way of displaying the linearity of the relationship between (external) B[a]P dose and DNA adduct levels is shown in Figure 6. Clearly, one can see a relative increase in adduct levels in the liver with increasing dose. This probably relates to EROD induction in this organ (see Appendix A, Range-finding study), which seems to have a clear

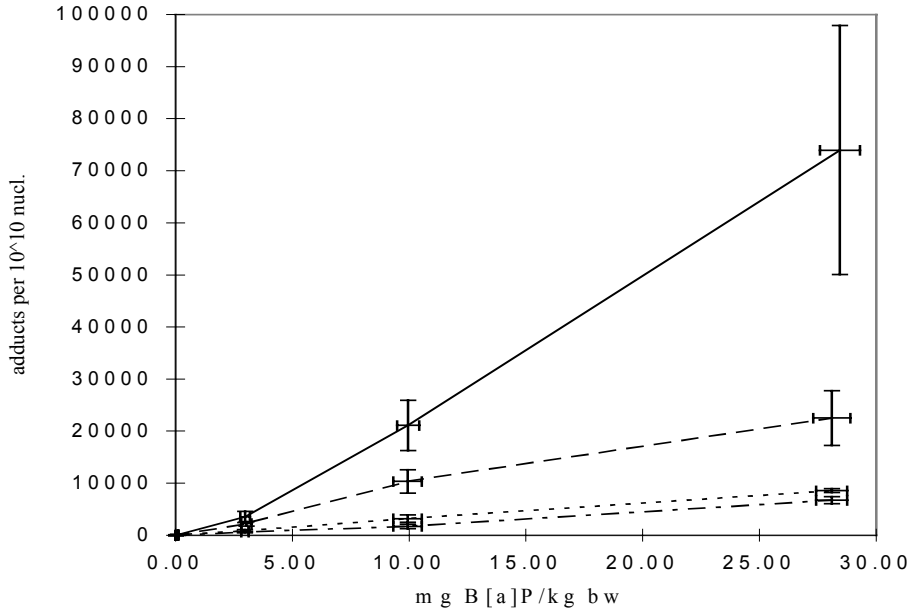

Figure 4. Relationship between B[a]P dose and steady state levels of DNA adducts in four organs in the female rat after 4 months. Depicted are total DNA adduct values as mean ± SD for 6 animals for liver (—) and forestomach (· – ·), and for 4 animals for lung (– –) and WBC (····).

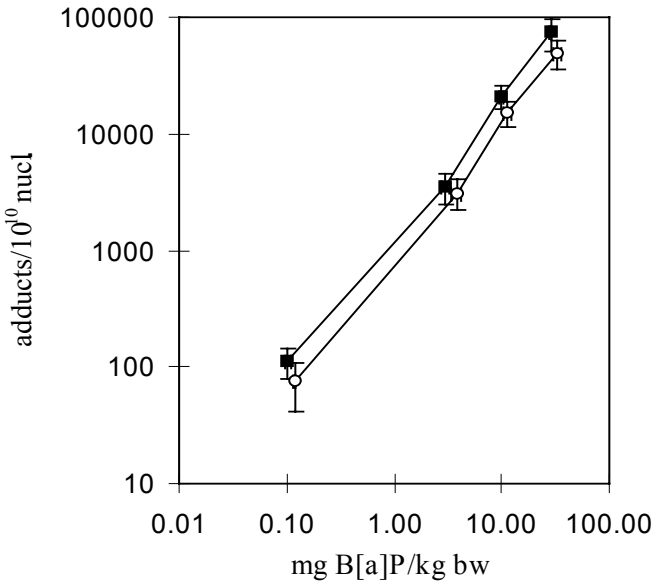

Figure 5. Relationship between B[a]P dose and steady state levels of DNA adducts in female and male rat liver after 4 months. Depicted are total DNA adduct values as mean ± SD for 6 females (■) and males (○).

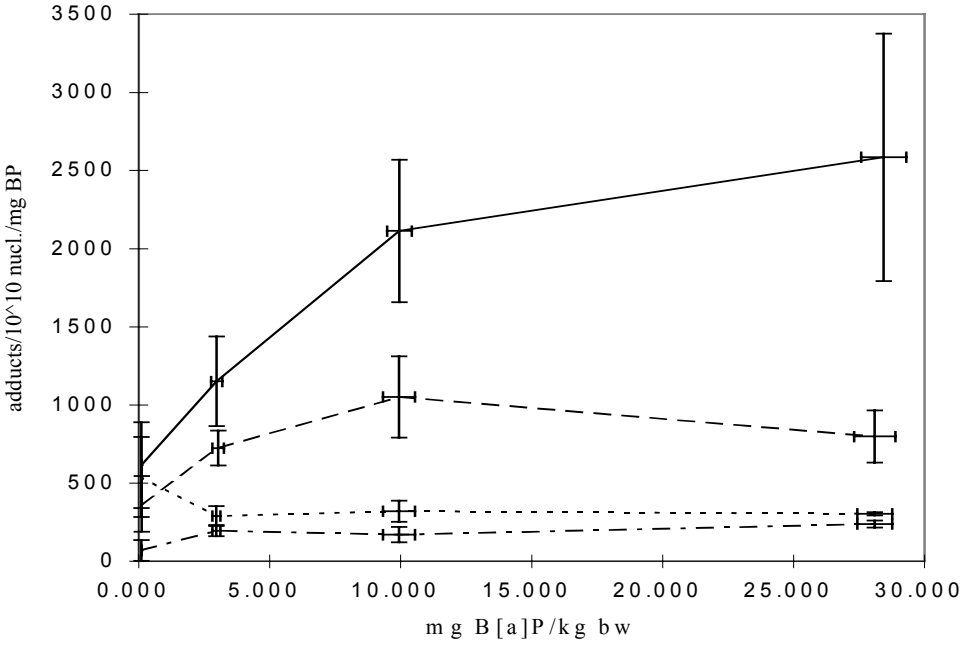

Figure 6. Efficiency of DNA adduct formation at different B[a]P dose levels in four organs in the female rat after 4 months of treatment. Depicted are total DNA adducts formed per mg B[a]P/kg bw. Values are mean ± SD for 6 animals for liver (—) and forestomach (· – ·), and for 4 animals for lung (– –) and WBC (- - -).

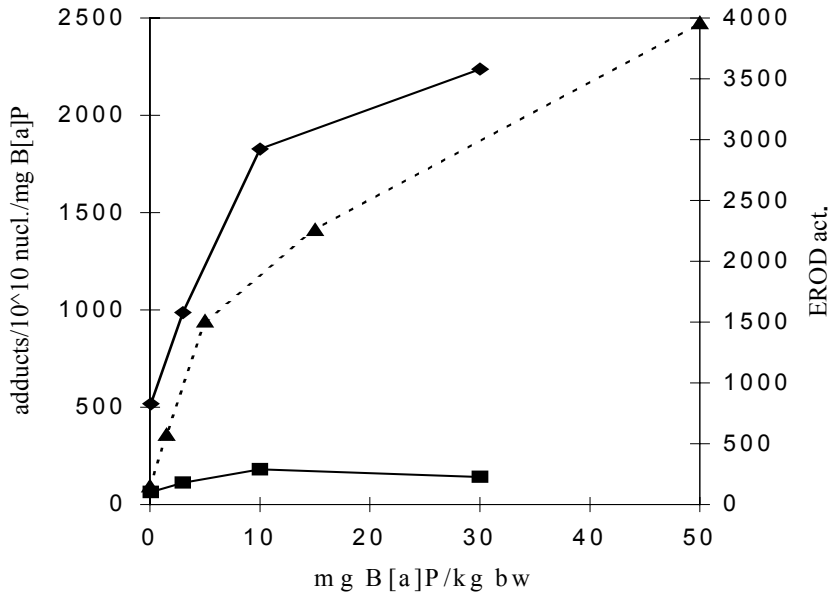

Figure 7. Relationship between efficiency of DNA adduct formation at different B[a]P dose levels and EROD induction in female liver. Depicted are mean DNA adduct 4 (◆) and 2 (■) values, and EROD activity (▲) as (nmol/min,g protein), respectively. DNA adduct levels are for 6 animals after 4 months of treatment, EROD activity is for 8 animals and is determined after 5 weeks of treatment (Dose range-finding study, Appendix A).

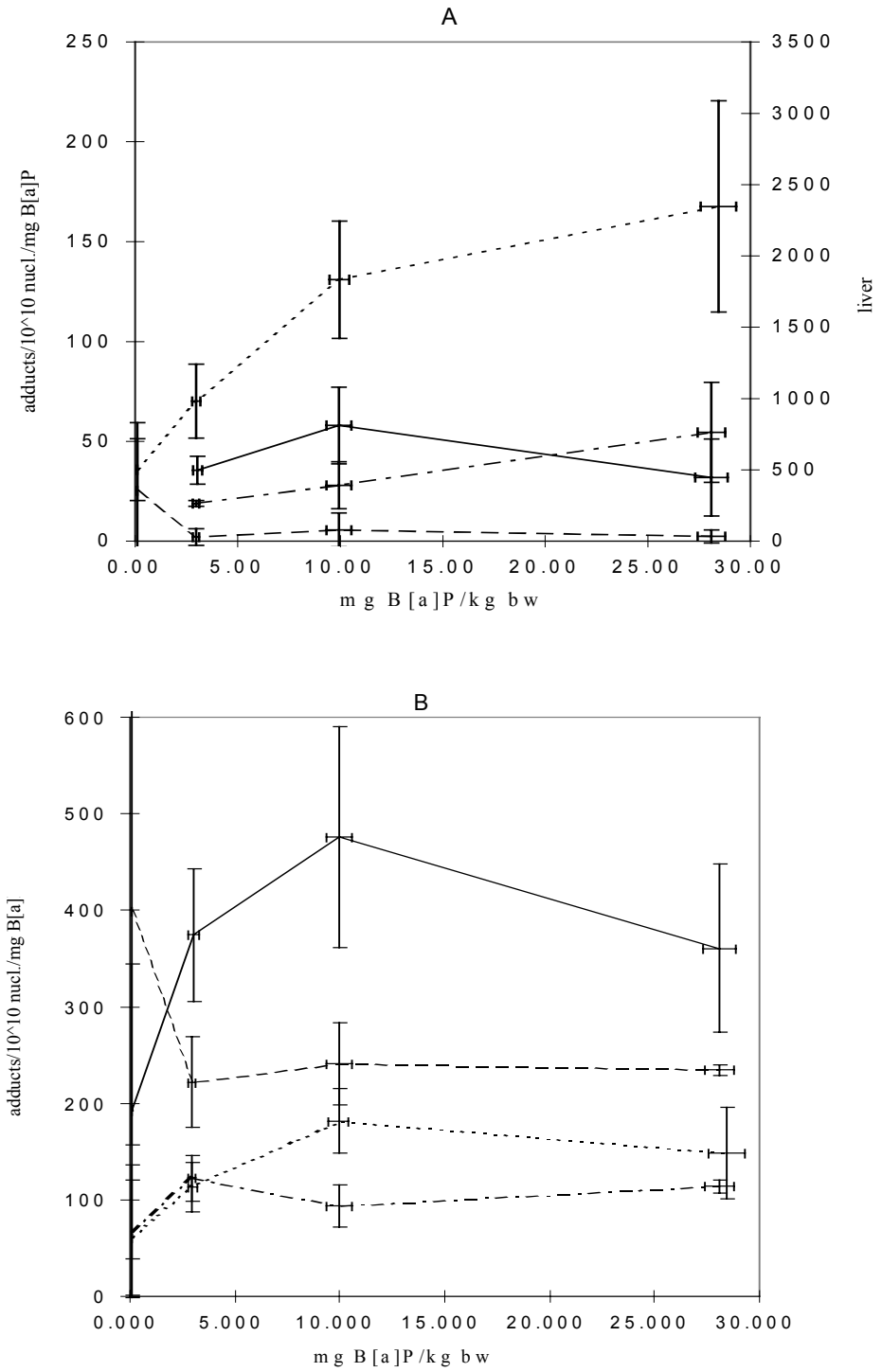

Figure 8. Efficiency of DNA adduct formation at different B[a]P dose levels in four organs in the female rat. Depicted are DNA adducts 4 (A) and 2 (B) formed per mg B[a]P/kg bw. Values are mean ± SD for 6 animals for liver (- - -), and forestomach (· – ·), and for 4 animals for lung (—), and WBC (– –).

impact on formation of adduct 4, not of adduct 2 (Figure 7). Figure 8 shows that this increased efficiency of DNA adduct formation with dose is more or less confined to adduct 4 in the liver, which constitutes about 80% of total DNA adduct levels in this organ.

Thus, taken together, within the dose range studied adduct formation seems to be fairly linear with the applied dose, except for adduct 4 in the liver.

3. Conclusions

B[a]P-induced DNA adducts were monitored by ^{32}P-postlabeling analysis, which enables a very sensitive detection of stable DNA adducts.

DNA adducts were found in all investigated organs and tissues, including blood cells. It should be remarked that the samples examined represented whole, thoroughly perfused organs and tissues, thus masking possible subcompartmental differences in adduct formation and/or repair, but excluding contamination by DNA adducts from captured blood cells. As tumours appeared in just a few organs, it may be concluded that the formation of stable DNA adducts by B[a]P *per se* is not sufficient for tumour-induction. Also, the data did not give support to the possibility that either the total number (i.e. intensity) of adducts or the presence of specific adducts are responsible for tumourigenesis. It should be remarked, though, that a critical role for regional differences in DNA adduct levels within an organ in this yet cannot be completely excluded here for which immunohistochemistry of DNa adducts could be instrumental. Based upon a few observations within the range-finding study and at the 90-day sacrifice, it is speculated that local cell proliferation may be the additional critical factor for tumour development.

As far as data could be analysed the relationship between applied dose and DNA adduct formation appears fairly linear down to the lowest investigated dose, which is 30-fold below the dose showing marginal carcinogenic effects upon chronic exposure. An exception here forms the liver DNA adduct 4, which shows a disproportionate increase in levels with increasing dose, in parallel with increased levels of EROD.

Literature

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APPENDIX C NCTR carcinogenicity study in mice

(Culp *et al.,* 1998)

Preface

1. Summary of the results observed in the mouse diet studies
2. Comments to the results obtained by Culp *et al.* (1998)
3. VSDs based on the NCTR mouse bioassays
4. Conclusions

NCTR carcinogenicity study in mice

Preface

This Appendix summarizes the relevant findings of a recent mouse diet study with B[a]P at NCTR (Culp *et al.*, 1994 and 1998). There are two main reasons for paying special attention to this study within the scope of this report. First of all, it is the only other chronic oral carcinogenicity study with B[a]P in rodents sofar that meets todays quality standards, thus providing a basis for a direct comparison with the results obtained in our rat study. Secondly, this NCTR project also incorporated two oral chronic studies with coal tar mixtures, thereby allowing a comparison between the effects of B[a]P solely, and that of B[a]P as PAH-mixture indicator.

The study will only be briefly described here, mainly focussing on the two issues mentioned above. For further details the reader is referred to the above mentioned papers by Culp *et al.*

1 Summary of the mouse diet studies

Design

Groups of 48 female 5-weeks old B6C3F1 mice were administered via the diet either B[a]P (5, 25, and 100 ppm), coal tar mixture 1 (100, 300, 1000, 3000, 6000, and 10000 ppm), or coal tar mixture 2 (300, 1000, and 3000 ppm) for 24 months. Two additional groups of 48 mice served as controls, i.e. one was fed the standard diet, the other received standard diet that had been treated with aceton in a manner identical to the B[a]P diets (Solvent control).

Results

With respect to B[a]P, at the highest dose tested a decrease in body weight was observed from week 50 onwards (not shown), most probably due to effects secondary to tumour-development in the upper digestive tract. As can be seen from Table 1 (which is adapted from Culp *et al.,*1998, and personal communications) B[a]P induces in these female B6C3F1 mice tumours in the epithelial layer of the tongue, the esophagus, and the forestomach. Also, increased incidences of sarcomas and of tumours in the squamous cell of the larynx were observed (not shown). Clearly, B[a]P treatment did not induce tumours in the liver and lung, nor hemangiosarcomas or histiocytic sarcomas (latter not shown).

Treatment with coal tar mixtures led to a decreased gain in body weight for 0.6 & 1.0% mixture 1, and 0.3% mixture 2 (not shown). These mice also showed a decreased food consumtion as compared to controls (see Table 1). Interestingly, increases in liverweight of about 40% were observed for both 0.3% mixtures over control values, whereas treatment with 25 ppm B[a]P did not result in an increased weight in this organ (liverweights of higher exposed animals were not determined due to tumour development and accompanied decreases in body weights; not shown). Interestingly, with respect to tumour induction (see Table 1), these 0.3% mixtures also lead to increased liver tumour incidences.

Table 1. Incidences of neoplasms in female B6C3F1 mice treated with B[a]P or coal tar mixtures 1 or 2. Number of tumour-bearing animals (TBA) per dose-group are indicated as: TBA/ number examined. Data taken from Culp et al.(1998).

agent	B[a]P[1]	Fd cons[2]	dose[3]	Tongue[4]	Oesophagus[5]	Forestomach[6]	Liver[7]	Lung[8]	Small intest.[9]	hemang.sarc.[10]
B[a]P	0			0/48	0/48	0/48	2/48	5/48	no	1/48
	5		0.6	0/48	0/48	3/47	7/48	0/48	no	2/48
	25		3	2/46	2/45	36/46	5/47	4/45	no	3/47
	100		12	23/48	27/46	46/47	0/45	0/48	no	0/48
Coal T1	0.22		0.03	0/47	0/47	2/47	4/48	3/48	0/46	0/48
	0.67		0.08	0/47	0/46	6/45	2/46	4/48	0/45	1/48
	2.4		0.3	0/47	0/47	3/47	3/48	4/48	0/47	1/48
	6.7	ns[11]	0.8	0/47	0/48	14/46	14/45	27/47	0/42	11/48
	13.0	25	1.2	0/46	0/47	15/45	1/42	25/47	22/36	17/48
	22.4	30	1.9	0/43	0/43	6/41	5/43	21/45	36/41	1/45
Coal T 2	1.1		0.15	0/47	0/48	3/47	7/47	4/48	0/47	1/48
	3.7	ns	0.4	0/48	0/46	2/47	4/47	10/48	0/47	4/48
	11.0	20	1.1	0/47	1/44	13/44	10/45	23/47	1/37	17/48
CT.control	0			0/48	0/48	1/48	0/47	2/47	0/47	1/48

[1]) B[a]P concentrations in the administered diets (in ppm); [2]) observed % reduction in foodconsumption as compared to controls (daily foodconsumption ≈ 3.8 g per animal; mean lifetime weight was about 32 g [personal communication]); [3]) (rounded) calculated daily intake of B[a]P in mg/kg bw, corrected for reductions in foodconsumption; [4]) Tongue (dorsal surface of the base) papillomas and/or squamous cell carcinomas; [5]) Oesophagus squamous cell papillomas and/or carcinomas; [6]) Forestomach papillomas and/or carcinomas of the squamous epithelium; [7]) Hepatocellular adenoma and/or carcinoma; [8]) Lung alveolar/bronchiolar adenomas and/or carcinomas; [9]) Small intestine adenocarcinomas; [10]) Hemangiosarcomas; [11]) ns: not significant.

Liver tumour incidence was not increased at the higher concentrations of mixture 1, which can be explained by the earlier onset of fatal tumours at other sites, e.g. the small intestine. Next to these two tumour sites, Table 1 further shows the other sites of carcinogenesis by these coal tar mixtures: i.e. the lung, forestomach, and endothelial cells of bloodvessels (hemangiosarcomas).

2. Comments to the results obtained by Culp *et al.* (1998)

Tumourigenicity of B[a]P and coal tars

When comparing the observed tumour sites in rats and mice orally exposed to B[a]P the common target sites of the upper digestive tract, i.e. oral cavity, oesophagus, and forestomach are noticed. They have also been recognized as such by others (Horie et a, 1965; Chouroulinkov *et al.,* 1967; Neal and Rigdon, 1967), clearly confirming B[a]P as a site-of-contact carcinogen. All these tumour sites in mice showed a remarkable steep dose-response relationship, also in line with previous observations by Neal and Rigdon (1967), and Weyand *et al.* (1995). Also, in concordance with the results obtained in our rat study, oral exposure to B[a]P did not lead to tumour development in the respiratory tract, a finding which conflicts earlier observations in mice of Rigdon and Neal (1966), and Weyand *et al.* (1995). In this latter case the A/J strain of mice were used that are known for their particular sensitivity to lung tumour development. Lung tumours are commonly observed after direct exposure of this tissue as demonstrated for mice by Furst *et al.* (1979), and for rats by Ishinishi *et al.* (1979), Deutsch-Wenzel *et al.* (1983), and Wenzel-Hartung *et al.* (1990). In rats, additionally, tumours are induced in the Zymbal gland and jejunum, though at dose levels exceeding those administered to the mice (on a weight per body weight-basis), i.e. 30 mg/kg bw.

Additionally, some remarkable findings can be noted in these mice studies by Culp *et al.*. First of all, the liver being one of the most sensitive target sites for carcinogenesis in the rat, does not seem to be a target-organ in the mouse. Livertumour development is induced in rats at dose-levels that also induce forestomach tumours in this species (see Appendix B,Table 3), and that are about comparable on a weight-to-unit-body weight-basis to those used in the mouse assay. The coincidental finding of both liver-growth and hepato-carcinogenesis at this site in mice exposed to either coal tar mixture (i.e. at a 0.3% diet concentration) is remarkable, and suggests a critical promotional role for mitogenic stimuli, evidently provoked by other coal tar ingredients. Also, the observation of liver tumours after B[a]P i.p. injection of infant male B6C3F1 mice may be taken as support for this (Rodriguez *et al.*, 1997). However, DNA adduct formation may also be critical here. As shown in Appendix B2, B[a]P-induced DNA adduct 4 is a major adduct found in rat liver; this adduct type was not detected in mouse liver upon B[a]P treatment (Culp *et al*., 1994). Also, analysis of the coal tar treated mice (Culp *et al.,* 1994) supported a possible role for DNA adduct formation *per se* in tumour development at this site: though about similar BPDE-N2-dG adduct levels were observed – for comparable intakes of B[a]P in B[a]P-diet and in coal tar mixed diet - total adduct levels showed about 10-fold higher after the coal tar mixture[11]. Thus, the absence of this tumour in a species known to be extra sensitive for tumour development at this site yet cannot be satisfactory explained. It should also be noted at this place that the liver tumours in these mice induced by coal tar mixtures predominantly (>80%)

[11] It should be noted, however that these binding levels were calculated based on a comparison to the [^{3}H]BPDE-DNA standard, which may have different effinciencies of hydrolosis or labeling than the DNA-adducted components found in coal tar-fed mice.

were designated adenomas, i.e. benign in nature, whereas those induced in the rat were almost exclusively in a malignant state.

Secondly, though B[a]P itself did not induce lung tumours in the mouse up to a daily dose of about 12 mg/kg bw, both coal tar mixtures clearly induced alveolar/bronchiolar tumours, though mainly (>90%) adenomas, already at B[a]P levels of about 0.5 mg/kg bw (see mixture 2, Table 1). Again, this suggests that factors additional to B[a]P are critical in tumour development at this site. DNA adduct analysis in male mice by the same group (Culp *et al.*, 1994) showed that DNA adduct levels in the lung considered to be BPDE-N2-dG adducts (co-eluting radioactivity) are about 10-fold higher after B[a]P as coal tar constituent, and that total DNA adduct levels were about 90-fold higher after coal tar exposure as compared to solely B[a]P exposure[11]. Weyand *et al.* (1995), who did find lung tumours upon feeding solely B[a]P, i.e. in sensitive A/J mice, also found a considerably increased incidence after coal tar feeding.

Thirdly, both coal tars, though most evidently mixture 1, induced small intestinal tumours in the mouse, whereas relatively high doses of solely B[a]P were ineffective in this respect. As noticed above, this site is a target for carcinogenesis in the rat, be it at clearly higher B[a]P exposures than those used in the mouse assay. From the mouse assay it is clear that the dose-response for this tumour is also strikingly steep. Because of the observation of a nearly 50% increase in the number of S-phase cells after 4 weeks of exposure to the two highest doses of mixture 1, and the absence of this effect up to and including the 0.3% mixture, these tumours most probably have arisen via locally induced cell proliferation instead of by DNA adduction (Culp *et al.,* 1998).

The absence of tumours in the oral cavity (tongue) and oesophagus upon treatment with coal tars, both target sites for B[a]P carcinogenesis, may be explained by the fact that these tumours only arise after relatively high local B[a]P exposure levels: since they were only observed at a daily B[a]P dose of about 12 mg/kg bw (100 ppm), not after 3 mg/kg bw (25 ppm), whereas daily B[a]P doses in the coal tar mixtures were at most 1.9 mg/kg bw (see Table 1).

B[a]P as PAH indicator

The availability of concurrent bioassays on orally administered B[a]P and these coal tars enables for the first time a comparison of the relative carcinogenic potency of these PAH mixtures with respect to B[a]P via this route (Culp *et al.,* 1998). For this, though, one has to assume that all effects induced by these coal tar mixtures and critical to carcinogenesis are solely due to PAH. One of the methods for establishing quantitative cancer risk estimates for mixtures is based on their relative potencies or toxic equivalency factors (TEF). In a TEF approach for PAH, the overall carcinogenic potency of the mixture is expressed relative to B[a]P and contributions of individual carcinogenic PAH are taken to be additive (Slooff *et al.*, 1989). Ideally, a reference or indicator chemical should induce a qualitatively similar spectrum of tumours in a specific animal species as found with the whole mixture. Implicit to this is the supposition that basically similar mechanisms of action are operative, which justifies such a surrogate approach. For the presently investigated PAH mixtures this would mean that B[a]P ought to induce tumours in lung, forestomach, liver, and small intestine, as well as to induce hemangiosarcomas. Of all these sites B[a]P *per se* appeared only able to induce tumours in the forestomach. From this point of view B[a]P really is not an ideal reference compound for these coal tar mixtures. One should bear in mind, though, that non-PAH constituents may be involved here for which B[a]P cannot be expected to substitute. As cited above, it is unclear what constituents of these coal tar mixtures - and by what

mechanisms - induce or promote tumour development at these other sites (see above and Culp *et al.*, 1998, Goldstein *et al.*, 1998). Although B[a]P may not be an ideal reference compound for these coal tars, alternative ways of establishing quantitative cancer risk estimates for PAH mixtures are problematic as well. One may take a unit dose of coal tar itself in risk assessment rather than that of a carcinogenic PAH constituent. However, there are clear qualitative and quantitative differences in the composition of the various coal tars (Goldstein *et al.,* 1998), not to speak of PAH mixtures from other sources. Therefore, taking one of the most potent carcinogenic PAH constituents, i.e. B[a]P (that is well studied, and extensively monitored as well), and a conservative TEF value for the other PAH constituents present, may not only be pragmatic but also justifiable for establishing quantitative cancer risk estimates for PAH mixtures. The absence of clear evidence for a critical non-PAH contribution or a non-PAH-like mechanism supports such an approach for the time being. Also, current risk assessment methodology does not ask for a well-established mechanism in this respect. The PAH indicator role of B[a]P will be further discussed in Appendix E.

3. VSDs based on the NCTR mouse bioassays

B[a]P bioassay

The results by Culp *et al.* (1994, 1998) allow the calculation of VSDs in a way as described in the main text for the rat study. Table 2 depicts the VSDs calculated from this mouse bioassay, which amount around 5 ng B[a]P/kg bw, when either taking a single tumour-site (i.e. forestomach) or the number of tumour bearing animals (TBA) as point of departure. A lifetime correction was not performed here as the default value of 750 days for this species (HCN, 1995) was more or less matched by the experimental period of 735 days and a survival of about 60%. Resembling observations in the rat study, a rather steep dose-response curve for the forestomach tumours was observed in this mouse study from low to mid dose (i.e. from 0.6 to 3 mg B[a]P/kg bw, as calculated from diet consumption; see Table 1). This suggests that by departing for linear extrapolation from the 3 mg/kg bw dose one might overestimate low-dose associated cancer risks. Nonetheless, for VSD derivation the most sensitive species/site combination for tumour development is taken, and as long as there is no reason to detract from this on the basis of human relevance for these tumours (HCN, 1996). Thus, from the present data a VSD of 5 ng B[a]/kg bw is derived.

Coal tar bioassays

As described above VSDs for B[a]P range around 5 ng B[a]P/kg bw. If individual tumour sites are taken for calculation of VSDs for the coal tar mixtures a range of 2 – 3 ng/kg bw is found for B[a]P as PAH-indictaor, when combining benign and malignant tumours (Table 3). If the TBA is taken instead a VSD-range of 1 – 3 ng/kg bw is found for coal tars (Table 3).

Comparing the absolute carcinogenic potency (i.e. without considering differences in target-tissues) for B[a]P itself and as coal tar indicator on the basis of the methodology used for deriving VSDs (see paragraph 4.2.2 main text), shows that the carcinogenic potency of the coal tar mixtures is about 2 to 5 times that expected purely on the basis of the amount of.

Table 2. VSDs based on the results of the oral carcinogenicity study with B[a]P in female B6C3F1 mice (modified from Culp et al., 1998).

Site	TD_{sign} [1]	I_{sign} [2]	VSD [3]
Forestomach squamous cell papillomas & carcinomas combined squamous cell carcinomas	 3 3	 0.76 0.65	 4 5
Tongue, papillomas and / or carcinomas carcinomas	 12 12	 0.48 0.15	 25 80
Oesophagus, Papillomas and / or carcinomas Carcinomas	 12 12	 0.59 0.24	 20 50
TBA, All treatment-related tumours [4] Benign and malignant tumours Malignant tumours	 3 3	 0.78 0.65	 4 5

[1]) TD_{sign}= the lowest dose level (mg/kg bw; 5 times a week) associated with a significantly increased tumour response; applied dose in mg B[a]P/kg bw, calculated from food consumption data (about 3.8 grams/day), and averaged body weights (about 32 grams); [2]) I_{sign}= observed incidence at the TD_{sign} minus background incidence;[3]) calculated VSD in ng B[a]P/kg bw (numbers rounded) as described in paragraph 4.2.2 main text; [4]) also oesophagus and tongue tumours included.

Table 3. VSDs based on the results of the oral carcinogenicity study with coal tar mixtures 1 and 2 in female B6C3F1 mice (modified from Culp et al., 1998).

Site	TD_{sign} [1]		I_{sign}		VSD [2]	
	CT 1	CT 2	CT 1	CT 2	CT 1	CT 2
Liver, Hepatocellular adenomas	 0.8	 -	 0.31	 -	 3	 -
Forestomach, Squamous cell papill. & carcinomas comb. Squamous cell carcinomas	 0.8	 -	 0.30 0.15	 -	 3 5	 -
Lung, Alveolar / bronch. adenomas & carcinomas Alveolar / bronch. carcinomas	 0.8	 0.44 -	 0.53 0.06	 0.17 -	 2 13	 3 -
TBA, liver & forestomach [3] Benign and malignant tumours Malignant tumours	 0.8	 -	 0.47 0.19	 -	 2 4	 -
TBA, All treatment-related tumours [4] Benign and malignant tumours Malignant tumours	 0.8	 0.44 -	 0.66 0.23	 0.17 [5] -	 1 4	 3 -

Only dose levels without reduced feed consumption are taken; [1]) applied dose in mg B[a]P/kg bw, calculated from food consumption data (about 3.8 grams/day), and averaged body weights (about 32

grams); [2]) calculated VSD in ng /kg bw for B[a]P as PAH indicator (numbers rounded), as described in paragraph 4.2.2 main text; [3]) for comparison with the VSDs calculated from the B[a]P bioassay; [4]) also lung tumours included; [5]) only lung tumours; response curve for the forestomach tumours was observed in this mouse study from low to mid dose (i.e. from 0.6 to 3 mg B[a]P/kg bw, as calculated from diet consumption). As argued above this would favour VSDs derived from the lower dose, which range from 15 to 30 ng B[a]P/kg bw.

B[a]P present. Obviously, this methodology has (implicit) assumptions with a high degree of uncertainty (see main text for discussion). For example, the noted steep dose-response for the induction of forestomach tumours by B[a]P suggests that higher VSD values would have been obtained if the next lower dose (0.6 mg/kg bw) was used as point of departure for low-dose extrapolation. Consequently, this would also have resulted in a higher factor for B[a]P as coal tar-indicator

3. Conclusions

The results of the NCTR studies with B6C3F1 female mice allow several conclusions to be made. First of all there is good agreement between the B[a]P dose levels – on a weight per unit body weight basis – that induce tumours in rats and mice. The VSDs (as ng B[a]P per kg body weight) derived from these studies, therefore, are also quite close, i.e. from around 10 for rats, and 5 for mice. Although there is some overlap in target-organs for carcinogenesis between these two species, remarkable differences are also observed: the liver is a major target-organ in the rat, whereas this site is not targeted in this susceptible mouse strain.

Quite remarkable also is the observation of rather steep dose-response relationships for tumour induction by B[a]P as well as by the coal tar mixtures in most major target-organs, i.e. forestomach, lung, oral cavity, small intestine, and liver. Though this is suggestive of a critical role within the mode of action of non-linear (perhaps even threshold-like) events, convincing evidence for this is lacking sofar. Evidently, conclusive evidence in this would be of prime interest for the risk assessment evaluation. As pointed above, by taking another TD_{sign} for linear extrapolation, both VSD values as well as the correction factor for B[a]P as coal tar indicator would substantially change (for further discussion on this point see main text).

The evaluation of B[a]P as PAH-indicator, i.e. for coal tar mixtures, has led to mainly two conclusions. Firstly, it is recognized that the spectrum of tumour-sites induced with the coal tars clearly differs with that induced by B[a]P solely. Thus, B[a]P is not an ideal guidance chemical for these coal tar mixtures, or one may also conclude that coal tars may represent more than only PAH. Anyway, constituents other than B[a]P (of PAH origin or not) contribute in a critical way. Evidence that these constituents contribute via both promotional as well as genotoxic events has been presented. Secondly, when ignoring these differences in target-sites, the cancer potency of the coal tar mixtures investigated in these mice is about 2 to 5 times that of B[a]P itself: i.e. VSDs based on B[a]P content range from 1 – 3 ng B[a]P per kg body weight.

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APPENDIX D Exposure of the general Dutch population to PAH in the diet

Preface

1. PAH in Dutch diet
2. PAH in diet studies in mice
3. Conclusions

Exposure of the general Dutch population to PAH in the diet

Preface

In order to be able to evaluate the cancer risk of the Dutch population by exposure to dietary PAH some critical data are needed. First of all, a clear picture of the PAH composition of this diet, i.e. especially those PAH considered carcinogenic. Next, their concentration and carcinogenic potency is required to estimate their relative contribution to the carcinogenicity of the PAH mixture as a whole. This is especially relevant for B[a]P, being by far the best studied PAH and often referred to as PAH indicator with respect to carcinogenic risks associated with this group of chemicals. This Appendix summarizes and comments the available data on the exposure of the general Dutch population to PAH in the diet.

Additionally, this Appendix also compares and comments the PAH composition of the Dutch diet with that of the only available animal studies on oral exposure to PAH mixtures, i.e. the coal tar studies in mice conducted at NCTR (Culp *et al.*, 1998; briefly discussed in Appendix C).

1. PAH in Dutch diet

In Table 1 the PAH contents in Dutch diet as determined by different procedures and methodologies is given. The data are from Vaessen *et al*.(1988), de Vos *et al*. (1990), and from Heisterkamp and Van Veen (1997). Vaessen *et al.* (1988) analysed 50 duplicate portions of 24-hour diets in Dutch home settings for 15 PAH collected in the years 1976, 1978, and 1984/1985. De Vos *et al.* (1990) analysed 17 PAH in 10 'market baskets' of 18 year old men sampled over a 2.5 year period (1984-1986). This group probably represents the population with the highest food consumption, and therefore, with the highest intake of contaminants like PAH. These foods were prepared by normal methods.

Of the PAH analysed by Vaessen *et al.* (1988) and de Vos *et al.* (1990) 11 were in common, of which 8 were also contained in the ICD of Slooff *et al.* (1989). Interestingly, Vaessen *et al* measure less B[a]P but more of the other PAH as compared to de Vos *et al.* Notwithstanding this, the differences in specific PAH content between the two studies may be considered small, i.e. within a factor 2 (except for F). These values are also in rather good agreement with dietary levels reported for the UK (Dennis *et al.*,1983), Austria (Pfannhauser, 1991), and Italy (Lodovici *et al.*, 1995; Turrio-Baldassarri *et al.*, 1996).

Heisterkamp and Van Veen (1997) analysed the dietary exposure of the general Dutch population as follows. They combined the outcome of the PAH composition of the examined food stuffs by de Vos *et al.* (1990) with the actual composition of the Dutch diet as determined by a national food consumption survey that was carried out in the Netherlands during a one-year period (VCP, 1990), using a statistical model, STEM, to estimate the interindividual variation in life-time intake (Slob, 1993). Another difference between the values of de Vos *et al.* (1990) and Heisterkamp and Van Veen (1997) is the fact that de Vos *et al.* interpreted 'not detectable' PAH in certain food products as not present (or present at detection levels), whereas Heisterkamp and Van Veen decided to take half the detection-limit value for these food items. This latter approach likely can overestimate real exposures by the examined food stuffs. An advantage of this latter approach is that it provides distribution characteristics of the experienced exposures. It appears that this distribution is lognormal with a 95%-distribution factor of 1.85. Thus (as indicated in Table 1, between brackets), this distribution is rather small: the 5% extremes of the distribution are within a factor of two

from the mean. However, as PAH content has only been determined for a few food products, this distribution factor clearly will be an underestimate of the (unknown) true factor. It should also be stressed that this distribution represents *averaged* lifetime values that are considered relevant from a toxicologist's point of view; among others US studies show that *daily* intake differences per individual or between individuals may span up to three orders of magnitude depending on differences in food habits and cooking methods, as well as the source of the foods (see Butler *et al.*, 1993).

These data, that represent the 1976-1986 period, show that the mean estimates of dietary levels of the PAH analysed by the various investigators do not differ substantially. If we take B[a]P for instance, values are within a factor of two to three, dependent upon the methodology and assumtions applied. Within some other countries in Europe (i.e.UK, Austria, and Italy), comparable averaged daily B[a]P intakes of 50 to 250 ng per person are reported (with daily intakes ranging from 15 to 360 ng per person; Dennis *et al.*, 1983; Pfannhauser, 1991; Lodovici *et al.*, 1995). More recent information i.e. since 1986, on Dutch dietary PAH, , is not available.

Main dietary sources of PAH were cereals, oils and fats (especially vegetable margarine), milk, some leafy vegetables, e.g. kale and spinach, mussels, and prepared meath, some of which showed clear associations with conditions of local pollution (de Vos *et al.*, 1990; Dennis *et al.*, 1991; Lodovici *et al.*, 1995).

Most PAH analysed in the diets have only very low carcinogenic potency as compared to B[a]P (see Appendix E). Those few that are considered to have higher or comparable carcinogenic activity as B[a]P, e.g. DB[ah]A, DB[ah]Py, 3-MC and B[b]F, respectively (analysed by de Vos *et al.,* 1990, Vaessen *et al.*, 1988, and by others: see Table 2), show dietary levels clearly lower or comparable to that of B[a]P. Similar relative dietary levels for DB[ah]A are reported for UK and Italien diets by Dennis *et al.* (1983), and Turrio-Baldassarri *et al.* (1996), respectively. Unfortunately, no data are available for other potent carcinogenic PAH, e.g. DB[al]Py, and 5-MCH. The available data on another representative of these dibenzopyrenes, i.e. DB[ai]Py, suggest that these PAH probably indeed have low dietary levels as compared to B[a]P. Thus, more data on these potent PAH could provide a more accurate view on the carcinogenic risks associated with oral exposure to dietary PAH, and also would allow a better verification of B[a]P as PAH indicator in this respect (see Appendix E).

2. PAH in diet studies in mice

Table 2 depicts the PAH contents in Dutch diet as determined by the different investigators, and the PAH composition of the coal tar mixtures fed to mice in studies conducted at NCTR (Culp *et al.,* 1998). This comparison is interesting as it may turn out that the PAH composition of coal tar is such, that it may be taken as surrogate for the average PAH composition of human diet. If so, this would enable the direct use of the cancer potency estimates derived from these coal tar studies in mice for calculating the cancer risk for humans associated with their exposure to dietary PAH.

Both coal tar mixtures administered to female B6C3F1 mice by Culp *et al*. (1998) are quite comparable with respect to their profile of examined PAH.

Table 1. Data on PAH exposure of Dutch people via the diet (ng/person, daily) as determined by different methods (see text, legend and references for details)

PAH[1]	Vaessen *et al* 1988[2]	Vos *et al* 1990[3]	Heisterkamp 1997[4]
A		30 (640)	
B[a]A	160	200 (360)	
B[k]F	600[5]	100 (140)	
B[a]P	80	120 (290)	207 (110-350)
B[ghi]Pe	160	200 (360)	
CH	1200	860 (1530)	
F	2700	990 (1660)	1405 (780-2240)
IP	160	80 (460)	
N	ND[6]	ND[6]	
Ph	1600	870 (4510)	
B[b]F	see B[k]F	310 (360)	
B[ghi]F		20 (1460)	
B[j]F	see B[k]F		
B[e]P	140		
B[c]Ph	50	110 (910)	
DB[ah]A	40		
DB[aj]A		540 (1030)	
DBF			
DB[ae]Py	nd[7]	10 (630)	
DB[ai]Py	nd[7]		
3,6-DMPh		110 (310)	
3-MC		90 (1680)	
6-MCH		580 (730)	

[1]) PAH shown in **bold** are those selected by Slooff *et al.* in their Integrated Criteria Document(ICD; 1989); For abbreviations see Appendix G; [2]) Analysed 50 duplicate diets in Dutch home settings for 15 PAH; these 50 were selected from 311 samples because of their expected "elevated" PAH-levels; [3]) Analysed 10 market baskets (23 food commodity groups) of 18 year old youngsters for 17 PAH: 'not detected' was taken as zero; figure between brackets represents outcome when 'not detected' is taken as equal to detection limit; [4]) From Heisterkamp and Van Veen (1997): they analysed diet exposure of the general population via data from [3]) and VCP (1990): 'not detected' was taken as half the detection limit; between brackets: figure represents 5-percentiles of population with low and high PAH exposures; [5]) Figure represents sum of B[k]F, B[b]F, and B[j]F; their ratio appears to be fairly constant within environmental compartments: 1, 70 & 29%, respectively (van Velze, 1996); [6]) ND: Not Determined; [7]) nd: not detected; detection limit was 30 ng.

Table 2. Comparison of PAH exposure via the diet of Dutch people and experimental animals. Values given are relative to B[a]P on a daily basis (weight/unit body weight).

PAH[1]	Vaessen *et al* 1988[2]	Vos *et al* 1988[3]	Heisterkamp[4] & others	Culp *et al, 1998*[5] CT 1	Culp *et al, 1998*[5] CT 2
A		0.3 (2.2)		1.4	1.1
B[a]A	2	1.7 (1.2)		1.3	1.2
B[k]F	7.5[6]	0.8 (0.5)		0.4	0.4
B[a]P	*1*	*1 (1)*	*1*	*1*	*1*
B[ghi]Pe	2	1.7 (1.2)		0.8	0.8
CH	15	7.2 (5.3)		1.3	1.1
F	34	8.3 (5.7)	6.8	2.7	2.3
IP	2	0.7 (1.6)		0.7	0.7
N	ND[7]	ND[7]		12.1	11.7
Ph	20	7.3 (15.6)		4.2	3.7
Ace				1.1	0.5
Acen				1.7	2.1
B[b]F	see B[k]F	2.6 (1.2)	0.72[12]	1.1	1
B[ghi]F		0.2 (5)			
B[j]F	see B[k]F				
B[e]P	1.8		0.68[12]		
B[c]Ph	0.6	0.9 (3.1)			
DB[ah]A	0.5, 0.5[8] , 0.1[9]	?[11]	0.1[12] / 0.3-0.5[13]	0.1	0.1
DB[aj]A		4.5 (3.6)		0.18[14]	
DBF				0.8	0.7
DB[ae]Py	nd[10]	0.08 (2.2)		?[11]	?[11]
DB[ai]Py	nd[10]			?[11]	?[11]
3,6-DMPh		0.9 (1.1)			
f				2	1.7
I				0.6	0.2
3-MC	?[11]	0.8 (5.8)		?[11]	?[11]
6-MCH		4.8 (2.5)			
1-MN				3.6	2.1
2-MN				6.2	3.9
Py			4.4[11] / 1.5[12]	2.8	2.6

Legend to Table 2 (previous page):
[1]) PAH shown in **bold** are those selected by Slooff *et al.* in their Integrated Criteria Document(1989); For abbreviations see Appendix G; [2,3,4]) see Table I; [5]) Modified from analytical data by Culp *et al* (1998), CT 1:coal tar mixture, CT 2: coal tar mixture 2; [6]) Figure represents sum of B[k]F, B[b]F, and B[j]F; their ratio appears to be fairly constant within environmental compartments: 1, 70 & 29%, respectively (Van Velze, 1996); [7]) ND: Not Determined; [8]) Turrio-Baldassarri *et al.*(1996); [9]) data from van Gastel (1994; page 60); [10]) nd: not detected; detection limit was 30 ng; ([11]) not determined;) [12]) Modified from results by Dennis MJ, *et al.* (1983); [13]) Modified from results by Lodovici M, *et al*. (1995); [14]) Modified from results presented by Muller *et al* (1997): Table C311221, page C-7.

This facilitates the comparison as it means that these animal studies can be taken as each others duplicate.

Overlap between animal and human exposure data is for 13 PAH only, of which 10 PAH were also evaluated in the Dutch ICD on PAH (Slooff et al., 1989). Of these 13 PAH, five have relatively low levels in the experimental mouse diets, i.e. CH, F, Ph, and B[k]F: 5 to 10 times (CH & F), and 2 to 5 times (B[ghi]Pe, PH & B[k]F), respectively. But due to their relatively low carcinogenic potency (as compared to B[a]P: see Appendix E), this is not considered critical with respect to the carcinogenic potency of the mixture as a whole. Of the relatively potent PAH DB[ah]A exposure of the animals was apparently also somewhat lower as compared to the levels humans experience in their diet. Some other PAH considered relevant with respect to their carcinogenic potency, e.g. DB[ah]Py, DB[al]Py, 3-MC, B[j]AA, and 5-MCH (see Appendix E), have not been analysed in these coal tar mixtures, unfortunately.

From this comparison it is clear that the analysed PAH profiles of the investigated coal tars and human diet do not accurately match. This conclusion may be less relevant in the present context, if this was mainly due to PAH of low carcinogenic potency: i.e. the comparison critically depends on PAH that substantially contribute to the carcinogenic potency of the mixture as a whole. As indicated, information on relative concentrations of these latter PAH is lacking. Thus, one cannot come to a sound conclusion yet concerning the comparability of the PAH exposures in coal tars and human diet, i.e. in terms of their carcinogenic potency. Although the available data do not suggest at forehand major differences in this respect, the extrapolation of the tumour findings in these animals to humans should for the time being be interpreted with caution.

3. Conclusions

The available data on PAH composition of the Dutch diet basically stem from only two types of measurements performed in the period 1976-1986. These data show no major differences in the mean estimates of dietary levels of the PAH as analysed by the various investigators. If we take B[a]P for instance, values are within a factor of two to three dependent upon the methodology and assumptions applied. Also, the approach resulting in a distribution of PAH exposures suggests relatively small variations in lifetime adjusted average exposures. Most PAH analysed in Dutch food have only a relatively low carcinogenic potency as compared to B[a]P; information on dietary levels of some relatively potent carcinogens is lacking. The available data on a few of them suggest their levels to be lower, or at the most comparable to that of B[a]P.

Comparison of the PAH analysed in human diet and those determined in coal tar diets administered to animals shows that there is overlap only for 13 PAH. Most of these are of

relatively poor carcinogenic potency as compared to B[a]P. As information on some more potent carcinogens is lacking, caution is needed when comparing the relative carcinogenic potency of the PAH exposures of the animals to that humans actually experience in their diet.

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APPENDIX E Methods for calculating cancer risks associated with PAH in Dutch diet

Preface

1. Use of carcinogenic potency of individual PAH
2. Use of carcinogenic potency of PAH mixtures
3. Conclusions

Methods for calculating cancer risks associated with PAH in Dutch diet

Preface

This Appendix focusses on the way how to estimate the cancer risks associated with exposure of the general Dutch population to the PAH encountered in the diet. The validity of taking B[a]P as an indicator for these carcinogenic risks as well as the possible use of the recent coal tar studies in mice in this context will be briefly discussed. Therefore, this Appendix will combine the available data on individual PAH with respect to their occurrence in human diet, and their relative carcinogenic potency as compared to that of B[a]P. The composition of PAH in human diet and the experimentally tested coal tars is described in Appendix D. Appendix C described the carcinogenicity of these coal tars in animal experiments.

1. Use of carcinogenic potency of individual PAH

One of the methods for establishing quantitative cancer risk estimates for mixtures is based on their relative potencies or toxic equivalency factors (TEF): in the TEF approach for PAH, the overall carcinogenic potency of the mixture is expressed relative to B[a]P and contributions of individual carcinogenic PAH are taken to be additive (US.EPA, 1986; Slooff *et al.*, 1989; Nisbet and Lagoy, 1992). B[a]P has been used in this way as an indicator for the estimation of cancer risks associated with inhalatory exposure to PAH. This was based on the notion that the calculated relative contribution of B[a]P to the carcinogenic potency of PAH exposures in epidemiological studies (showing their carcinogenic effects) was rather substantial, and fairly constant for various PAH mixtures encountered in the environment (Slooff *et al.*, 1989). The choice for this concept, therefore, was not a pragmatic one only (i.e. simply monitoring B[a]P concentrations).

The validity of applying a similar approach to estimate human cancer risks associated with PAH exposure via food seems obvious. The average PAH composition in the diet represents a balanced integration of all encountered PAH profiles via this route; the change in average PAH profile over the years should then, consequently, be verified at regular intervals. However, there are some other aspects that seriously hinder such an approach. First of all, there are no data on human cancer risks associated with dietary exposure to PAH or a PAH indicator such as B[a]P, as there are for the inhalatory route. Secondly, because the exposure route is different, the tumour response probably differs as well, i.e. both qualitatively as well as quantitatively; note that inhalatory exposure to PAH resulted in local tumour development in the lung. Also, the composition of PAH in human diet may differ quite well from that experienced by inhalation (PAH in the diet stem among others from both environmental pollution and from the storage and processing of foods), which may impact upon the relative contribution of B[a]P to the carcinogenic potency of the PAH mixture as a whole. And, finally, the available animal data for this exposure route were – untill recently – only for individual PAH like B[a]P, instead of PAH mixtures.

In order to estimate the contribution of B[a]P to the carcinogenic potency of dietary PAH (which also would allow its use as indicator for PAH associated cancer risks via this route; be it possibly on the basis of another unit cancer potency as applied for inhalatory exposure) available data on concentrations and carcinogenic potencies for PAH were

evaluated. Table 1 updates the relative carcinogenic potency as compared to that of B[a]P of the 10 PAH evaluated in a previous RIVM report, i.e. Integrated Criteria Document on PAH (Slooff *et al.,* 1989). Based on the available data at that time the carcinogenic potency of the examined PAH were scaled to that of B[a]P, both in an absolute way and a relative way (Table 1, first two columns). The new proposal, i.e. a relative scaling, incorporates more recent data / evaluations by others (details are outlined in Table 2 of Appendix G). Note the differences in the amount of available data / evaluations for the different PAH (Table 1). Only potencies of F and CH are changed, i.e. they were judged as being less potent.

Table 1. Relative carcinogenic potencies of PAH as compared to B[a]P: PAH selected in the ICD PAH (Slooff et al., 1989). Both 1989 and new summary are presented. PAH are ordered according to estimated relative potency to B[a]P.

PAH	ICD 1989[1] sf-abs.	sf-rel.	Availability[2] data / evaluations	summary[3] sf-rel.
B[a]P	*1.00*	*1.00*		*1.00*
IP	0.08	0.1	+++(+)	0.1
B[a]A	0.04	0.1	+++	< 0.1
B[k]F	0.09	0.1	+++	< 0.1
CH	0.89	0.1	++++	0.1 - 0.03
B[ghi]Pe	0.03	0.1	+++	≈ 0.03
F	0.06	0.1	+	0.01
Ph	0.01	0.1	+(+)	< 0.01
A	0	0	+	0
N	0	0	+	0

[1]) Data from ICD report of Slooff *et al.* (1989); scaling factor (sf), absolute (abs) or relative (rel); [2]) Qualification of relative richness of data source for the specific PAH: see Table 2 of Appendix G for further details; [3]) Summary based on available data (see Table 2 of Appendix G).

Table 2 shows that the available data on occurence and relative carcinogenic potency of PAH in the diet is far from complete. As already stated (Appendix D) most knowledge concerns PAH of relatively low carcinogenic potency (i.e. those selected in the ICD on PAH, Slooff *et al.*, 1989). In order to compensate for this lack of data on the carcinogenic potency of PAH in human diet, their total contribution was estimated at that time to be about 10 times (Slooff *et al.,* 1989), or even up to 25 times (Kramers and van der Heyden, 1988) that of B[a]P alone.

Especially critical within the present context, however, are those PAH judged to be at least 0.1th the potency of B[a]P itself, i.e. from B[j]AA down to CPP (or when including heterocyclic PAH, down to DNPy's). Of the few PAH in this selection whose concentrations have been determined, only B[b]F apparently exceeds levels of B[a]P; taking its estimated relative carcinogenic potency, around 0.1 (see Table 3, Appendix G), its contribution to the

Table 2. Relative concentration and relative estimated carcinogenic potency of dietary PAH as compared to B[a]P (B[a]P set at ≡ 1.0). The relative potency was ordened into 4 groups based on the results of various carcinogenicity tests as published in the literature (see Appendix G). Residual PAH not shown here (but listed in Appendix G) are of an estimated potency of at least two orders lower than that of B[a]P.

PAH[1]	Vaessen *et al.*, 1988[2]	Vos *et al,.* 1990[3]	others[4-7]	Culp *et al., 1998*[8] CT 1	CT 2
group 1: potency (probably) > B[a]P[9]					
B[j]AA					
DB[ah]A	0.5		0.08- 0.5[5-7]	0.1	0.1
DB[ah]Py	<0.38[15]				
DB[al]Py					
3-MC		0.8			
5-MCH					
group 2: 1/10 B[a]P < potency ≤ B[a]P[9]					
An					
B[e]AA					
B[l]AA					
B[b]F	'5.3'[10]	2.6		1.1	1
B[rst]pp (?)[11]					
CPP (?)					
7H-DB[cg]C (?)					
6-NCH [12]					
DNF's (?)					
DNPy's[13] (?)					
group 3: potency ≤ 1/10 B[a]P[9]					
IP	2	0.7		0.7	0.7
B[a]A	2	1.7		1.3	1.2
B[k]F	'0.1'[10]	0.8		0.4	0.4
CH	15	7.2		1.3	1.1
B[ghi]Pe	2	1.7		0.8	0.8
B[j]F	'2.1'[10]				
DB[aj]A		4.5		0.18[14]	
DB[ah]Ac					
DB[aj]Ac					
DB[ae]F					
DB[ae]Py		0.08			
DB[ai]Py	<0.38[15]				
DB[el]Py (?)					
2-MCH					
1-NP					
2-NF					
DNPy 1,3-					
group 4: potency ≤ 1/100 B[a]P[9]					
F	34	8.3	6.8[4]	2.7	2.3

Legend to Table 2 (previous page):
[1]) PAH shown in **bold** are those selected by Slooff *et al* in their Integrated Criteria Document (1989); For abbreviations see Appendix G; If not depicted in the Table PAH is considered hardly if at all carcinogenic (e.g. **Ph**, **A**, and **N**); [2]) Analysed 50 duplicate diets in Dutch home settings for 15 PAH; [3]) Analysed 10 market baskets (23 food commodity groups) of 18 year old youngsters for 17 PAH: 'not detected' was taken as zero; figure between brackets represents outcome when 'not detected' is taken as equal to detection limit; [4]) From Heisterkamp and Van Veen (1997): they analysed diet exposure of the general population via data from [3]) and VCP (1990):): 'not detected' was taken as half detection limit; between brackets: figure represents 5-percentiles of population with low and high PAH exposures; [5]) Modified from results by Dennis MJ, *et al.* (1983); [6]) Modified from results by Lodovici M, *et al* (1995); [7]) Turrio-Baldassarri *et al.* (1996); [8]) Modified from analytical data by Culp *et al* (1998), CT 1:coal tar mixture, CT 2: coal tar mixture 2; [9]) The potency ordening is based on the results of various carcinogenicity tests as published in the literature (see Appendix E). [10]) figure calculated from sum of {B[k]F, B[b]F, and B[j]F} (see Table 2 Appendix D) based on their ratio within environmental compartments which appears to be fairly constant: 1, 70 & 29%, respectively (Van Velze, 1996); [11]) ?: belonging to this potency group?: based on only one test or mean of various tests showing wide variation in results; [12]) *italics*: indicates heterocyclic PAH; [13]) i.e. 1,6- and 1,8-DNPy's; [14]) Modified from results presented by Muller *et al* (1997): Table C311221, page C-7; [15]) actually not detectable; detection limit was 30 ng per kg;

potency of dietary PAH will be of about the same order as that of B[a]P. Unfortunately, there are no data for the other potent PAH DB[al]Py; representatives of these dibenzopyrenes examined, i.e. the DB[ah]Py and DB[ai]Py, show dietary levels (substantially) lower than that of B[a]P.

One pragmatic way out to value the PAH of unknown potency could be to group the PAH into chemically related subclasses like the dibenzo-anthracenes, -pyrenes, and benzo-aceanthrylenes and take the data of one member as representative for all. Unfortunately, the available data suggest that members within these structurally related subclasses (may) have quite divergent potencies: compare e.g. DB[ah]A, DB[ah]Py and DB[al]Py, and 5-MCH with DB[aj]A, DB[ae]Py and DB[ai]Py, and 2-MCH, respectively (Table 2).

Table 2 also includes some heterocyclic PAH. It appears that those nitro-PAHs that are examined for their relative potency, are in most cases clearly less potent as B[a]P. However, target-organs for carcinogenesis may also differ for these heterocyclic PAH subgroups[12], suggesting that different mechanisms may be operative, and, consequently, signifying that their incorporation into some general PAH TEF approach actually does not seem justified.

Another critical point to note here, is that most data on relative carcinogenic potency stem from animal experiments with non-oral exposure routes, i.e. intrapulmonary injection and instillation, skin painting and intraperitoneal injection. In fact, the generally considered adequate data here, i.e. from long-term oral studies with individual PAH, are not available. In the studies performed mainly locally-induced tumours are evaluated, and it appears that the order of carcinogenic potency of some investigated PAH changed with the tissue examined (Larssen, 1995). Thus, for the route of interest here, i.e. the oral route, the actual relative carcinogenic potency may even differ from that depicted in the Table.

Taken together, the available data yet do not allow some TEF approach for the quantification of the carcinogenic risk associated with dietary exposure to PAH. Consequently, it is

[12] Upon oral exposure PAH induce tumours of the upper digestive tract, liver, lungs and mammary gland, whereas nitro-PAH induce tumours only in pituitary and mammary gland.

based on this concept also not possible to use B[a]P as an indicator for this purpose. The next paragraph discusses another way of using B[a]P as a PAH indicator. For this, the recently described coal tar studies in mice are used, which are the first animal experiments describing the carcinogenic effects of oral exposure to PAH mixtures (Culp *et al.*, 1998). Fortunately, these studies also included concurrent experiments with exposure to B[a]P solely, enabling a direct comparison between the carcinogenic effects of these PAH mixtures to that of B[a]P.

2. Use of carcinogenic potency of PAH mixtures

The most direct way to quantify human cancer risks for oral exposure to dietary PAH in the absence of human data, would be to examine the carcinogenic potency of this mixture in animals. Unfortunately, that data are not available. What ís available, though, are data on carcinogenic effects for this route of another PAH mixture, namely that of coal tars (Culp *et al.*, 1998). Whether this PAH mixture could be taken as a surrogate for the PAH encountered in human diet depends upon the comparability of their PAH profiles. This comparison was briefly discussed in Appendix D; in Table 2 (this Appendix) the available data on relative carcinogenic potencies are included. Unfortunately, levels of the relevant PAH in this respect, i.e. those with relatively high potencies (like DB[al]Py, 3-MC, B[j]AA, and 5-MCH), are mostly not available for either the coal tar mixtures or human diet, or both. Therefore, from the data in Table 2 one can only come to a very rough qualitative conclusion concerning the relative carcinogenic potency of the PAH exposures of the animals fed coal tar to that humans actually experience in their diet: the examined PAH of relatively high potency (i.e. higher than 0.1th that of B[a]P) all seem to have somewhat lower levels in the coal tars (i.e. scaled to B[a]P). This would imply that the PAH composition of human diet has a higher carcinogenic potency as compared to that of coal tar when standardized to the amount of B[a]P present. Given this, human cancer risks associated with exposure to dietary PAH can be estimated from these mice studies in two ways, be it semi-quantitatively only.

First, the carcinogenic potency of coal tar in mice is calculated and related to the amount of B[a]P (as one of the most potent PAH present in both coal tars and human diet): i.e. B[a]P is used as PAH indicator. Subsequently, the amount of B[a]P in human diet is taken to calculate the cancer risks associated with dietary exposure to PAH using this potency value derived for coal tar. Next, one could introduce some uncertainty factor at this point to compensate for the above identified uncertainties in the database, e.g. the general lack of comparative data on concentrations and carcinogenic potencies of relevant PAH in both PAH mixtures.

The other way of estimating human cancer risks associated with exposure to PAH in the diet (using these mice studies) includes in the analysis the results of the concurrently performed carcinogenicity assay with B[a]P itself. In expressing the carcinogenic potency both for coal tar mixtures and B[a]P in dose units B[a]P, the contribution of other PAH within the coal tar mixture is reflected in the difference between the two estimates. Subsequently, the amount of B[a]P in human diet is taken to calculate the cancer risks associated with dietary exposure to B[a]P. Then, the difference-factor is applied for extrapolation to the cancer risk associated with all PAH present in the diet. Again, one could introduce at this point some uncertainty factor to compensate for the above identified uncertainties in the database, i.e. the general lack of comparative data on concentrations and carcinogenic potencies of relevant PAH in both PAH mixtures. The advantage of this approach is that it allows the inclusion of other performed animal experiments on B[a]P, e.g. that with the rat at our Institute. Thus, one can derive at a some estimate for the carcinogenic potency of B[a]P by combining adequate animal data on this PAH, and, subsequently, use the

above mentioned difference factor (i.e. potency-ratio of B[a]P in B[a]P- and coal tar-diets, respectively), and an uncertainty factor.

It is clear, as outlined above, that the described extrapolations should all be interpreted with caution: basically, there are no adequate data for a solid estimation of risks associated with dietary exposure to PAH. When incorporating the recently performed coal tar studies in this estimation, there are two further arguments that warrant caution (and that were already stated previously in Appendix C). First of all, the carcinogenic effects observed in coal tar fed mice may in part be due to constituents other than PAH. Clearly, when used as such, this might lead to overestimation of risks associated with exposures to PAH solely; obviously, this cannot be compensated for (i.e. in the absence of further data on this point). Secondly, it is recognized that the spectrum of tumour-sites induced with the coal tars clearly differs with that induced by B[a]P itself. Thus, B[a]P is not an ideal guidance chemical for these coal tar mixtures (note: it may still be so for pure PAH mixtures): constituents other than B[a]P – either PAH, non-PAH or both – can contribute in a critical way.

3. Conclusions

Untill recently there were no empirical data to support the concept of taking B[a]P as an indicator for human cancer risks associated with oral exposure to PAH: for this route only animal experiments were available for solely B[a]P. It was more or less by educated guess that in order to compensate for the carcinogenic potency of the other PAH present in the diet a factor of 10 should be used (Slooff *et al.*, 1989), or even higher numbers (Kramers and Van der Heyden, 1988).

An analysis of the database available for estimating human cancer risks associated with dietary exposure to PAH shows that there are still critical inadequacies. Probably not all carcinogenic PAH have been identified so far, and some of these may be quite potent. Moreover, data for most identified carcinogenic PAH are based on only one or just a few animal data, are derived from various experimental designs, and notably mainly concern non-oral exposure routes. Also, attention in these experiments was paid almost exclusively to local tumour development. Thus, based upon this, it is hardly possible to quantify the relative carcinogenic potency of the various PAH relative to that of B[a]P, not to mention under the condition of oral exposure. The use of a TEF-like approach for estimating human risks asssociated with dietary exposure to PAH, therefore, is yet not considered feasible.

Despite this incompleteness of the database, however, the estimation of human cancer risks associated with dietary exposure to PAH seems somewhat more justified today since important data have appeared since 1989: the recently published mice studies performed at NCTR (Culp *et al.,* 1998), and the rat study reported here. The described estimation lends on B[a]P as a PAH-indicator, which can be critisized, but which is also not disaffirmed by the available data sofar: B[a]P still appears to be one of the most important carcinogenic PAH examined in human diet, when comparing its relative concentration as well as its relative carcinogenic potency to that of other examined PAH. The approach outlined here, however, clearly is still imperfect and should be applied with caution because of the next uncertainties: the possible substantial contribution to the carcinogenicity of dietary PAH of yet unidentified PAH (and the possible absence of these in the investigated coal tars), and the possible role for non-PAH in coal tar.

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APPENDIX F Alternative VSD calculations based on the present rat carcinogenicity study

Preface

1.1 VSD via next lower dose to the TD_{sign} for rat liver tumours

1.2 Results of using next lower dose to the TD_{sign} for linear extrapolation

2.1 VSD via the two--stage clonal expansion model

2.2 Model description

2.3 Results of applying the two-stage model to rat liver tumour data

2.4 Validity of using two-stage model for extrapolation to low dose risks

3. Conclusions

Alternative VSD calculations

based on the present rat carcinogenicity study

Preface

A VSD is derived from linear extrapolation from the TD_{sign} in an animal experiment (see paragraph 4.2.2, main text); this method is considered conservative, i.e. it is recognized that it may overestimate risks at low doses in certain cases (HCN, 1995, 1996). Here we will shortly describe and comment two alternative approaches for deriving a VSD: one is by taking the next lower dose to the TD_{sign} as point of departure for linear extrapolation, the other is by using the so-called biologically-based Moolgavkar -Venzon-Knudson (MVK) model that incorporates cell kinetics, a parameter that is considered critical to carcinogenesis.

1.1 VSD via next lower dose to the TD_{sign} for rat liver tumours

The VSD for B[a]P was derived by linear extrapolation from the TD_{sign} for liver tumours or for TBA in the rat study (both resulted in a similar VSD; see Table 4 main text). However, some data support a sublinear behaviour of the dose response curve for this major tumour by B[a]P (as outlined in paragraph 5.3.2). Evidence for this also directly stems from an analysis of the observed dose-response relationship for liver tumours: if the next lower dose to the TD_{sign}, i.e. 3 mg/kg bw, is chosen for linear extrapolation, a VSD of 9 ng B[a]P is derived.

1.2 Results of using next lower dose to the TD_{sign} for linear extrapolation

Table 1 shows the incidence of liver tumours in the rat study for the TD_{sign} (10 mg/kg bw), and the next lower dose (3 mg/kg bw), together with their confidence intervals.

Table 1. Confidence intervals to the incidence of liver tumours induced in female rats by B[a]P. (for explanation see text).

Site	Dose[1]	tumour incidence (%) (% CI)[2]	VSD[3]
Liver, adenomas	3	3.8 (0.47 - 13.2)	30 (9 - 246)
linear extrapolation of 10 → 3[4]	'3' ↑	*23 (18 - 26)*	
adenomas & carcinomas combined	10	75 (61.1 - 86)	5 (4 - 6)

[1]) dose level (mg/kg bw; 5 times a week); [2]) percentual tumour incidence (minus background incidence) and between brackets: 5% to 95% confidence interval (CI) on this response; [3]) calculated daily dose in ng B[a]P/kg bw (numbers rounded); [4]) values calculated via linear extrapolation from response and CI for 10 mg/kg bw: e.g. 18% = 3/10 x 61.1%.

The data show that the response observed at this lower dose significantly differ from that obtained by linear extrapolation from the TD_{sign}: i.e. the upper limit on the observed incidence at 3 mg/kg bw (13.2%) is below the calculated incidence at this dose derived from linear extrapolation of the lower limit on the TD_{sign} (18%). As the liver tumours observed at 3 mg/kg bw are assumed to be induced by B[a]P (the spontaneous incidence obviously is much lower in this species, Wester *et al.*, 1985a, 1985b, 1990), one may, as an alternative to the methodology prescribed by the HCN, use this lower non-significant response, i.e. its upper confidence limit, as point of departure for VSD calculation. In this way a VSD of 9 ng/kg bw is obtained (or 0.9 ng /kg bw when B[a]P is taken as PAH indicator), i.e. not substantially different from the VSD estimate obtained by the standard methodology.

2.1 VSD via the two-stage clonal expansion model

An approach that has gained more attention in the field of quantitative cancer risk assessment since the late 1980s is the so-called biologically-based modeling as introduced by several investigators (a.o. Moolgavkar *et al.* 1988, 1990; Cohen and Ellwein, 1990; Chen and Farland, 1991). These models owe their growing popularity to their attempt to incorporate real biological phenomena regarded essential elements in the carcinogenic process, e.g. cell kinetics and mutation rates. Here we will shortly describe one of these models, the so-called Moolgavkar-Venzon-Knudson (MVK) model, that we applied to the carcinogenicity data of the presented rat study.

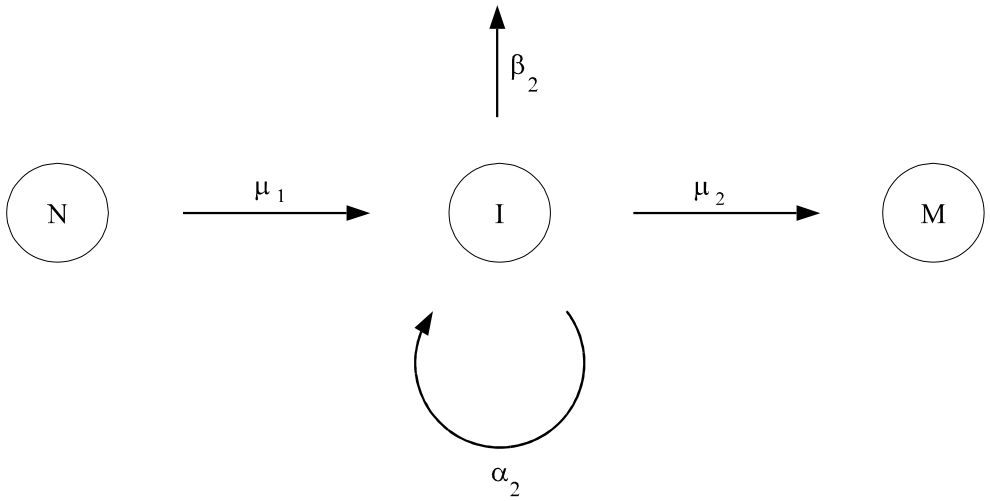

Figure 1. Schematic representation of the two-stage clonal growth model for cancer, with five parameters: number of normal cells N, mutation rates μ_1 and μ_2, intermediate cell division rate α_2 and intermediate cell death rate β_2. I and M represent the number of intermediate and malignant cells, respectively.

2.2 Model description

Figure 1 schematically depicts the concept of the so-called partial stochastic two-stage model developed by Moolgavkar *et al.* (1988, 1990). In this model the concept of carcinogenesis is that essentially two (rate-limiting) mutation events (μ_1 and μ_2) are needed to transform a normal cell within an organ into a fully malignant tumour cell. The first mutation event transforms a normal cell into a so-called 'initiated' cell, the second mutation event transforms an 'initiated' cell into a malignant cell. The division and death rates of normal cells are described deterministically, i.e. by a function describing the growth of the organ. Intermediary cells are assumed to divide at rate α_2, and assumed to die (or differentiate) at rate β_2. In the absence of further data μ_1 and μ_2 are assumed to be equal[13].

2.3 Results of applying the two-stage model to rat liver tumour data

The MVK model was fitted to the time-to-tumour data for liver tumours as observed in the chronic rat study. Data on cell number per unit liver were adapted from Moolgavkar *et al.* (1990a). Essentially no differences were found for data from males or female; therefore, these were combined in the analysis. The assumption of a linear relationship between administered B[a]P dose and proliferation-rate of intermediary cells appeared to result in a much better fit to the data as compared to relating dose with mutation rate: see Figure 2.

[13] This increased reflection of biology in these models has a price in the demand for additional data on these various biological parameters, which are in most instances not available and, besides, difficult to unequivocally identify and quantitate (Crump, 1994). This also hampers their use in low-dose risk estimation (Crump, 1996).

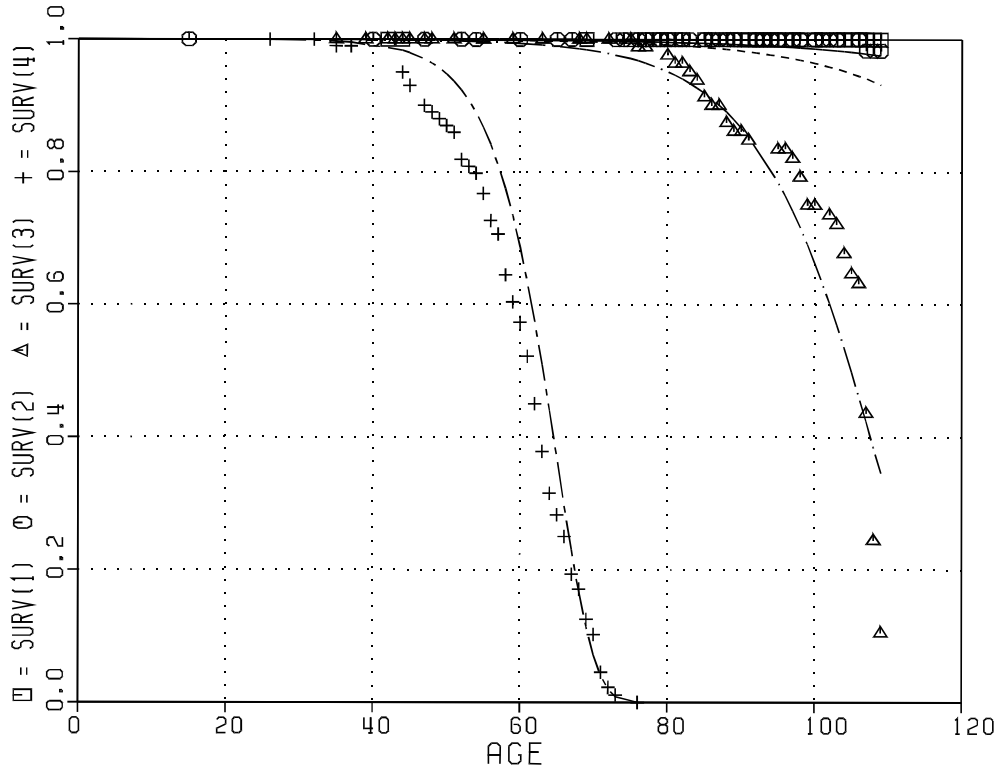

Figure 2. MVK model fitted to time-to-tumour data for liver tumours in B[a]P-exposed rats, indicated by the marks, which are the Kaplan-Meier estimates. The four survival functions are (from right to left) associated with dose levels 0 (□), 3 (O), 10 (Δ), and 30 (+) mg B[a]P/kg bw. In the group receiving 10 mg/kg bw a number of animals appeared to have tumours when killed at termination; these data were treated as censored data in fitting the model.

Fixed parameters:	Estimated parameters were:
N = 9.66E8	$\varepsilon = \alpha_2 - \beta_2$ = 8.69E-3 + 6.38E-4 x dose (mg/kg bw)
	$\mu_1 = \mu_2$ = 1.59E-9

The result of the fitted model is shown in Figure 2.

Incorporating the observed dose-related increased liver weights at the 90-day sacrifice, by making the number of cells N dose-dependent, had a negligible effect on the likelihood. Furthermore, making the number of normal cells age dependent, i.e. in proportion to the growing body weight, did not have much influence on the likelihood function as well. Also, setting both parameters ε and μ dose-dependent could not improve the fit. No reasonable fit could be obtained by setting ε fixed at zero; this agrees with the generally adopted idea that cells having undergone a transforming mutation (intermediate cells I in the model) generally have a growth advantage compared to normal cells.

From the model a potency factor (i.e. a low-dose slope factor) is obtained for the dose-response function of 1.1E-1 $(\text{mg/kg bw})^{-1}$ (including a correction to a lifespan 1000

days)[14]. From this factor a VSD, i.e. a daily dose associated with a lifetime cancer risk of 10^{-6}, can be estimated to be 17.5 ng/kg bw.

3. Conclusion

None of the alternative approaches for deriving a VSD result in a substantial change from the one arrived at via adopted standard methodology. The 'next lower dose' approach results not even in a doubling of the regular VSD, whereas 'the MVK' approach resulted in a three-fold higher VSD only.

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[14] Note: when a lifespan of 750 days was taken instead the potency factor would amount to 1.0E-2, i.e. 11.1 times less potent than the estimate obtained for a lifespan of 1000 days; The correction factor for this applied by the Health Council of The Netherlands (HCN, 1995) amounts to 1.8 (1000/750 x 1000/750) only.

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APPENDIX G Carcinogenic potency of PAH relative to B[a]P

(Tables)

Carcinogenic potency of PAH relative to B[a]P (Tables)

Table 1. Relative carcinogenic potencies of PAH as compared to B[a]P: PAH selected in the ICD PAH by Slooff et al. (1989). Both data evaluated by Slooff et al. (1989) [upper part], as well as those of others [lower part] are presented. All these data are summarized in a semi-qualitative way in the last column (which is also presented as Table 1 in Appendix E).

PAH	IARC[1]	lung-impl[2] *lung*	dermal; long-term[2] *skin*	dermal/initiation[2] *skin*	i.p. inj.[2] *lung*	i.p. inj.[2] *liver*	ICD PAH[3] sf-abs.	ICD PAH[3] sf-rel.
A	3			0.007[7]			0	0
B[a]A	2A		0.00	0.04			0 - 0.04	0.1
B[k]F	2B	0.03	0.001		0	0.09	0.03 - 0.09	0.1
B[a]P	2A	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***
B[ghi]Pe	3	0.01	0.02	0.006			0.01 - 0.03	0.1
CH	3		0.1-.08	0.05	0.02	0.8	0.05 - 0.9	0.1
F	3		0.00		0.06		0 - 0.06	0.1
IP	2B	0.08			0	0	0 - 0.08	0.1
N	ne						0	0
Ph	3			0.02[5]	0.01	0	0.01	0.1

PAH	Chu & Chen[4]	Clement[5]	Rugen et al[6]	Wenzel-H.*et al.*[7]	Nisbet & Lagoy[8]	Muller[9]	Larsen[10]	summary[11]
A					0.01		0 - (+)	0
B[a]A	0.013	0.15	0.0045		0.1	0.014	weak	< 0.1
B[k]F	0.004	0.066			0.1	0.037		< 0.1
B[a]P	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	***strong / ++***	*1*
B[ghi]Pe		0.022			0.01	0.012	+	0.03
CH	0.001	0.0044		0.03	0.01	0.026	weak	0.03
F					0.001		0 - (+)	0.01
IP	0.23	0.017	0.0060		0.1	0.067		0.1
N					0.001			0
Ph				0.001	0.001	0.00064		< 0.01

[1]) Classification (if available) according to IARC, IARC Monographs, *supplement 7*, 1987; [2]) data from Montizaan *et al.* (1989); [3]) from: Slooff *et al.* (1989); [8]) from Chu and Chen (1984 [5]) Clement (1988); [6]) Rugen *et al.* (1989); [7]) Wenzel-Hartung *et al.* (1990); [8]) Nisbet and Lagoy (1992); [9]) Muller *et al*. (1997); [10]) data adapted from Larsen (1995): +++: very potent; ++: potent; +: moderate; (+): weak; o: no activity; ?: possibly ≈ B[a]P potency, further verification needed; [15]) summary of all depicted evaluations.

Table 2. Relative carcinogenic potencies as compared to B[a]P of PAH not selected in the ICD PAH by Slooff et al. (1989).

PAH	IARC[1]	Wynder[2]	Chu & Chen[3]	Clement[4]	Rugen etal [5]	Wenzel-H *et al.*[6]	Nisbet & Lagoy [7]	Muller [8]	Larsen [9]	Nesnow *et al.*[10]
Ace	ne						0.001			
Acen	ne						0.001			
An	ne	0.316		0.32[11]				0.28		
B[a]Ac								0.00	0 ?	
B[c]Ac								0.00	(+)	
B[e]AA								0.12	++ ?	
B[j]AA										DBahA- 5MCH[12]
B[l]AA								0.77	+++ ?	
B[a]f	3								0	
B[b]f	3								0	
B[c]f	3								0	
B[a]F	ne								moderate	
B[b]F *	2B	0.12	0.08	0.14	0.036		0.1	0.11	strong	0.43 (0.56)
B[ghi]F	3								0 ?	
B[j]F	2B	0.052		0.06	0.078			0.045	+	
BNT	ne					0.02		0.028	(+) ?	
B[e]P	3	0.007		0.004				0.00		
B[rst]pp								1.1		
B[c]Ph	3							0.023	(+)	
B[b]tp								0.014		
C									(+)	
CPP	3							0.012	+	2.9 (1.2)
DB[ac]A	3								+ ?	
DB[ah]A	2A		0.69	1.1	0.59	1.91	5 (1)[13]	0.89	strong	118 (16.5)
DB[aj]A	3								(+)	
DB[ah]Ac								0.11	+	
DB[aj]Ac								0.00	+	
7H-DB[cg]C	2B								strong / ++	

(Table continued on next page)

(Table 2 Continued I)

PAH	IARC[1]	Wynder[2]	Chu & Chen[3]	Clement[4]	Rugen etal [5]	Wenzel-H *et al.*[6]	Nisbet & Lagoy [7]	Muller [8]	Larsen [9]	Nesnow *et al.*[10]
DBF	ne									
DB[aj]A	3								(+)	
DB[ah]Ac								0.11	+	
DB[aj]Ac								0.00	+	
7H-DB[cg]C	2B								strong / ++	
DBF	ne									
DB[ae]F	3								+	
DB[ae]Py	2B								+	
DB[ah]Py	2B							1.2	strong / ++	
DB[ai]Py	2B								+	
DB[al]Py	2B								strong/ +++?	
DB[el]Py									0	
3,6-DMPh	ne									
f	3						0.001		0 ?	
I	ne									
x-MB[a]A									(+)	
1-MCH									0	
2-MCH									+	
3-MCH	ne								(+)	
5-MCH	2B								strong/+++?	8.8 (2.9)
6-MCH	3								(+)	
3-MC								2.6		118 (16.5)
1-MN	ne									
2-MN	ne						0.001			
Pe	3									
Pi									(+)	
Py	3			0.081			0.001	0.00		
T									0 ?	

(Table continued on next page)

(Table 2 Continued II)

PAH	IARC[1]	Wynder[2]	Chu & Chen[3]	Clement[4]	Rugen etal [5]	Wenzel-H *et al.*[6]	Nisbet & Lagoy [7]	Muller [8]	Larsen [9]	Nesnow *et al.*[10]
nitro-PAH										
9-NA	3									
6-NB[a]P	3								(+)	
1-NCH									0 ?	
2-NCH									0 ?	
3-NCH									0 ?	
6-NCH	3								strong	
2-NF									+ ?	
3-NF	3								(+)	
1-NPy	3							0.00	0 - (+)	
1,3-DNPy									+	
1,6-DNPy									+ / ++ ?	
1,8-DNPy	(3)								strong; +/++?	
DNFs									strong; +/++?	

[1]) Classification (if available) according to IARC, IARC Monographs, *supplement 7*, 1987; [2]) Wynder *et al.* (1959); [3]) Chu and Chen (1984); [4]) Clement (1988); [5]) Rugen *et al.* (1989); [6]) Wenzel-Hartung *et al.* (1990); [7]) Nisbet and Lagoy (1992); [8]) Muller P (1997); [9]) data adapted from Larsen (1995): +++: very potent; ++: potent; +: moderate; (+): weak; o: no activity; ?: possibly ≈ B[a]P potency (single or questionable experiments), further verification needed [10]) Nesnow *et al*. (1995): numbers represent potency relative to B[a]P; Between brackets: relative potency to B[a]P for skin painting from Habs *et al.* (1980); [11]) note: based on only a single study; [12]) means: potency in between that of DB[ah]A and 5MCH according to Nesnow *et al*. (1998); [13]) TEF proposed for DB[ah]A by Nisbet and Lagoy (1992); *note:* data suggest equipotency with B[a]P at high doses, but higher potency of DB[ah]A at low doses.

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