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REVIEW

# Biomonitoring of arylamines and nitroarenes

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Arylamines and nitroarenes are very important intermediates in the industrial manufacture of dyes, pesticides and plastics, and are significant environmental pollutants. The metabolic steps of *N*-oxidation and nitroreduction to yield *N*-hydroxyarylamines are crucial for the toxic properties of arylamines and nitroarenes. Nitroarenes are reduced by microorganisms in the gut or by nitroreductases and aldehyde dehydrogenase in hepatocytes to nitrosoarenes and *N*-hydroxyarylamines. *N*-Hydroxyarylamines can be further metabolized to *N*-sulphonyloxyarylamines, *N*-acetoxyarylamines or *N*-hydroxyarylamine *N*-glucuronide. These highly reactive intermediates are responsible for the genotoxic and cytotoxic effects of this class of compounds. *N*-Hydroxyarylamines can form adducts with DNA, tissue proteins, and the blood proteins albumin and haemoglobin in a dose-dependent manner. DNA and protein adducts have been used to biomonitor humans exposed to such compounds. All these steps are dependent on enzymes, which are present in polymorphic forms. This article reviews the metabolism of arylamines and nitroarenes and the biomonitoring studies performed in animals and humans exposed to these substances.

*Keywords*: Aromatic amines, nitroarenes, haemoglobin adducts, albumin adducts, DNA adducts, urine metabolites, biomonitoring

*Abbreviations*: AAF, 2-acetylaminofluorene; 3- and 4-ABP, 3- and 4-aminobiphenyl; AcBz, *N*-acetylbenzidine; AcMDA, *N*-acetyl 4,4′-methylenedianiline; AcMOCA, *N*-acetyl 4,4′-methylenebis(2-chloroaniline); 1- and 2-AF, 1- and 2-aminofluorene; BAT value, biological acceptable tolerance value for occupational exposures; 2- and 4-CA, 2- and 4-chloroaniline; CBI, chemical binding index; CFA, 3-chloro-4-fluoroaniline; CFA-S, 4-chloro-5-fluorophenol sulphate; 2-, 3- and 4-CNB, 2-, 3- and 4-chloronitrobenzene; CYP, cytochrome P450; dA = 2′-deoxyadenosine; dA-N6-2,6-DMA, 4-(deoxyadenosin-N6-yl)-2,6-dimethylaniline; 2,4- and 2,6-DAT, 2,4- and 2,6-diaminotoluene; 2,6-DCA, 2,6-dichloroaniline; DcBz, 3,3′-dichlorobenzidine; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; 2,4-DFA, 2,4-difluoroaniline; DFP, [$^3$H]diisopropyl-fluorophosphate; dG, 2′-deoxyguanosine; dG-C8-4-ABP, *N*-(deoxyguanosin-8-yl)-4-ABP; dG-N2-2,6-DMA, 4-(deoxyguanosin-N2-yl)-2,6-dimethylaniline; dG-O6-2,6-DMA, 4-(deoxyguanosin-O6-yl)-2,6-dimethylaniline; DiAcBz, *N*,*N*′-diacetylbenzidine; 2,4- and 2,6-DMA, 2,4- and 2,6-dimethylaniline; 1,2- and 1,3-DNB, 1,2- and 1,3-dinitrobenzene; DNBA, dinitrobenzoic acid; 2,4- and 2,6-DNT, 2,4- and 2,6-dinitrotoluene; 3-EA, 3-ethylaniline; ESI-MS, electrospray ionization mass spectrometry; ETS, environmental tobacco smoke; FMO, flavine adenine dinucleotide-containing monooxygenase; GC-MS, gas chromatography-mass spectrometry; GST, glutathione S-transferase; Hb, haemoglobin; HBI, haemoglobin binding index; HFBA, heptafluorobutyric anhydride; HPLC, high pressure liquid chromatography; IARC, International Agency for Research on Cancer; LC-MS, liquid chromatography-mass spectrometry; LC-MS-MS, liquid chromatography with tandem mass spectrometry; 2-, 3, and 4-MA, 2-, 3- and 4-methylaniline; MAK, maximum allowable workplace concentrations; MDA,

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4,4′-methylenedianiline; MDI, 4,4′-methylenediphenyl diisocyanate; metHb, methaemoglobin; MOCA, methylenebis(2-chloroaniline); MPO, myeloperoxidase; NADH, reduced nicotinamide-adenine dinucleotide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; NAT, *N*-acetyltransferase; 4-NBP, 4-nitrobiphenyl; NCI, negative chemical ionization; NCTR, National Center for Toxicological Research, US Food and Drug Administration; 2-NF, 2-nitrofluorene; nitro-PAH, nitrated polyaromatic hydrocarbon; 1- and 4-NP, 1- and 4-nitropyrene; 2-, 3- and 4-NT, 2-, 3- and 4-nitrotoluene; ODA, 4,4′-oxydianiline; OSHA, Occupational Safety and Health Administration; PAH, polyaromatic hydrocarbon; PBI, protein binding index; PCA, 2,3,4,5,6-pentachloroaniline; 3′-P-dG-AcBz, *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine; PFPA, pentafluoropropionic anhydride; PGHS/COX, prostaglandin H synthase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; QSAR, quantitative structure–activity relationship; SCE, sister chromatid exchange; SULT, sulphotransferase; TDA, 4,4-thiodianiline; TNT, 2,4,6-trinitrotoluene; UGT, uridine diphosphate glucuronosyltransferase.

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## Introduction

### *Occurrence and toxicity of arylamines*

Arylamines are widely used as dye intermediates, especially for azo dyes, pigments and optical brighteners, as intermediates for photographic chemicals, pharmaceuticals and agricultural chemicals, in polyurethanes, and as antioxidants in polymers (Lawrence and Marshall 1985, Schwenecke and Mayer 1985, Vogt and Gerulius 1985). The manufacture of isocyanates, primarily 4,4′-methylenediphenyl diisocyanate (MDI), accounts for the largest share of aniline consumption. Analgesics and sulphonamides are examples of important pharmaceuticals derived from arylamines. Arylamines are used directly in inks for pens, in shoe cream, in dyes for inkpads and in hair dyes (IARC 1993). In the past the bicyclic diamino compounds such as benzidine, 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine and 3,3′-dichlorobenzidine (DcBz) were used for the production of azo dyes. However, since benzidine can be metabolically released from azo dyes, all dyes based on benzidine have been banned.

The main source of exposure to arylamines for the general population is from cigarette smoke or products that contain compounds synthesized from arylamines. Among other compounds, arylamines are present in cigarette smoke as 2,3-, 2,4-, 2,5- and 2,6-dimethylaniline, 2-, 3- and 4-ethylaniline, 2-, 3-, and 4-methylaniline, 1- and 2-naphthylamine, and 2-, 3-, and 4-aminobiphenyl (Patrianakos and Hoffmann 1979, Luceri *et al*. 1993). In Taiwan, extracts from three cooking oil fumes contained 2-naphthylamine and 4-aminobiphenyl (Chiang *et al*. 1999). Another source is food dyes containing aromatic amines as contaminants (Ramseier *et al*. 1987, Lancaster and Lawrence 1992). Arylamines are moderately to extremely toxic. The main hazards associated with arylamine exposure are methaemoglobinaemia and carcinogenesis. Both of these effects are attributed to the products of *N*-oxidation. Among the arylamines, the chloroanilines and nitroanilines are the strongest methaemoglobin (metHb) inducers (Kiese 1974).

## Occurrence and toxicity of nitroarenes

Nitroarenes play an important role in the chemical industry (Hartter 1985). In 1980 5% of the total benzene–toluene–xylene production was used to produce nitroaromatic compounds. Nitrobenzenes, dinitrobenzenes, dinitrotoluenes and mono- and dichloronitrobenzenes are important industrial intermediates in the

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production of plastics, rubber chemicals, polyurethanes, dyes and pesticides. Nitrotoluenes are used as intermediates for the dye industry and for the manufacture of explosives. Dinitrotoluenes are intermediates for the production of 2,4- and 2,6-diaminotoluene (2,4 and 2,6-DAT), which are widely used for the production of toluenediisocyanates. During World War II several soils became contaminated with deposits of 2,4,6-trinitrotoluene (TNT) and its byproducts. Soil, rivers and lakes close to ammunition plants have been found to be highly contaminated with several derivatives and byproducts of TNT (Feltes *et al*. 1990, Gowik *et al*. 1994). Nitrobenzene, nitrotoluene isomers and chloronitrobenzenes have repeatedly been observed in samples from the river Rhine, Germany (Feltes *et al*. 1990, Dieter 1994, Gowik *et al*. 1994, Hoering *et al*. 1994). Nitroarenes are also important air pollutants due to combustion motors (Howard *et al*. 1990) and to cigarette smoke (Hoffmann *et al*. 2001). Other significant pollutants are the nitroaromatic fragrances and perfumes, which are now ubiquitous in the environment (Ippen 1994, Käfferlein *et al*. 1998). Musk xylene, a synthetic musk often used in different fragrances and soaps as a substitute for natural musk, has been found in human breast milk (Rimkus *et al*. 1994), in blood (Angerer and Kafferlein 1997) and in adipose tissue (Rimkus *et al*. 1994). In general, the same acute toxic effects are seen after nitroarene exposure as after arylamine exposure, since the same reactive intermediates – *N*-hydroxyarylamine and nitrosoarene – are biologically available (Rickert *et al*. 1984, Rickert 1985, 1987). In humans, nitrobenzene and other nitroarenes generate metHb more slowly than aniline, dinitrobenzene or nitroaniline, but cyanosis is more persistent. The symptoms produced by inhalation of nitrobenzene are headache, vertigo, nausea, vomiting, depressed respiration, disturbed vision, coma and death from respiratory failure. The skin is bluish grey or intensely cyanotic. Repeated exposure may be followed by liver impairment, leading to yellow atrophy, haemolytic jaundice and varying degrees of anaemia, with Heinz inclusion bodies seen in the red cells. Nitrotoluenes are toxic by inhalation, ingestion and dermal absorption. In TNT-exposed humans, the notable toxic manifestations have included aplastic anaemia, toxic hepatitis and cataracts (Ahlborg *et al*. 1988).

The toxicological and chemical data of arylamines and nitroarenes can be obtained from various online databanks. The Hazardous Substances Data Bank produced by the National Library of Medicine, Bethesda, Maryland, USA (http://www.nlm.nih.gov) has the broadest data set for arylamines and nitroarenes, and includes all chemical names, manufacturing methods, applications, consumption patterns, physical and chemical properties, personal safety precautions, shipping, storage, disposal, metabolism, toxicities, environmental impacts and analytical laboratory methods. Toxicological data are listed in the Registry of Toxic Effects of Chemical Substances, which is produced by the National Institute for Occupational Safety and Health, Cincinnati, Ohio, USA (http://www.niosh.gov). In Germany, the maximum allowable workplace concentrations (MAK) and the biological acceptable tolerance (BAT) value are defined by a commission of the Deutsche Forschungsgemeinschaft (DFG) (http://www.dfg.de). The DFG publish the toxicological data collected for the definition of the MAK values, the methods used to biomonitor exposure to amines, and the methods used to measure air concentrations. Data on the mutagenicity and carcinogenicity of compounds are available online (http://www.iarc.fr) or in the scientific publications of the International Agency for Research on Cancer (IARC). Some monographs on

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arylamines and nitroarenes have been published by the World Health Organization (http://www.who.ch) in the monograph series 'Environmental Health Criteria'. Permissible exposure limit values for work or ambient atmospheres have been established by the Occupational Safety and Health Administration (OSHA) and the National Institute of Occupational Institute and Safety and Health, and threshold values have been set by the American Conference of Governmental Industrial Hygienists (http://www.acgih.org).

## Metabolism of arylamines

Arylamines are taken up through the gastrointestinal tract, the respiratory system and the skin. The metabolism of arylamines has been studied intensively (Garner *et al*. 1984, Kadlubar and Beland 1985, Gorrod and Manson 1986, Beland and Kadlubar 1990, Miller 1994, Delclos and Kadlubar 1997). Ring oxidation, *N*-glucuronidation, *N*-acetylation and *N*-oxidation are the major metabolic pathways of arylamines in mammals. *N*-Oxidation is a crucial step in the metabolism of arylamines and aromatic amides to toxic products. Four classes of enzymes are mainly responsible for *N*-oxidation: (1) cytochrome P450 (CYP) (Guengerich 2001); (2) flavine adenine dinucleotide-containing monooxygenase (FMO) (Cashman 1995, Whetstine *et al*. 2000); (3) prostaglandin H synthase (PGHS/COX) (O'Brien 2000, Wiese *et al*. 2001); and (4) myeloperoxidase (MPO) in breast and lung tissue (O'Brien 2000). The CYP isoenzyme CYP1A2 is mainly responsible for the *N*-oxidation of planar arylamines such as 4-aminobiphenyl (4-ABP) and 2-aminofluorene (2-AF) (Hammons *et al*. 1991, Guengerich 2001), whereas CYP3A4 has been shown to be the main enzyme that catalyses the *N*-oxidation of non-planar arylamines such as methylenebis(2-chloroaniline) (MOCA) and dapsone (Yun *et al*. 1992). The two other enzyme systems (FMO and PGHS/COX) play only a minor role in the toxicity and the metabolism in the liver of arylamines (O'Brien 2000). FMO can catalyse the formation of *N*-hydroxyarylamines from secondary arylamines as *N*-methylbenzidine and *N*-methyl-4-ABP (Ziegler *et al*. 1988, Ziegler 1993). Recently Wiese *et al*. (2001) investigated the role of PGHS/COX, which exists in two distinct isoforms: a constitutively expressed isoform (COX-1) and an inducible isoform (COX-2). COX-1 is expressed in all extrahepatic tissue, whereas COX-2 is present constitutively in a few tissues (brain, testis, kidney) but its expression can be induced by a variety of mediators (e.g. cytokines, growth factors, tumour promoters). The ability of human COX to activate several arylamines was demonstrated by Wiese *et al*. (2001). Earlier work had been performed either with ovine sources of enzyme or with microsomal preparations of human tissue, which may have contained either COX-1 or COX-2. In organs such as the bladder, where little CYP is present, PGHS/COX could be important for the formation of toxic and genotoxic intermediates. Following immunological and/or chemical insult, neutrophils release MPO and undergo respiratory burst, which is characterized by a massive increase in oxygen consumption and a consequent increase in the reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent production of superoxide and other free radicals. MPO activates a wide range of arylamines (Tsurata *et al*. 1985).

In figure 1, the *N*-oxidation and subsequent reaction pathways are presented. Arylamines are metabolized in the liver by monooxygenases to yield highly reactive *N*-hydroxyarylamines. *N*-Hydroxyarylamines can be detoxified by a

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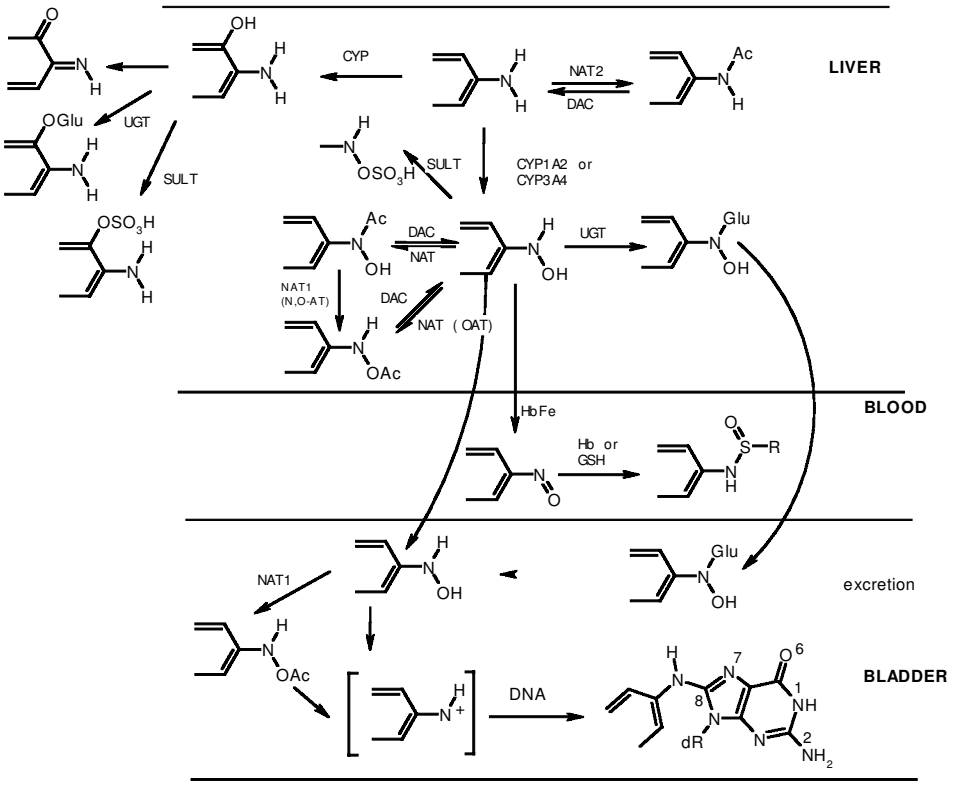


Figure 1. Short summary of arylamine metabolism (Beland and Kadlubar 1990, Delclos and Kadlubar 1997). *N*-hydroxylation by CYP (CYP1A2 for planar arylamines and CYP3A4 for bulky arylamines) is the predominant mode of activation of arylamines. *C*-Oxidation yielding phenolic metabolites is generally involved in detoxification. In some cases, phenols can be metabolized to reactive iminoquinones. Acetylases and deacetylases (DAC) have an important role in arylamine metabolism. Arylamines are detoxified by polymorphic NAT2 and excreted in the urine. NAT1 appears to function as an *O*-acetyltransferase (OAT) and as an *N,O*-acetyltransferase (*N,O*-AT) when using acetyl coenzyme A or hydroxamic acids, respectively, as acetyl donors. NAT2 appears to act preferentially as an OAT and NAT. Direct *N*-sulphation to yield sulphamates is a minor pathway of metabolism for most arylamines. The arylamines are oxidized to the potentially genotoxic *N*-hydroxyarylamines. The arylamines or the *N*-hydroxyarylamines can be glucuronidated. The *N*-hydroxyarylamines and the *N*-glucuronide are transported from the blood in the bladder. The acidic pH of the bladder catalyses the formation of the nitrenium ion, which forms a DNA adduct with guanine. In the blood the *N*-hydroxylamines are taken up in the erythrocytes and oxidized to nitrosoarenes, forming metHb; this process is known as the Kiese redox cycle (Kiese 1974).

microsomal reduced nicotinamide-adenine dinucleotide (NADH)-dependent reductase that rapidly converts the *N*-hydroxyarylamine back to the parent amine (King *et al.* 1999). *N*-Hydroxyarylamines can be further metabolized to *N*-sulphonyloxyarylamines, *N*-acetoxyarylamines or *N*-glucuronide *N*-hydroxylarylamine. These highly reactive intermediates are responsible for the genotoxic and cytotoxic effects of this class of compounds. *N*-Hydroxyarylamines and *O*-activated hydroxyarylamines react with DNA and proteins of the organs and the blood (haemoglobin [Hb] and albumin). Other products besides those of *N*-oxidation can also lead to toxic reactions. For example, the formation of quinoneimine intermediates after the oxidation of arylamines in the para and ortho

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positions can yield cytotoxic substances (Vermeulen *et al*. 1992). The toxic effects seen may be the result of the reaction of quinoneimine with the thiols of glutathione or protein, or the formation of superoxide anion radicals in a redox cycle with oxygen. The possible reactions that can evolve from benzoquinoneimine intermediates have been investigated by many research groups in studies on paracetamol (Vermeulen *et al*. 1992).

In humans there are toxicologically important, individual polymorphisms of *N*-acetyltransferase (NAT) (Meyer and Zanger 1997, Hirvonen 1999), CYP (McLeod *et al*. 1997, Landi *et al*. 1999, Wormhoudt *et al*. 1999) and glutathione *S*-transferase (GST) (Wormhoudt *et al*. 1999, Landi 2000); these are important for the individual susceptibilities that lead to disease (d'Errico *et al*. 1996, 1999). Slow acetylators have a higher bladder cancer risk but a lower colon cancer risk than fast acetylators (Lang *et al*. 1994). In the urinary bladder, putative arylamine DNA adducts are predominant and are significantly elevated in current smokers. Rapid CYP1A2 and slow NAT2 phenotypes are implicated in the activation (*N*-oxidation) and detoxification (*N*-acetylation) of arylamines in human bladder carcinogenesis (Yu *et al*. 1995, Badawi *et al*. 1995, Landi *et al*. 1996, Vineis and Martone 1996, Dallinga *et al*. 1998, Peluso *et al*. 1998, Probst-Hensch *et al*. 2000). Recent data have indicated that NAT1, which is expressed in human urothelium and catalyses the *O*-acetylation of *N*-hydroxyarylamines, is significantly correlated with DNA adduct levels and is bimodally distributed in this tissue (Badawi *et al*. 1996, Hirvonen 1999). The expression of NAT varies, and studies have been performed to detect the level of NAT1 and NAT2 mRNA in different tissues (Debiec-Rychter *et al*. 1999). Intratissue differences in NAT mRNA were observed: the most abundant NAT2 transcripts were found in hepatocytes, while NAT1 dominated in the urothelium and in colon epithelial cells. Specific NAT1 and NAT2 mRNAs were also present in the epithelial lining of the lung bronchi, the mammary gland and the small intestine epithelial cells, the outer layer of placental syncytiotrophoblast cells, the kidney tubules, and the pineal gland. Qualitative differences in the sites of the mRNA of these two enzymes were seen only in the kidney specimens, in which NAT2 was expressed in both proximal and distal tubules, and the NAT1 was detected only in the former. In addition, the level of expression of human NAT genes and *N*-acetylation rate markedly differed among individuals (Williams 2001). Preliminary studies suggested that the differences in NAT activity could not be attributed to NAT polymorphism. Thus, the level of mRNA expressed in the liver did not markedly differ between individuals with heterogeneous genotypes of the NAT2 gene. Therefore, in order to correlate the target dose with the disease, it would be necessary to make a quantitative assessment of the contribution of metabolism to the activation of the carcinogen in the organs of interest.

Introducing substituents in the ortho position can sterically hinder the oxidation of the amino group. This reduces the toxic potential of the amino group. For example, 3,3′,3,3′-tetramethylbenzidine is much less toxic than benzidine (Ashby *et al*. 1982). For the same steric reason, 2-naphthylamine (2-NA) is genotoxic and 1-NA is not, since animals dosed with the corresponding *N*-hydroxy-1-NA developed tumours. *N*-Acetylation is also hindered by substituents to the amino group in the ortho position. This has been shown in experiments with rabbits and with rats (Andres *et al*. 1987, Sabbioni 1992). The glucuronidation (Ritter 2000, Babu *et al*. 1996) or sulphation (Glatt 2000) of the amino or hydroxy group are

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further important pathways of toxification and detoxification. *N*-Glucuronides are excreted via the bile in the faeces, depending on the size of the amine. The *N*-sulphate is excreted in the urine. Eleven distinct sulphotransferase (SULT) forms are known, and differ greatly in their tissue distribution and substrate specificity. Functionally relevant genetic polymorphisms are known for two of the forms, SULT1A1 and SULT1A2, which can activate *N*-hydroxyarylamines and hydroxamic acids (Glatt 2000, Nagata and Yamazoe 2000). The different isoenzymes of uridine diphosphate glucuronosyltransferases (UGT) have been reviewed recently (Ritter 2000, Tukey and Strassburg 2000), and the discovery of polymorphisms is presently the research topic of several laboratories. *N*-Glucuronidation of arylamines competes with *N*-oxidation and is therefore a detoxification mechanism. UGT catalyses the *N*-glucuronidation of *N*-hydroxyarylamines and is likely to be responsible for their transport to the colon, where the pH or bacterial glucuronidases can release free amine or *N*-hydroxyarylamine, which can be further activated. UGT shows a widely varied but unimodal distribution in humans (Kadlubar *et al*. 1992). Recent results suggest marked differences in the substrate specificity of different UGT isozymes for arylamines and their *N*-hydroxy derivatives (Kadlubar *et al*. 1992, Orzechowski *et al*. 1994). Non-carcinogenic 1-NA was conjugated at a higher rate and higher affinity than 2-NA in cell-expressed rat UGT1.6. UGT1.6 showed poor activity towards *N*-hydroxy-4-ABP and 4-ABP. The substrate specificity of human UGT1.6 also appeared to be limited to planar 1-NA, 2-NA and its *N*-hydroxy derivative, whereas UGT1.7 showed a broader substrate specificity, including the bulky arylamines 4-ABP and its *N*-hydroxy derivative.

UGT, SULT and NAT2 can form more toxic products with *N*-hydroxyarylamines by introducing a glucuronide, sulphate or acetyl group on the hydroxy function. The leaving group properties of the hydroxy group are increased, and the ultimate carcinogen can be obtained at physiological pH. It has been shown in dogs that about 70% of *N*-hydroxy-4-ABP reaches the bladder in unconjugated form, while the remaining 30% is in the form of the *N*-glucuronide (Kadlubar *et al*. 1991). The glucuronides of 4-ABP and *N*-hydroxy-4-ABP are both acid labile, with half-life values of 10.5 and 32 min, respectively, at pH 5.5. The glucuronide of *N*-hydroxy-*N*-acetyl-4-ABP was not acid labile, with half-life values at pH 5.5 and 7.4 of 55 and 68 min, respectively. Thus, the glucuronide of 4-ABP was the most acid-labile conjugate. A large portion of the *N*-hydroxy-4-ABP will be oxidized in erythrocytes to nitrosobiphenyl, which then forms a sulphinamide with the cysteine of Hb or glutathione. In animals and in humans it has been shown that *N*-hydroxy-4-ABP reacts with urethelial bladder DNA and with Hb (figure 1).

The metabolism of benzidine is atypical for arylamines, since it is a substrate for extrahepatic peroxidases rather than CYP (reviewed in Whysner *et al*. 1996, Lakshmi *et al*. 2000). However, after *N*-acetylation benzidine becomes a substrate for CYP, resulting in the production of *N*-hydroxy-*N*-acetyl-benzidine (Frederick *et al*. 1985, Delclos and Kadlubar 1997). The two pathways yield different reaction products. Presently, only the products resulting from the CYP pathway have been found in humans. This in agreement with the fact that fast acetylators have a higher risk for bladder cancer when exposed to benzidine (Hayes *et al*. 1993).

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## Metabolism of nitroarenes

Nitroarenes are taken up through the gastrointestinal tract, the respiratory systems and the skin. The first metabolic step for nitroarenes is reduction of the nitro group or oxidation of the aromatic ring (Beauchamp *et al.* 1982, Rickert 1987). After nitro reduction, the same metabolites as after arylamine exposure are found. Nitroarenes are reduced by the microflora in the gut and by several enzymes in the liver to nitro radical anions, nitrosoarenes, *N*-hydroxyarylamines and/or the amine. Several studies have been performed to establish which is the more important organ for the reduction of nitroarenes. In rats treated with antibiotics, the covalent Hb binding of nitrobenzene, 4-nitrobiphenyl (4-NBP) and 2-nitrofluorene decreased by factors of 7, 30 and 2, respectively (Suzuki *et al.* 1989). This demonstrates that the reductive activation of nitrobenzene and 4-NBP is largely dependent on their metabolism by the intestinal microflora. The reduction of 2-nitronaphthalene was not affected by antibiotic treatment. In rabbits, antibiotics did not affect the nitro reduction of 1,3-dinitrobenzene (1,3-DNB) (Rickert *et al.* 1984). In *in vitro* experiments it has been shown that nitroarenes with strong electron-withdrawing groups such as 1,3-DNB, 1,2-DNB, 1,4-DNB and TNT can be reduced in erythrocytes. Therefore, easily reducible nitroarenes such as dinitrobenzenes, trinitrobenzenes and TNTs can be reduced in sites other than the gut or the liver. The reducibility of nitroarenes can be predicted from the calculated energy level of the lowest unoccupied molecular orbital (Debnath *et al.* 1992b, Sabbioni and Sepai 1995). In nitrobenzene the nitro group is in the same plane as the benzene ring. By introducing a bulky substituent in the ortho position, the nitro group is forced out of the plane of the ring. This decreases the reducibility and, in general, the toxicity of the compounds, for example TNT versus musk xylene. However, musk xylene is much more lipophilic than TNT; this will reduce the elimination rate and increase the accumulation in fat tissue.

In male Fisher 344 rats the urinary metabolites after an oral dose of nitrobenzene were 4-hydroxyacetanilide, 4-nitrophenol and 3-nitrophenol (Beauchamp *et al.* 1982). These metabolites were excreted as sulphate ester conjugates. Metabolism and excretion of 2-nitrotoluene (2-NT), 3-NT and 4-NT was studied in male Fischer 344 rats (Chism and Rickert 1991). The major metabolites excreted 72 h after administration were the corresponding benzoic acids, acetamidobenzoic acids and nitrohippuric acids. However, the percentage of these metabolites varied between the isomers. The most abundant excreted metabolites of 2-NT were *S*-(2-nitrobenzyl)-*N*-acetylcysteine (12%), 2-nitrobenzylglucuronide (14%) and a metabolite tentatively identified as a sulphur-containing conjugate of 2-aminotoluene, conjugated through the benzyl group (16%). When radiolabelled 2-NT was incubated with isolated hepatocytes from the same species of rat, the following metabolites were detected: 2-nitrobenzyl alcohol, 2-nitrobenzyl alcohol glucuronide, 2-nitrobenzoic acid and an unidentified metabolite. However, when incubated with isolated microsomes, only the nitrobenzyl alcohol was detected.

Nitrated polyaromatic hydrocarbons (nitro-PAHs) have been subject of several studies, and excellent reviews have been published recently (Fu and Herreno-Saenz 1999, Purohit and Basu 2000). 1-Nitropyrene (1-NP) (van Bekkum *et al.* 1999) and 2-nitrofluorene (Moller 1994) are used in biomonitoring studies as lead compounds for exposure to nitro-PAH. After intragastric and intraperitoneal

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administration and after inhalation of 1-NP, the majority (50–60%) of the administered dose is excreted in the faeces and 15–20% of the dose is excreted in the urine. The major pathways in the biotransformation of 1-NP are nitro-reduction with subsequent acetylation or *N*-oxidation, ring-hydroxylation and conjugation. The main metabolite identified in the urine of rats given 1-NP was 6-hydroxy-*N*-acetyl-1-aminopyrene, which accounted for approximately 20% of the total radioactivity observed in the urine (Ball *et al*. 1994). 2-Nitrofluorene is metabolised *in vivo* by two different routes (Moller 1994). After inhalation, potent mutagenic metabolites, hydroxylated nitrofluorenes, are formed. The metabolites are distributed by the systemic circulation. After oral administration, 2-nitro-fluorene is reduced to the corresponding amine and then acetylated, yielding 2-acetylaminofluorene (AAF), which is a potent carcinogen. Further ring-hydroxylation of AAF leads to detoxification and excretion. Induction of CYPs affects the metabolism, and more hydroxylated nitrofluorenes are formed and more mutagenic metabolites are found in the circulation. Hydroxylated nitrofluorenes are excreted in the bile as non-mutagenic glucuronide conjugates. When these conjugates are excreted via the bile, intestinal $\beta$-glucuronidase can liberate direct-acting mutagens into the intestine. Thus, inhalation of 2-nitrofluorene can lead to formation of potent mutagens in the intestine.

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## Mutagenicity and carcinogenicity of arylamines

Several arylamines are mutagenic and carcinogenic (Debnath *et al*. 1992a). Many amines are mutagenic in the *Salmonella* tester strains TA98 and TA100, but metabolic activation with the S9 microsomal preparation mix is required for activity for most of the active ones. 2,4-DAT, 2,4-diaminoethylbenzene and a few amines containing a nitro group are direct mutagens (Chung *et al*. 1997). In general, the mutagenic potency increases with the oxidizability (Sabbioni and Sepai 1995) and the lipophilicity of the arylamine. Several models have been developed to predict the mutagenicity of arylamines (Debnath *et al*. 1992a, Benigni *et al*. 1994, 2000). The quantitative structure–activity relationship (QSAR) has been derived for the mutagenic activity of 88 aromatic and heterocyclic amines acting on *Salmonella typhimurium* TA98 with the S9 microsomal preparation and 67 amines acting on TA100 with the S9 microsomal preparation (Debnath *et al*. 1992a). Mutagenic activity can be predicted with equations containing a parameter for hydrophobicity (log P) and the calculated energy of the highest occupied molecular orbital of the amine. The dependence of mutagenic activity on hydrophobicity and electron effects are similar in TA98 and TA100. Benigni *et al*. (1994) discovered that there is a dramatic difference between the QSARs for mutagenic potency and those for yes/no mutagenic activity: hydrophobicity plays a major role in determining the potency of the active compounds, whereas mainly electron factors differentiated the actives from the inactives. The electron factors are those expected on the basis of the hypothesized metabolic pathways of the chemicals. It appears that these factors (possible together with size/shape) determine the minimum requirement for the chemicals to be metabolized, whereas the hydrophobicity determines the extent of activity of chemicals that can be metabolized (the actives). The differences in the QSARs found for the *Salmonella* strains TA98 and TA100 are probably a consequence of the different molecular mechanisms of mutagenicity in these organisms.

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The carcinogenicity of arylamines has been tested in mice and rats (Garner *et al*. 1984). The National Toxicology Program (NTP) study (http://ntp-server. niehs. nih.gov) included several arylamines (Gold *et al*. 1993, http://potency. berkeley. edu/cpdb.html). Arylamines induce tumours at different sites in different species. Ashby compared the mutagenicity and carcinogenicity of the compounds from the NTP studies with his predictive model (Ashby and Paton 1993). Most carcinogenic arylamines were mutagenic, except for two compounds (Ashby and Tennant 1991). However in five cases the compounds were mutagenic but not carcinogenic. In humans (Case *et al*. 1954, Parkes and Evans 1984) benzidine, 2-naphthylamine, 4-chloro-2-methylaniline and 4-ABP have been classified by the IARC as clearly carcinogenic in humans (http://193.51.164.11/monoeval/ crthall.html).

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## Mutagenicity and carcinogenicity of nitroarenes

Most nitroarenes are mutagenic in the *Salmonella* TA100 or TA98 test. Including nitroheteroaromatic compounds, 30 out of 162 were not mutagenic (Debnath *et al*. 1992b, Benigni *et al*. 1994). For the nitroarenes and arylamines, the *N*-hydroxyarylamine is believed to be the crucial intermediate that is activated to give an electrophilic nitrogen species capable of reacting with DNA. The major difference between the two classes of mutagens lies in their transformation to *N*-hydroxyarylamines. The nitroarenes are reduced to the critical *N*-hydroxyarylamine by cytosolic reductases present in the bacterium. Thus, nitroarenes must initially penetrate through the cell membrane, but the unstable *N*-hydroxyarylamine is then close to its activation site and to the DNA. In contrast, arylamines are oxidized by enzymes found in S9, principally CYP. Thus activation takes place outside the bacterium, and it is the unstable *N*-hydroxyarylamine that must penetrate the cell in order to reach the DNA and cause mutagenesis. A QSAR has been derived for the mutagenic activity of 117 aromatic and heteroaromatic nitro compounds acting on *S. typhimurium* TA100. Relative mutagenic activity is bilinearly dependent on hydrophobicity, with an optimal log P of 5.44, and is linearly dependent on the energy of the lowest unoccupied molecular orbital of the nitro compound. The dependence of mutagenic activity on hydrophobicity and electron effects is very similar for TA98 and TA100. Mutagenic activity in TA100 does not depend on the size of the aromatic ring system, as its does in TA98. The effect of the choice of assay organism, TA98 versus TA100, on the QSAR of nitroarene is similar to the effect previously found for arylamines. Bulky substituents to the nitro group in the ortho position decrease the mutagenicity (Klein *et al*. 2000a), the reducibility of the nitro group and the toxicity. Recently the effect of bulky alkyl substituents far away from the nitro group on the mutagenicity was systematically investigated (Klein *et al*. 2000b, Haack *et al*. 2001) using derivatives of 4′-NBP bearing bulky substituents in the para 4′ position. In the absence of S9 in TA98 and TA100, the mutagenicity of the compounds decreased with the increasing steric demand of the attached alkyl groups, in the order of 4′-H > 4′-methyl > 4′-ethyl > 4′-*n*-butyl > 4′-*iso*-propyl > 4′-*tert*-butyl-adamantyl.
Changes in the molecular form from planar to non-planar caused by the bulk of the introduced substituents may be responsible for this effect by interfering with an efficient intercalation into DNA.

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The cytotoxicity of several nitroarenes has been studied in cells. The cytotoxicity of nitroarenes is related to their ease of reduction to nitroradical anions and nitrosobenzenes (O'Brien *et al.* 1990). The most cytotoxic compounds have the strongest electron-withdrawing substituent (e.g. 14-DNB). Biaglow *et al.* (1977) postulated that for nitroarenes with oxidation–reduction potentials more positive than $-0.35$ V, cellular oxygen utilization was stimulated and the chances of oxidative stress are increased. The IARC (http://193.51.164.11/monoeval/crthall.html) has classified 2,6-dinitrotoluene (2,6-DNT), 2,4-DNT, nitrobenzene and 4-methoxy-nitrobenzene as possible carcinogens in humans. All other monocyclic and bicyclic nitroarenes were unclassifiable as to carcinogenicity to humans. Only 3,7-dinitro- and 3,9-dinitrofluoranthene, 1,6- and 1,8-dinitropyrene, 6-nitrochrsene, 2-nitrofluorene, 1-NP and 4-NP have been classified as possibly carcinogenic to humans.

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## Biomonitoring

Biomarkers may be used to assess the exposure and effects of chemicals and the susceptibility of individuals. They can also be used to elucidate cause–effect and dose–effect relationships in health risk assessment, in clinical diagnosis and for monitoring purposes. Molecular biomarkers have been identified for several chemicals. The rationale for the use of biomarkers (Skipper and Groopman 1991, IPCS 1993, Timbrell 1998, Albertini *et al.* 2000) is represented in figure 2. This model classifies molecular biomarkers according to the discrete steps in the progressive nature of disease. The scientific community distinguishes between (1) markers of susceptibility, (2) markers of the internal dose, (3) markers of the biologically effective dose, (4) markers of early biological effects, (5) markers of altered function, and (6) markers of clinical disease. This review will focus the progress made on the development of markers of the internal dose and the biologically effective dose.

## Biomonitoring of arylamines and nitroarenes in human breast milk

Debruin *et al.* (1998, 1999) measured the monocyclic arylamines aniline, 2-methylaniline (2-MA) and *N*-methylaniline at parts per billion levels in human milk samples using a new solid-phase microextraction technique coupled with gas chromatography-mass spectrometry (GC-MS). All samples had quantifiable concentrations of aniline (0.05–5.2 ng $l^{-1}$). Eleven of the samples contained both 2-MA and *N*-methylaniline. For 2-MA, levels ranged from $< 0.01$ to 0.26 ng $l^{-1}$. For *N*-methylaniline, levels ranged from $< 0.01$ to 7.44 ng $l^{-1}$. The levels of 2-MA and its mean value (0.04 ng $l^{-1}$) were approximately 10-fold smaller than those of aniline (0.36 ng $l^{-1}$) and *N*-methylaniline (0.55 ng $l^{-1}$). The level of aniline in the milk (0.05–5.8 ng $l^{-1}$) was comparable to levels previously detected in the urine (see below). For 2-MA, levels in the milk ($< 0.01$–0.26 ng $l^{-1}$) were approximately one order of magnitude lower. This indicates that human breast ductal epithelial cells are directly exposed to arylamines, including 2-MA, which is a mammary carcinogen in female rats. Peroxidases present in milk can oxidize arylamines to reactive electrophiles, which bind to DNA and induce mutations. Hydrogen peroxide, required for peroxidase-dependent oxidations, is supplied by milk xanthine oxidase and by the respiratory burst of neutrophil cells that are present

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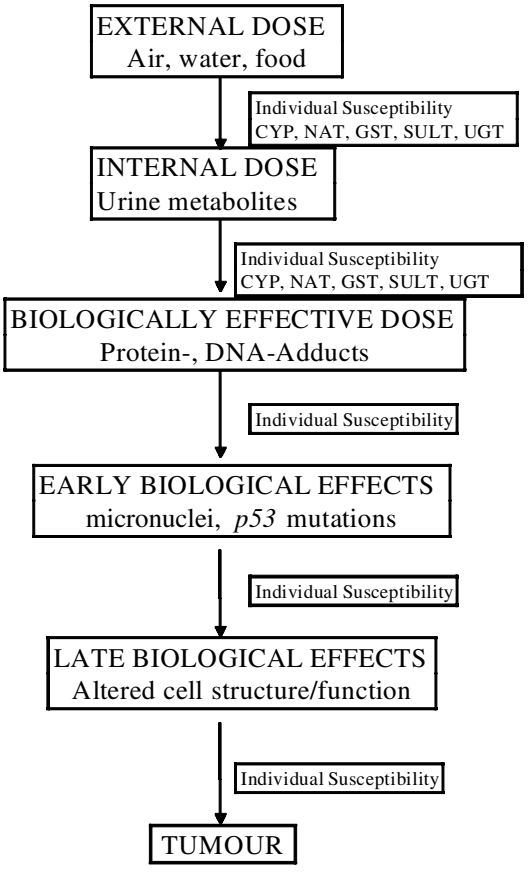


Figure 2. Molecular biomarker paradigm.

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in milk and activated by exposure to it. Josephy (1996) proposed that lactoperoxidase and MPO activate arylamines within the breast ducts, and that these enzymes play a crucial role in the chemical induction of breast cancer. The activation of arylamines and genotoxic damage was confirmed in exfoliated cells (Martin *et al.* 2000, Williams and Phillips 2000) isolated from breast milk samples.

The levels of musk xylene, musk ketone, musk ambrette, musk moskene and musk tibetene were investigated in 32 human adipose tissue samples and 23 human milk samples from northern Germany (Rimkus *et al.* 1994, reviewed in Käfferlein *et al.* 1998). The residue levels for musk xylene and musk ketone were 0.02–0.22 mg kg$^{-1}$ fat and 0.01–0.09 mg kg$^{-1}$ fat, respectively. In a few samples musk ambrette and musk moskene were detected at low levels. Musk tibetene was not detected.

## Biomonitoring of arylamines and nitroarenes in human urine

In most biomonitoring studies urine analysis involved determination of the parent arylamine or of the *N*-acetylated metabolites. Determination of the arylamine only provides information on the uptake of the compound. Compounds in urine are an indicator of recent exposure (about 48 h). The additional determi-

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nation of the *N*-acetylamines gives an indication of the *N*-acetylator phenotype (Lewalter and Korallus 1985). The presence of arylamines in urine has been investigated in several studies. The arylamines studied include benzidine (Rothman *et al*. 1996a), 3,3′-dimethylbenzidine, 3,3′-dimethoxybenzidine (Neumeister 1991), 2-MA (Ward *et al*. 1996), 4,4-methylenedianiline (MDA) (Cocker *et al*. 1988a, Schütze *et al*. 1995), MOCA (Ward *et al*. 1986, 1987, Osorio *et al*. 1990) and diaminotoluenes (Lind *et al*. 1996). In a few studies DNA adducts have been found in urinary bladder exfoliated cells (Talaska *et al*. 1991a, b, Kaderlik *et al*. 1993, Rothman *et al*. 1997). BAT values for occupational exposures have been established for aniline (1 mg $l^{-1}$) and for nitrobenzene (0.1 mg $l^{-1}$) (Lewalter 1986). In both cases aniline is measured.

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### Monocyclic arylamines

Aniline and 2-, 3-, and 4-MA, which are representative of arylamines in cigarette smoke, were identified and quantified in human urine following base hydrolysis under reflux (El-Bayoumi *et al*. 1986). Smokers excreted 3.1 μg/24 h of aniline and 6.3 μg/24 h of 2-MA ($n = 16$). Non-smokers excreted 2.8 μg/24 h of aniline and 4.1 μg/24 h of 2-MA ($n = 12$). 3-MA and 2-MA were detected in the urine of two of the 11 smokers and four of the nine non-smokers. The observed intra- and interindividual variations in the amounts of urinary aniline and 2-MA were relatively large. Thus sources other than cigarette smoke contribute significantly to the concentrations of aniline and methylanilines in human urine.

A urinalysis method for detecting 2-MA and aniline was developed for the biomonitoring of workers (Brown *et al*. 1995). Urine specimens were made to 4.7 M in NaOH and heated at 80°C for 2 h to convert the metabolites acetanilide and *N*-acetyl-2-methylaniline, to the free amines. Extraction of the hydrolysate with butylchloride and back extraction with 0.1 M HCl gave an amine fraction, which was analysed by reversed phase high pressure liquid chromatography (HPLC) with electrochemical detection. For 2-MA and aniline the limit of detection was 0.6 and 1.4 μg $l^{-1}$, respectively. This method was applied to 171 urine specimens from chemical plant workers. The median 2-MA levels were 11 μg $l^{-1}$ exposed pre-shift, 65 μg $l^{-1}$ exposed post-shift, 0.7 μg $l^{-1}$ non-exposed pre-shift, and 2.6 μg $l^{-1}$ non-exposed post-shift. For aniline the median levels were 11 μg $l^{-1}$ exposed pre-shift, 23 μg $l^{-1}$ exposed post-shift, 2.0 μg $l^{-1}$ non-exposed pre-shift, and 3.2 μg $l^{-1}$ non-exposed post-shift. The urinary levels of the two amines, especially 2-MA, demonstrated significant uptake of the amines during the work shift and an accumulation of part of the dose with each passing work shift.

In both these studies the method used did not distinguish between acetylamines and the corresponding arylamines.

### Aromatic diamino compounds

#### Benzidine congeners

Meal *et al*. (1981) investigated the presence of benzidine and its conjugates in the urine of workers exposed to benzidine-derived dyes. Urine samples were collected from 29 workers in three textile dyehouses, two tanneries, and two dyestuff quality control laboratories in England. In 200 samples obtained over a

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period of 15 months and analysed by GC-MS, no free benzidine or monoacetylbenzidine was detected. The detection limit was $0.92\,\mu g\,l^{-1}$. Nevertheless, after strong acid hydrolysis of urine samples taken on two separate occasions from textile dye weighers, trace amounts of benzidine were detected.

A sampling and analytical procedure for the measurement of benzidine, 3,3′-dimethoxybenzidine, 3,3′-dimethylbenzidine, and 4,4′-MDA in urine was developed for use in industrial hygiene investigations by Neumeister (1991). After hydrolysis of urine metabolites, the free diamines are isolated using a C18 solid sorbent. The free diamines are eluted, concentrated, and analysed by HPLC, monitoring the ultraviolet (UV) absorbance and the electrochemical response. Using UV detection, the limit of detection for these aromatic diamines was less than $2\,\mu g\,l^{-1}$, while the limit of quantification was less than $6\,\mu g\,l^{-1}$. For electrochemical detection, the limit of detection was less than $0.3\,\mu g\,l^{-1}$ and the limit of quantification was less than $0.9\,\mu g\,l^{-1}$.

Rothman coordinated a large biomonitoring project in workers from India exposed to benzidine and benzidine azo dyes (Rothman *et al*. 1996a, b, 1997, Zhou *et al*. 1997, DeMarini *et al*. 1997). For the analyses of the urine samples Hsu *et al*. (1996) developed an isotope dilution GC-MS method with negative ion chemical ionization to quantify urine concentrations of benzidine (Bz) and of its acetylated metabolites *N*-acetylbenzidine (AcBz) and *N*,*N*′-diacetylbenzidine (DiAcBz). Urine samples were purified by solid-phase extraction, reduced with $LiAlH_4$, and derivatized with pentafluoropropionic anhydride (PFPA). The derivatives were measured by selected ion monitoring relative to deuterium-labelled internal standards. Urinary concentrations of AcBz ($2.9–732\,\mu g\,l^{-1}$) substantially exceeded those of benzidine ($0.1–37\,\mu g\,l^{-1}$) and DiAcBz ($0.2–17\,\mu g\,l^{-1}$) in the six workers exposed to benzidine or benzidine-based dyes.

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### *MDA congeners*

Most urine biomonitoring studies have been published for MOCA. Since the late 1970s, MOCA users have relied on urinary monitoring as the primary method of assessing MOCA exposure in the workplace (Ward *et al*. 1986, Clapp *et al*. 1991). Urinary concentrations of $70–1500\,\mu g\,l^{-1}$ were found in samples from workers in one area of a MOCA production facility (Ward *et al*. 1987). Between 1980 and 1983, 16.9% of 3323 urinary samples submitted by 54 USA companies contained MOCA at concentrations $>50\,\mu g\,l^{-1}$. MOCA is absorbed primarily through the skin. Due to concerns that airborne monitoring would not truly assess the degree of worker absorption of MOCA, an association of MOCA users implemented a voluntary biomonitoring programme to assess and reduce worker exposure to MOCA. The design of closed transfer systems and production of a fused, hardened MOCA pellet are examples of actions taken to reduce worker exposure. After implementing these controls, the percentage of urine specimens with MOCA levels $<25\,\mu g\,l^{-1}$ increased from 77% to 86%. During this time, the percentage of samples with levels $>50\,\mu g\,l^{-1}$ decreased from 12% to 8%.

Urine samples from workers exposed to MOCA contain labile metabolite that, on hydrolysis, yields the parent compound at concentrations two to three times that of free MOCA (Cocker *et al*. 1990, Cocker *et al*. 1996). Evidence has now been obtained that the major labile metabolite is an *N*-glucuronide of MOCA. In view of the lability of this compound and the fact that its concentration in urine is two to

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three times that of free MOCA, it is essential that any strategy for the biomonitoring of exposed workers takes into account the *N*-glucuronide.

Cocker *et al.* (1988a, b) reported a GC-MS assay for the determination of MOCA, *N*-acetyl-4,4′-methylenebis(2-chloroaniline) (AcMOCA), MDA and *N*-acetyl 4,4′-methylenedianiline (AcMDA) in urine. The method is based on the solvent extraction of the compounds together with deuterium-labelled internal standards. The compounds are separated and detected by GC-MS as their PFPA derivatives. The method has been applied to the detection of MDA and AcMDA in the urine of workers occupationally exposed to MOCA and MDA. The results show that, whilst AcMOCA is a relatively minor urinary metabolite, a significant proportion of MDA is excreted as the *N*-acetylated compound.

Recently Robert *et al.* (1999a, b) investigated the best way to store and hydrolyse urine from MOCA-exposed workers, and reviewed all extraction and analysis procedures used to date for the determination of MOCA. The addition of sulphamic acid is proposed for the storage and extraction of urine with isooctane. MOCA was quantified by HPLC using electrochemical and UV detection.

Cocker *et al.* (1994) examined exposure to MDA in 411 workers from 45 factories. All urine samples were analysed for MDA and its acetyl metabolites, and results were reported as total MDA. In this study, 91% of post-shift urine samples and 88% of pre-shift samples had less than 50 nmol MDA $mmol^{-1}$ creatinine. Some data indicated that when exposure to MDA was through inhalation (as solid material or contaminated dust), post-shift urine samples had higher MDA concentrations than samples taken pre-shift the next day. When exposure was most likely to be through the dermal route, urine samples taken pre-shift the next day tended to have higher MDA concentrations than urine samples collected immediately post-shift on the day of exposure. Therefore, a biological monitoring sampling strategy for MDA must take account of the route of entry into the body. If exposure is likely to be via inhalation, post-shift samples should be collected, and if exposure is likely to be via the skin, pre-shift samples taken the next day are more appropriate.

MDA and AcMDA have also been found in workers exposed to MDI (Sepai *et al.* 1995b, Dalene *et al.* 1996).

### *Diaminotoluenes*

In urine, 2,4- and 2,6-DAT were determined in workers exposed to the corresponding diisocyanates 2,4- and 2,6-toluene-diisocyanate. Urine samples were hydrolysed in HCl or $H_2SO_4$ for several hours. The arylamines were then extracted, derivatized with PFPA and analysed by GC-MS with negative chemical ionization (NCI). Skarping's group have published several studies on this topic (Lind *et al.* 1996). Interestingly, the best NCI results were obtained using ammonia rather than methane as the reactant gas. Diaminotoluenes have also been found in females with polyurethane-covered breast implants (Chan *et al.* 1991, Sepai *et al.* 1995a).

### *Nitroarenes*

Rickert's group have performed several metabolism studies with nitroarenes in animals (reviewed in Rickert *et al.* 1984, Rickert 1985, 1987). The meta-

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bolic profile was determined in a small number of workers. Urine specimens were collected over a 72 h period from workers at a dinitrotoluene (DNT) manufacturing plant. Samples were analysed for 2,4- and 2,6-DNT and putative metabolites by GC-MS (Turner *et al*. 1985). Urine was treated with glucuronidase, then extracted, and derivatized with bis(trimethylsilyl)trifluoroacetamide and diazomethane. Urine from workers exposed to DNT contained 2,4- and 2,6-DNT, 2,4- and 2,6-dinitrobenzoic acid (DNBA), 2,4- and 2,6-dinitrobenzylalcohol (cleaved from the glucuronide), 2-amino-4-nitrobenzoic acid, and 2-(*N*-acetyl)amino-4-nitrobenzoic acid. Excretion of these metabolites peaked near the end of the shift, but declined to either very low or undetectable concentrations by the start of work the following day. The calculated half-times were 1.0–2.7 h for the elimination of total DNT-related material detected in urine and 0.8–4.5 h for the elimination of individual metabolites. The most abundant metabolites were 2,4-DNBA and 2-amino-4-nitrobenzoic acid, which together accounted for 74–86% of the metabolites detected. These data indicate that urinary DNT metabolites in humans are qualitatively similar to those found in rats, but quantitative differences exist in the relative amounts of each metabolite excreted.

Woollen *et al*. (1985) examined the urine of workers in an explosives factory exposed to technical grade DNT. Levels of the DNT metabolite 2,4-DNBA were extremely low or non-detectable in urine samples prior to starting work at the beginning of the working week, but had a mean of $17\,mg\,l^{-1}$ in post-shift urine samples. There were wide variations in the concentrations excreted in urine by different workers and by individual workers on consecutive days. Atmospheric levels of DNT (detected by personal monitoring) were well below the recommended limit and therefore could not account for the observed excretion of 2,4-DNBA. The skin may have been the major route of absorption of DNT during this process. Uptake of DNT is rapid; the highest 2,4-DNBA levels were normally seen in the end-of-shift specimens. 2,4-DNBA was shown to be the major known DNT metabolite excreted in human urine.

A total of 82 urine samples from persons working in explosives disposal were investigated in Germany (Angerer and Weismantel 1998). After acid hydrolysis in order to release the conjugated part of the 2,4-DNBA, the analyte was selectively separated from the urine matrix via various extraction steps and then derivatized with HCl/methanol to the methylester. Quantitative analysis was carried out using GC-MS. 3,5-DNBA was used as an internal standard. The detection limit was $1\,\mu g\,l^{-1}$ urine. The concentrations of 2,4-DNBA detected ranged from the detection limit to $95\,\mu g\,l^{-1}$ urine.

Metabolites of TNT have been found in the urine of rats fed TNT, in the urine of rats exposed to TNT via the skin, and in the urine of TNT munitions workers. The metabolites found included untransformed TNT, 2-amino-4,6-DNT, 4-amino-2,6-DNT, 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene (Yinon and Hwang 1986, Ahlborg *et al*. 1988, Yinon 1990, Rosenblatt 1991).

Since World War II, exposure to TNT derivatives is also an environmental problem. In order to monitor workers involved in the clean up of ammunition sites from World War I and II, Bader *et al*. (1998) developed two GC-MS methods for the detection of 12 nitroarenes in urine (nitrobenzene, 1,2- and 1,3-DNB, 1,3,5-trinitrobenzene, 2-, 3- and 4-NT, 2,4- and 2,6-DNT, TNT, 2-amino-4,6-DNT, and 4-amino-2,6-DNT). Urine samples were obtained from nine workers from an ammunition-dismantling workshop and from 12 controls. TNT was detected in

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six samples at concentrations between 4 and 43 $\mu g l^{-1}$. The main metabolites of TNT, 4-amino-2,6-DNT and 2-amino-4,6-DNT, were found at concentrations ranging from 143 to 16 832 $\mu g l^{-1}$ and from 24 to 5787 $\mu g l^{-1}$, respectively. Non-conjugated aminodinitrotoluenes were present at varying percentages of the total amount. 2,4-DNT and 2,6-DNT were found in two samples (2–9 $\mu g l^{-1}$). Nitroarenes were not detectable in urine specimens from the controls.

Urinary metabolites of human subjects acutely poisoned with 4-chloronitrobenzene (4-CNB) were identified by Yoshida *et al.* ( 1993). Very large amounts of *N*-acetyl-(4-nitrophenyl)-cysteine, relatively large quantities of 4-chloroaniline (4-CA) and 2-chloro-5-nitrophenol, small amounts of 2-amino-5-chlorophenol and 2,4-dichloroaniline (2,4-DCA), and traces of 4-chloro-2-hydroxyacetanilide were found. All the 4-CNB was metabolized, since the parent compound was not found in urine. *N*-Acetylated metabolites of 4-CA and 2-amino-5-chlorophenol were found in only one of the eight individuals, indicating that this pathway is weak or possibly absent in some humans.

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## Blood protein adducts of arylamines and nitroarenes

*N*-Hydroxyarylamines and *O*-activated hydroxyarylamines react with the blood proteins Hb and albumin. To date, except for a 4-ABP adduct with albumin, only the formation of hydrolysable adducts has been studied for arylamines. The formation of hydrolysable adducts of nitrosofluorene with the cysteine of peptides was discovered by Lotlikar *et al.* (1965). The formation of nitrosoarenes in erythrocytes and adduct formation with Hb and reduced glutathione was studied for several years by Eyer (Eyer 1979, Eyer and Gallemann 1996) and Neumann (Gaugler and Neumann 1979, Neumann 1986). Briefly, *N*-hydroxyarylamines are oxidized in the erythrocytes to nitrosoarenes, which react with the thiol group of cysteine to semimercaptals. Elimination of hydroxide to the sulphenamide cation, which partly allocates its positive charge to the less electronegative sulphur atom, and addition of a water molecule with subsequent proton rearrangement yields the sulphinamide (Kazanis and McClelland 1992). Hb adducts are stable, but glutathione adducts are reduced by glutathione reductases back to the parent compounds (Eyer 1979). Arylamines react with cysteine 93 of the $\beta$-chain of Hb. The structure of the Hb adduct with 4-ABP has been determined by X-ray crystallography of an *in vitro* modified Hb with 4-nitrosobiphenyl (Ringe *et al.* 1988). Albumin adducts have only been analysed for a few compounds (see below).

### *Blood protein adducts of arylamines and nitroarenes in rodents*

For a few aromatic amines it has been shown that Hb adducts are surrogate markers for the modification of DNA in the target organ. A linear dose–response relationship was demonstrated in rats 24 h after a single oral dose of AAF (Pereira *et al.* 1981) and [$^3$H]-trans-4-dimethylaminostilbene (Neumann 1980). After chronic exposure to DcBz (Sagelsdorff *et al.* 1996) and 2,4-DAT (Wilson *et al.* 1996), linear relationships between DNA and Hb adducts were found. In the case of 2,4-DAT, Hb and DNA binding showed a saturation effect at higher doses. One important advantage of using protein adducts of carcinogens rather than urine metabolites is the longer availability of the protein adducts in the biological system. The exposure history over the lifetime of the proteins can be studied by measuring stable albumin

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or Hb adducts. An important premise of protein dosimetry is the stability of the protein adducts to be analysed. The lifetime of the adducted protein should be equal to the lifetime of the unadducted protein. The lifetimes of several protein adducts have been studied (reviewed in Skipper and Tannenbaum 1990). The following factors can influence the lifetime of protein adducts: (1) the chemical bond between the protein and the xenobiotic may be unstable; (2) functional alterations in the proteins might cause earlier elimination of the proteins; (3) Hb damaged by oxidants is digested by proteases present in the erythrocytes (Fagan and Waxman 1991); and (4) the presence of adducts in proteins is known to be a signal for the catabolism of proteins (Stadtman 1990).

The lifetime of Hb adducts has mainly been studied in rats, and conflicting results have been obtained. Only 4-ABP adducted Hb has similar a lifetime to control Hb in rats (Green *et al*. 1984). The 4-ABP adduct levels decreased by 2.5% per day, indicating that the adduct is removed more rapidly than unmodified Hb, which is removed at a rate of 1.7% per day. With regard to benzidine (Neumann 1984a), MOCA (Cheever *et al*. 1990, 1991) and 2-MA (DeBord *et al*. 1992), the decay of the adducted Hb followed a first-order kinetic with a half-life of 14, 11 and 12.6 days, respectively. Additional kinetic evaluations of the 2-MA and MOCA adduct half-lives have been published recently (Troester *et al*. 2001). In a further experiment with benzidine, Neumann *et al*. (1993) labelled erythrocytes by injecting [$^3$H]diisopropylfluorophosphate (DFP) into 18 female Wistar rats. After 3 days the rats were divided in two groups: one received [$^{14}$C]benzidine and the other one only solvent. Using a semilogarithmic plot, half-lives of 14.7 and 14.4 days were found for the DFP label and the benzidine label, respectively. The benzidine adduct is therefore considered stable *in vivo*. On the basis of further experiments and by applying an equation proposed by the International Committee for Standardization in Haematology, Neumann concluded that the lifespan of erythrocytes in female rats given benzidine is 65 days, but in addition 3% of the erythrocytes are eliminated per day independent of age. For 2,4-difluoroaniline (2,4-DFA) the half-life was only 6.4 days (Sabbioni 1992). This very short half-life suggests instability of the adduct or an increased elimination rate of the adducted Hb in the spleen. Fast adduct elimination was also noted in humans for 2,4-DFA (Boogard *et al*. 1994a). Compared with aniline sulphinamide adducts, 2,4-DFA adducts are more likely to be hydrolysed under physiological conditions, since 2,4-DFA is the better leaving group than aniline (p$K_a$ of 2,4-DFA minus p$K_a$ of aniline = 1.2). 2,4-DFA adducts to cysteine might be biodehalogenated via the formation of a fluoroquinoneamine, as described for 2,4-DFA in microsomal preparations (Rietjans *et al*. 1990). The formation of a fluoroquinoneamine would cause breakage of the nitrogen–sulphur bond.

Eyer *et al*. (1983) showed that simple adducts of 4-dimethylaminophenol with the $\beta$-93 cysteine of human Hb do not change the functional properties of Hb. Only the additional reaction with $\beta$-146 histidine causes crucial alteration of the functional properties. Therefore, the pharmacokinetics that lead to the steady state of the Hb adduct level might not be the same for all arylamines. The stability of albumin adducts have been determined for a few compounds. [$^{14}$C]MOCA, [$^{14}$C]2-MA and [$^3$H]4-ABP have half-lives of 4.6 days (determined after chronic dosing of 28.1 $\mu$mol kg$^{-1}$ for 28 days), 2.6 days (after single dose of 930 $\mu$mol kg$^{-1}$) and 3 days (single dose), respectively. In the case of 4-ABP, the elimination half-life of albumin containing the characterized adduct [3-(tryptophan-*N*1-yl)-4-

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acetylaminobiphenyl] was determined to be 4.7 days (Skipper *et al.* 1984, 1985). The albumin half-life in rats is about 4 days.

### Animal treatments

*Single exposure.* Single dose experiments with animals have been performed by administering the chemical orally or intraperitoneally. For both methods peak levels of Hb adducts were mostly found after 24 h. For example, in the study by Feng *et al.* (1994), following administration of a single intraperitoneal dose of [$^3$H]2-AF, peak 2-AF–Hb adduct levels were achieved at 12–18 h and reached a plateau at 72 h post-injection in rapid and slow acetylator congenic hamsters. In rats given [$^{14}$C]MOCA orally, maximum binding was found after 24 h (Cheever *et al.* 1990). In rats given [$^{14}$C]2-MA intraperitoneally, maximum binding with globin was found after 24 h. Therefore, most Hb binding and DNA studies have been performed with biological material obtained 24 h after dosage. Albumin adducts were found for a few compounds. With albumin it appears that maximum adduct levels are reached 4 h after dosing with [$^{14}$C]2-MA (DeBord *et al.* 1992).

*Exposure routes.* Animals have been dosed orally, intraperitoneally and dermally. The difference in the levels of protein adducts produced has been studied for [$^{14}$C]2-MA and [$^{14}$C]MOCA. Approximately a two-fold increase in radioactivity bound to Hb was observed after intraperitoneal administration of 100 mg kg$^{-1}$ [$^{14}$C]2-MA versus oral intubation. Significant differences related to the route of administration of [$^{14}$C]MOCA were detected for 24 h globin covalent binding, with intraperitoneal > oral > dermal (Cheever *et al.* 1990, 1991). After dermal administration of MOCA a large proportion of the dose (86.2%) remained at the application site throughout the study. The 24 h globin covalent binding was 7.84 and 0.02 pmol mg$^{-1}$ globin after oral and dermal exposure, respectively. The difference between oral and peritoneal administration was approximately two-fold for globin adducts.

### Hb adducts of monocyclic arylamines and 4-ABP

The methods for the animal experiments, the isolation of Hb, and the quantification of arylamines and nitroarenes bound to Hb (table 1) have been investigated by several research groups (Green *et al.* 1984, Albrecht and Neumann 1985, Birner and Neumann 1988, Suzuki *et al.* 1989, Sabbioni 1992, Sabbioni 1994b).

As preliminary work for the analysis of human samples, Tannenbaum's group dosed male Fischer rats with 4-ABP by intraperitoneal injection (0.5 µg kg$^{-1}$ to 5 mg kg$^{-1}$) (Green *et al.* 1984). Twenty four hours after a single dose of 5 mg kg$^{-1}$, rats showed 8% of the administered dose in their blood and 0.8% in the plasma. After dialysis of the washed, lysed, membrane-free erythrocytes, 7.3% of the dose remained. After treatment with acidic acetone, only 0.13% of the dose precipitated with globin, while 6.6% of the dose was recovered in the acetone fraction. After further purification this fraction contained 5% of the dose of 4-ABP. The method was repeated using an internal standard for the work-up loss; according to this experiment, 11% of the dose was covalently bound to Hb. 4-ABP chronic dosing

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Table 1. Hb adducts of monocyclic arylamines and nitroarenes in rats and humans, and biomonitoring of arylamines in urine of workers.

| Arylamine | Rat HBI[a] | Carcino-genicity[b] | Nitroarene | Rat HBI[a] | Carcino-genicity[b] | Products[c] | References | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Human, urine[d] | Human, blood[e] |
| Aniline (A) | 22.0[f] | 3 | Nitrobenzene (NB) | 60.0[g] | 2B | Propham, *N*,*N*-dimethylaniline, *N*-ethylaniline | El-Bayoumi *et al.* 1986 | Lewalter and Neumann 1996, Ward *et al.* 1996, Bryant *et al.* 1988 |
| 4-Methylmercapto-A | 3.8 | | | | | | | |
| 4-Fluoro-A | 33.0 | | 4-Fluoro-NB | 39.5 | | | | |
| 2-Chloro-A | 0.5 | | 2-Chloro-NB | 2.1 | 3 | | | |
| 3-Chloro-A | 12.5 | | 3-Chloro-NB | 54.2 | 3 | Chlorpropham | | |
| 4-Chloro-A | 569.0[f] | 2B | 4-Chloro-NB | 215.4 | 3 | Monuron, monolinuron | Riffelmann *et al.* 1995 | Riffelmann *et al.* 1995, Lewalter and Neumann 1996 |
| 4-Bromo-A | 341.0 | | 4-Bromo-NB | 225.8 | | | | |
| 4-Iodo-A | 296.0 | | | | | | | |
| 4-Trifluoromethyl-A | 148.0 | | | | | | | |
| 2-Methyl-A | 4.0[f] | 2B | 2-Methyl-NB | 0.72 | 3 | | Brown *et al.* 1985, Lewalter and Neumann 1996, Ward *et al.* 1996 | Lewalter and Neumann 1996, Ward *et al.* 1996, Bryant *et al.* 1988 |
| 3-Methyl-A | 4.9[f] | | 3-Methyl-NB | 1.0 | 3 | | | Bryant *et al.* 1988 |
| 4-Methyl-A | 4.3[f] | | 4-Methyl-NB | 0.43 | 3 | | Lewalter and Neumann 1996 | Bryant *et al.* 1988 |
| 2-Ethyl-A | 5.1 | | 2-Ethyl-NB | 0.26 | | | | Bryant *et al.* 1988 |
| 3-Ethyl-A | 12.7 | | | | | | | Bryant *et al.* 1988 |
| 4-Ethyl-A | 5.8 | | 4-Ethyl-NB | 0.12 | | | | Bryant *et al.* 1988 |
| 2,4-Dimethyl-A | 2.3[f] | 3 | 2,4-Dimethyl-NB | NB | | | Lewalter and Neumann 1996 | Bryant *et al.* 1988 |
| 2,5-Dimethyl-A | 7.3 | 3 | | | | | | Bryant *et al.* 1988 |
| 2,6-Dimethyl-A | 1.1 | 2B | 2,6-Dimethyl-NB | NB | | Lignocaine | | Bryant *et al.* 1988, 1994 |
| 3,4-Dimethyl-A | 0.7 | | 3,4-Dimethyl-NB | NB | | | | Bryant *et al.* 1988 |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3,5-Dimethyl-A | 14.0 | | 3,5-Dimethyl-NB | 0.63 | | | | Bryant *et al.* 1988 |
| 2,4,6-trimethyl-A | 0.2 | 3 | 2,4,6-Trimethyl-NB | NB | | | | |
| 2,4,5-Trimethyl-A | 0.7[f] | 3 | | | | | | |
| 2,4-Difluoro-A | 32.0 | | 2,4-Difluoro-NB | 2.3 | | | Boogard *et al.* 1994a | Boogard *et al.* 1994a |
| 3-Chloro-4-fluoro-A | 30.7 | | 3-Chloro-4-fluoro-NB | 10.0 | | | Boogard *et al.* 1994b | Boogard *et al.* 1994b |
| 2,4-Dichloro-A | 0.6 | | 2,4-Dichloro-NB | NB | | | | |
| 2,6-Dichloro-A | NB | | | | | | | |
| 3,4-Dichloro-A | 9.0[f] | | | | | Linuron, propanil | Lewalter and Korallus 1986 | Lewalter and Korallus 1986, Pastorelli *et al.* 1998 |
| 3,5-Dichloro-A | 0.6 | | | | | Vinclozolin | | |
| Pentachloro-A | NB | | Pentachloro-NB | NB | | | | |
| 2-Chloro-4-methyl-A | 1.0 | | | | | | | |
| 4-Chloro-2-methyl-A | 28.0[f] | 2A | | | | Chlordimeform | Riffelmann *et al.* 1995, Lewalter and Neumann 1996 | |
| 5-Chloro-2-methyl-A | 28.0[f] | 3 | | | | | | |
| 6-Chloro-2-methyl-A | 0.8[f] | | | | | | | |
| 3-Cyano-A | 1.5 | | | | | | | |
| 3-Trifluoromethyl-A | 28.4 | | | | | | | |
| 4-ABP | 344.0[f] | 1 | 4-Phenyl-NB | 177.1 | 3 | | | Ward *et al.* 1996, Bryant *et al.* 1988, 1994, Del Santo *et al.* 1991 |

NB, not bound: no arylamine was released after basic treatment of Hb.

[a] Hb binding index (HBI) = (mmol arylamine per mol Hb)/(mmol compound per kg body weight). Most HBIs of nitroarenes and arylamines given are from Sabbioni (1992) and Sabbioni and Sepai (1995).

[b] Evaluations of carcinogenicity to humans as evaluated in IARC (http://www.iarc.fr).

[c] Hb adduct of the parent arylamine that had been metabolically released from the product was found in rats (see text).

[d] BAT = 1000 $\mu g\,l^{-1}$.

[e] BAT = 100 $\mu g\,l^{-1}$.

[f] From Birner and Neumann (1988).

[g] From Albrecht and Neumann (1985).



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led to an accumulation of the adduct to a level 30 times greater than that found after a single dose (Green *et al.* 1984, Skipper *et al.* 1985). *In vitro* hydrolysis (0.1 M NaOH) of the adduct regenerated 4-ABP, allowing detection at the sub-nanogram level. Human Hb was also readily adducted using *N*-hydroxy-4-ABP *in vitro*; the predominant site of adduction appeared to be the $\beta$-93 cysteine residue (Ringe *et al.* 1988).

Covalent binding of 13 monocyclic arylamines to Hb (table 1) was studied in female Wistar rats by Neumann's group (Birner and Neumann 1988). The animals were dosed by gavage and sacrificed after 24 h. The Hb adducts were hydrolysed under alkaline conditions (1 M NaOH). In all cases the parent amine could be identified by GC coupled to a nitrogen-sensitive detector. Five compounds were also studied in female mice. Hb binding was expressed as the Hb binding index (HBI): HBI = (mmol compound bound to Hb)/(mol Hb)/(dose in mmol $kg^{-1}$). Hb binding was lower than in rats, but to varying degrees: the rat (HBI)/mouse (HBI) values were 569/132, 28/1, 28/2.5, 4/2.1 and 22/2.2 for 4-CA, 5-chloro-2-methylaniline, 4-chloro-2-methylaniline, 2-MA and aniline, respectively. Hb binding in rats correlated remarkably well with the maximum metHb level achieved with the five examples studied (4-CA, 4-ABP, 5-chloro-2-methylaniline, aniline and 2,4-dimethylaniline [2,4-DMA]), with HBI/metHb(%) values of 569/49, 344/52, 28/2, 22/1.7 and 2.3/1.0, respectively.

Our group (Sabbioni 1992, 1994b) investigated the Hb binding of arylamines in female Wistar rats (table 1). The arylamines were given to female Wistar rats by gavage and the rats were sacrificed 24 h later. Hb was hydrolysed with 0.1 M NaOH in the presence of appropriate internal standards and extracted with hexane. The hexane fraction was analysed by GC-MS using electron impact ionization in the single ion mode. Structure identification was based on the retention time and on the mass spectrum or the ratio of the main mass fragments. Except for 2,3,4,5,6-pentachloroaniline (PCA) and 2,6-DCA, all the arylamines tested ($n = 36$) formed hydrolysable Hb adducts. The highest Hb binding was obtained with compounds with a halogen in the para position, whereas a chlorine in the ortho position reduced the formation of Hb adducts drastically (1000-fold for 2-CA compared with 4-CA). An additional ortho chlorine atom, as in 2,6-DCA or PCA, abolished Hb binding totally. All alkyl-substituted amines had a lower HBI than aniline. The HBI of 3-ethylaniline (3-EA) was higher than that of 2-EA and 4-EA. This might be explained by the fact that the oxidation of alkyl groups in the ortho or para position to an amino group is facilitated compared with that of alkyl groups in meta positions. Two methyl groups in the ortho position, as in 2,6-DMA or 2,4,6-trimethylaniline, almost abolished Hb binding.

In QSAR studies we compared the Hb binding, mutagenicity and carcinogenicity of over 30 arylamines (Sabbioni 1994a, Sabbioni and Sepai 1995). In general, the level of Hb binding decreased with the oxidizability of the arylamines, except for 4-ABP, which binds extremely well to Hb, and compounds that are substituted with halogens in the ortho and/or meta position. For halogen-substituted arylamines, the level of Hb binding was directly proportional to the p$K_a$. However, in general, the mutagenicity or carcinogenicity of arylamines increased with their oxidizability. This first set of data suggests that the levels of Hb binding, mutagenicity, and carcinogenicity of arylamines are not determined by the same electron properties of the compounds, or not by these properties alone. These

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results indicate that Hb binding may not prove to be a useful index of the genotoxic potency of arylamines.

### *Hb adducts of aromatic diamino compounds*

Birner *et al.* (1990) performed Hb binding studies with aromatic bicyclic diamino compounds. Benzidine and some congeners were administered orally at a dose of 0.5 mmol $kg^{-1}$ to female Wistar rats (table 2). The rats were sacrificed after 24 h. Hb adducts were hydrolysed under alkaline conditions (0.1 M NaOH). The arylamines were extracted with C18 reversed phase cartridges and analysed by HPLC with an electrochemical detector. With benzidine, three cleavage products were observed, the major component being *N*-acetylbenzidine (AcBz). This indicates that 4-nitroso-4′-*N*-acetyl-ABP is the major reactive metabolite in erythrocytes. In addition, benzidine and 4-ABP were identified. The latter indicates a new metabolic pathway of benzidine. With DcBz, two cleavage products – 3,3′-dimethoxybenzidine and 3,3′-dimethylbenzidine – were observed, the parent diamines being present in excess to or in amounts comparable to the monoacetyl derivatives. With 3,3′,5,5′-tetramethylbenzidine, no Hb adduct could be found.

In another study female Wistar rats were given DcBz (0.006%, 0.0012% or 0.00024%) via drinking water for 4 weeks (Joppich-Kuhn *et al.* 1997). After Hb hydrolysis in the presence of the internal standards DcBz-$d_6$ and AcDcBz-$d_6$, and derivatization with heptafluorobutyric anhydride (HFBA), DcBz and AcDcBz were determined. The adduct levels increased during the first 3 weeks and remained constant thereafter. A dose-proportional increase in the total amount of DcBz– and AcDcBz–Hb adducts from 8.1 ng DcBz $g^{-1}$ Hb at 0.3 mg $kg^{-1}$ body weight per day (0.00024% in drinking water) to 159.9 ng DcBz $g^{-1}$ Hb at 5.8 mg $kg^{-1}$ body weight per day (0.006% in drinking water) was observed in Hb samples isolated from animals treated for 4 weeks with DcBz. The ratio of the DcBz adduct to the AcDcBz adduct was strongly dose dependent. At low DcBz doses the AcDcBz and DcBz adducts were formed at similar levels, whereas at high DcBz doses the DcBz adduct was predominant.

Bailey *et al.* (1990) studied Hb adducts of MDA in female Wistar rats from England; the $^{14}$C-ring-labelled compound was given intraperitoneally (25 mg $kg^{-1}$) and unlabelled material was given by gavage (1, 3, 7 and 12 mg $kg^{-1}$). The animals were sacrificed after 24 h. Two adducts were released from Hb on hydrolysis under mildly basic conditions (0.1 M NaOH for 2 h); these were identified as MDA and AcMDA and accounted for between 36 and 45% of the total radioactivity bound to the protein. A quantitative assay procedure using deuterium-labelled analogues of MDA and AcMDA as internal standards was subsequently developed for measuring both of the base-released arylamines in rat Hb. Extraction with ethyl acetate was followed by derivatization with PFPA and analysis by GC-MS. In rats given 1–12 mg $kg^{-1}$ the amount of the Hb adducts of MDA and AcMDA increased with dose. However, a dose-dependent ratio, as seen in rats dosed with DcBz, for AcMDA/MDA was also found (Joppich-Kuhn *et al.* 1997). At the lowest dose the ratio was 11 and at the highest dose 2.8. The same experiment was repeated with female Wistar rats from Germany with the highest dose (Sabbioni and Schütze 1998). The AcMDA/MDA ratio in this case was 0.7. In total, a two-fold higher

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Table 2. Hb adducts of polyaromatic amino and nitro compounds and aromatic diamino, dinitro and trinitro compounds in rats and human, and analyses of urine in workers. Only human Hb adducts of compounds that were found to bind to animal Hb are listed.

| Compound | HBI[a] | Carcino-genicity[b] | Nitro-compound | HBI[a] | Carcino-genicity[b] | Products[c] | References | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Human, urine | Human, Hb |
| Benzidine | 21.3[d] | 1 | | | | Azodyes | Lewalter and Neumann 1996[i], Rothman *et al.* 1997, Hsu *et al.* 1996, Meal *et al.* 1981 | Sabbioni and Beyerbach 2000 |
| 3,3′-Dichlorobenzidine | 3.5[d] | 2B | | | | Azodyes | Lewalter and Neumann 1996[i], Hatfield *et al.* 1982 | |
| 3,3′-Dimethoxybenzidine | 2.7[d] | 2B | | | | | | |
| 3,3′-Dimethylbenzidine | 1.9[d] | 2B | | | | | | |
| 4,4′-Oxydianiline | 16.6[e] | 2B | | | | | | |
| 4,4′-Thiodianiline | 15.6[e] | 2B | | | | | | |
| 4,4′-Methylenedianiline | 3.7[e] | 2B | | | | Polyurethane | Lewalter and Neumann 1996[i], Cocker *et al.* 1994, Schütze *et al.* 1995, Dalene *et al.* 1996 | Lewalter and Neumann 1996[i], Cocker *et al.* 1994, Sepai *et al.* 1995b |
| MOCA | 5.1[e] | 2A | | | | | Lewalter and Neumann 1996[i], Ward *et al.* 1986, Clapp *et al.* 1991, Cocker *et al.* 1988b | Lewalter and Neumann 1996[i], Vaughan and Kenyon 1996 |
| Dapsone | 0.13[e] | 3 | | | | | Irudaya *et al.* 1983, Horai and Ishizaki 1985, Zuidema *et al.* 1986 | |
| 1,3-Diaminobenzene | 0.7[f] | 3 | 1,3-Dinitrobenzene | 69.0[f] | | | Lewalter and Neumann 1996[i] | Lewalter and Neumann 1996[i] |
| 1-Amino-3-nitrobenzene | 3.1[f] | | 2,4,6-Trinitrotoluene | 6.0[f] | 3 | | Ahlborg *et al.* 1988, Yinon and Hwang 1986 | Liu *et al.* 1995, Sabbioni *et al.* 1996 |
| | | | Musk xylene | | 3 | | | Riedel *et al.* 1999 |
| 2,4-Diaminotoluene | 2.8[g] | 2B | 2,4-Dinitrotoluene | 0.7[f] | 2B | Polyurethane | Rosenberg and Savolainen 1986, Lind *et al.* 1996, Chan *et al.* 1991, Sepai *et al.* 1995a | |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2-Amino-4-nitrotoluene | 1.0[f] | | | | | | | |
| 4-Amino-2-nitrotoluene | 0.1[f] | | | | | | | |
| 2,6-Diaminotoluene | 0.15[f] | | 2,6-Dinitrotoluene | 1.2[f] | 2B | Polyurethane | Rosenberg and Savolainen 1986, Lind *et al.* 1996, Chan *et al.* 1991, Sepai *et al.* 1995a | |
| 2-Aminofluorene | 11.2[h] | | 2-Nitrofluorene | 0.10[h] | 2B | Fuel combustion | | Zwirner-Baier and Neumann 1998 |
| 1-Aminopyrene | 0.014[h] | | 1-Nitropyrene | NB[h] | 2B | Fuel combustion | | Zwirner-Baier and Neumann 1998 |
| 1-Naphthylamine | | 3 | | | 3 | | Lewalter and Neumann 1996[i] | Lewalter and Neumann 1996[i] |
| 2-Naphthylamine | 0.65[h] | 1 | 2-Nitronaphthalene | 0.14[h] | 3 | | Lewalter and Neumann 1996[i] | Lewalter and Neumann 1996[i], Stillwell *et al.* 1987, Bryant *et al.* 1987 |

NB, not bound.
[a] Hb binding index (HBI) = (mmol arylamine per mol Hb)/(mmol compound per kg body weight).
[b] Evaluations of carcinogenicity to humans as evaluated in IARC (http://www.iarc.fr).
[c] Hb adduct of the parent arylamine that had been metabolically released from the product.
[d] Birner and Neumann (1988); total HBI of the *N*-acetyl diamino and the diamino compound.
[e] Sabbioni and Schütze (1998); total HBI of the *N*-acetyl diamino and the diamino compound.
[f] Zwirner-Baier and Neumann (1994); the sum of all adducts is given.
[g] Wilson *et al.* (1996).
[h] Suzuki *et al.* (1989); Hb was not in solution during hydrolysis, therefore these values are probably too low.
[i] Reviewed in Lewalter and Neumann (1996); cited references are not peer-reviewed journals.



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amount of MDA plus AcMDA was bound to Hb in female Wistar rats from England.

Sabbioni and Schütze (1998) studied Hb binding of aromatic diamino compounds (table 2), MDA and congeners. Female Wistar rats dosed with 0.5 mmol kg$^{-1}$ diamine by gavage were sacrificed after 24 h. Hb from dosed animals and from controls was isolated and hydrolysed in basic conditions. The released diamines and monoacetyldiamines were quantified by HPLC with electrochemical detection and/or GC-MS. MDA, 4,4′-oxydianiline (ODA), 4,4′-ethylenedianiline, and 4,4′-thiodianiline (TDA) were bound to Hb as diamines and monoacetyldiamines. 4,4′-Methylenebis(2,6-dimethylaniline), 4,4′-methylenebis(2,6-diethylaniline), MOCA (see also Bailey *et al*. 1993) and 4,4′-sulphonyldianiline (dapsone) bound only as diamines to Hb. 4,4′-Methylenebis(2,6-dichloroaniline) did not bind to Hb. Thus, the presence of two substituents in the ortho position and the presence of electron-withdrawing groups in the para position in the amino group drastically reduced the formation of Hb adducts. The level of Hb adducts was compared with their carcinogenic potency. The extent of Hb binding of the bicyclic diamines (dapsone, DcBz, MDA, MOCA, TDA, ODA and benzidine) increased with their carcinogenic potency.

Hb binding of diaminotoluenes was studied by Neumann's group (Zwirner-Baier *et al*. 1994) and Froines' group (Wilson *et al*. 1995, 1996). 2,4-DAT (0.5 mmol kg$^{-1}$) and 2,6-DAT were given by gavage to female Wistar rats (Zwirner-Baier *et al*. 1994) and the animals were sacrificed after 24 h. Hb was hydrolysed in 1 M NaOH or 10% $H_3PO_4$ for 1 h. Unfortunately it is not stated which conditions were used for every experiment, and which internal standards were used. 2,4-DAT did not form hydrolysable Hb adducts, whereas 2-acetylamino-6-aminotoluene was released from the Hb of rats dosed with 2,6-DAT. Wilson *et al*. (1996) investigated the formation of Hb adducts following intraperitoneal administration of 2,4- and 2,6-DAT at different doses to male Fischer 344 rats. The rats were sacrificed after 24 h and in a single experiment after 30 days. 2,4-DAT and 2,6-DAT bound to the same extent to Hb. However, only the non-acetylated parent amines were found in this case. This is a consequence of the harsh hydrolysis used (see Wilson *et al*. 1995). Hb was hydrolysed in saturated NaOH, sonicated for 3 h at 37C, and extracted with toluene after the addition of NaCl . The toluene extract was derivatized with HFBA, washed with phosphate buffer and spiked with the internal standard 2,6-DMA. Quantification was performed by GC-MS with electron impact ionization. In male Fischer 344 rats given 0, 0.5, 5, 15, 50, 150 and 250 mg kg$^{-1}$, the amount of Hb binding did not increase linearly (results of 0, 46.7, 83, 108.3, 143, 245 and 274 ng 2,4-DAT g$^{-1}$ Hb, respectively). Similar amounts of 2,6-DAT bound to Hb. Further experiments with diaminotoluene derivatives are presented in the section on Hb adducts of nitroarenes and in the section on DNA adducts.

### *Hb adducts of metabolically released arylamines*

Arylamines are building blocks for several industrial products. Therefore the metabolic release of arylamines from different products has been studied. After administration of the azo dye Direct Red 28 to female Wistar rats, benzidine, AcBz and 4-ABP could be cleaved after mild base hydrolysis from Hb (Birner *et al*. 1990). In this case, the fraction of 4-ABP was greater than after giving benzidine to

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the animals. Indeed, the same amount of 4-ABP (HBI = 2.2) was found to be bound as the sum of benzidine and AcBz (HBI = 0.3 + 1.8). The metabolic release of DcBz from azo dyes was investigated in two independent studies with the same rat strain (female Wistar rats) and dosage protocol (Zwirner-Baier and Neumann 1994, Sagelsdorff *et al*. 1996). Sagelsdorff *et al*. (1996) studied the release of DcBz from a insoluble azo pigment (Pigment Yellow 17) and from a soluble azo dye (Direct Red 46) in female Wistar rats given 0.2% (w/w) Colour Index Pigment Yellow 17 in the diet or 0.06% (w/v) Colour Index Direct Red 46 in the drinking water for 4 weeks. Steady-state DcBz–Hb adduct levels were determined by GC-MS with NCI as well as DcBz–DNA adduct levels in the liver by $^{32}$P-postlabelling. The Hb and DNA adduct levels were compared with the respective adduct levels obtained in animals after treatment for 4 weeks with DcBz in the drinking water (see above and Joppich-Kuhn *et al*. 1997). A dose-proportional increase in adduct levels from 8.1 ng g$^{-1}$ Hb and 2.6 ng g$^{-1}$ DNA (3.3 adducts/10$^9$ nucleotides) to 160 ng g$^{-1}$ Hb and 45.4 ng g$^{-1}$ DNA (56.1 adducts/10$^9$ nucleotides) was observed in the DcBz-treated rats. In rats treated with Direct Red 46, total adduct levels of 17.7 ng (9.2 ng DcBz + 8.2 ng AcDcBz) g$^{-1}$ Hb and 5.2 ng g$^{-1}$ DNA (6.4 adducts/10$^9$ nucleotides) were found. No Hb or DNA adducts were found in rats treated with Pigment Yellow 17 in the diet, at a limit of detection of 0.1 ng g$^{-1}$ Hb and 0.08 ng g$^{-1}$ DNA (1 adduct/10$^{10}$ nucleotides). The results of this study demonstrate the lack of bioavailability of DcBz from the Pigment Yellow 17. This is in contrast to results obtained earlier with the same rat strain and the same dose, but with a different analytical procedure (Zwirner-Baier and Neumann 1994). In rats given Direct Red 46 and Pigment Yellow 17, 27 and 15 ng DcBz g$^{-1}$ Hb, respectively, were found. No acetylated DcBz was detected. In this study a HPLC method with electrochemical detection and *N*-acetyl-3,3′,5,5′-tetramethylbenzidine as the internal standard was used. Sagelsdorff used deuterated DcBz and AcDcBz (the ideal choice) as internal standards and GC-MS with NCI for quantification.

Hb adducts of aniline were found after giving female Wistar rats acetanilide (Albrecht and Neumann 1985), paracetamol (Albrecht 1985, Neumann 1984a,b), *N*-methylaniline, *N*,*N*-DMA (Birner *et al*. 1990), acetoanilide and *N*-EA (Beyerbach and Sabbioni 1999). The levels of Hb binding of all the aniline derivatives were of the same order of magnitude as in rats given aniline. 2,4-DMA adducts were found after giving rats acetoacet-*m*-xylidide (Beyerbach and Sabbioni 1999).

Arylamines are building blocks of pesticides (Sabbioni and Neumann 1990b), and the Hb adducts of arylamines released from pesticides have been investigated. Female Wistar rats were dosed orally with urea and carbamate pesticides (up to 1 mmol kg$^{-1}$). Blood was obtained after 24 h, and Hb isolated and hydrolysed in 1 M NaOH. The amines were extracted and quantified by GC using nitrogen-specific or mass-selective detection. The HBI values obtained with various pesticides were as follows (arylamines are given in parentheses): linuron and diuron (3,4-DCA), 0.8 and 4.5, respectively; monuron and monolinuron (4-CA), 39 and 558, respectively; chloropropham (3-CA), 2.8; chlordimeform (4-chloro-2-methylaniline), 2.4; and propham (aniline), 2.4. No adducts were found with vinclozoline and iprodione (3,5-DCA) and quintozene (PCA). These results demonstrate the possible use of arylamine–Hb adducts for measuring the bioavailability of potentially hazardous components of pesticides, and the extent to which they are released and metabolically activated.

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*Hb adducts and plasma protein adducts of arylamines*

Both Hb adducts and plasma protein adducts have only been determined in a few studies. Neumann's group (Neumann *et al.* 1993) investigated Hb adducts and plasma protein adducts after giving female Wistar rats radiolabelled arylamines. Results were compared in terms of the protein binding index (PBI) (i.e. pmol compound per mg protein per dose in mmol $mg^{-1}$). For 4-CA, acetanilide, benzidine and DcBz, the PBI ratios of Hb/plasma proteins (% hydrolysable adduct) were 2388 (93%)/82 (24%), 177(88%)/70(16%), 940(84%)/1830(0%) and 737(32%)/537(26%), respectively. Therefore, the ratio was different for different compounds. In the case of 4-CA the difference was 30-fold, but in all other cases the differences were below a factor of 3. Most of the Hb adducts are hydrolysable to the arylamines (84–93%). For plasma protein adducts, the hydrolysable amount varied from 0–26%. According to current knowledge, the hydrolysable fraction results from the reaction products of the nitroso-derivatives yielding sulphinamide adducts with cysteine (Eyer and Gallemann 1996, Kazanis and McClelland 1992). The non-hydrolysable fraction probably results from the reaction products of activated *N*-hydroxyarylamines. Adducts resulting from nitrosoarenes are much higher with Hb than with plasma proteins. However, the absolute values for non-hydrolysable adducts are higher with albumin than with Hb. Therefore, it appears that nitroso-derivatives are a minor component in the plasma compared with the erythrocytes. This can be explained by the fact that *N*-hydroxyarylamines can be transformed to nitrosoarenes in the erythrocytes but not in the plasma. In female Wistar rats given MOCA (1 mg $kg^{-1}$) by gavage a PBI ratio of Hb/albumin (% hydrolysable adduct) of 279(54%)/277(0%) was found (Sabbioni and Neumann 1990a). In this case albumin was isolated by affinity chromatography. In male Sprague-Dawley rats given radiolabelled 2-MA (50 mg $kg^{-1}$) intraperitoneally, a PBI ratio of Hb/albumin of 280(63%)/319.4 (0%) was found (DeBord *et al.* 1992). However, in this case albumin was isolated by fractional acid precipitation; under such conditions sulphinamide adducts are partially cleaved (Green *et al.* 1984). In the same study, animals were treated with different amounts of 2-MA. 2-MA–albumin binding was not linear; but 2-MA–Hb binding appeared to increase linearly in a dose-dependent manner. The biological half-lifes of 2-MA bound to albumin or Hb were observed to be 2.6 and 12.3 days, respectively, after rats were given a single dose of [$^{14}$C]2-MA and sacrificed after 4 h to 28 days. In subsequent experiments with unlabelled 2-MA, Hb and albumin adduct levels were determined by HPLC analysis of the cleavage product using fluorescence detection (Cheever *et al.* 1992). Mean adduct levels for Hb increased rapidly over the first 4 h, with the highest level (3.7 ng $mg^{-1}$ Hb) detected 24 h after 2-MA administration at a dose of 50 mg $kg^{-1}$ body weight. In contrast, adduct levels for pooled albumin samples increased from 0.7 ng $mg^{-1}$ albumin at 2 h to 2.5 ng $mg^{-1}$ albumin at 4 h, but were not detectable 24 h after dosing. Although Hb and albumin adducts differ in stability, the ratio of 2-MA adducts may be useful in long-term industrial biomonitoring for the evaluation of 2-MA exposure. However, it appears that the hydrolysable adduct can only be detected about 6 h after exposure.

To date, only the structure of 4-ABP with serum albumin has been elucidated (Skipper *et al.* 1985). Serum albumin was isolated from male Sprague-Dawley rats dosed by gavage at 27 h after administration of [$^{3}$H]4-ABP. Pronase digestion of the purified albumin yielded a mixture of radiolabelled materials, which was resolved into five major components by reverse phase liquid chromatography

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From detailed UV, $^{1}$H-nuclear magnetic resonance and mass spectral analyses, four of these components were determined to be 4-ABP, 4′-hydroxy-4-acetyl-ABP and two other metabolites, all of which are presumed to be non-covalently associated with the serum albumin. The fifth component, however, resulted from covalent bond formation and was identified as a tetrapeptide containing 3-tryptophanyl-4-acetyl-ABP, the amino acid sequence of which was $H_2N$-Ala-Trp-Ala-Val. Since rat serum albumin contains only a single tryptophan residue in a hydrophobic drug-binding site, its high selectivity for carcinogen binding suggests a unique role for this protein in the detoxification and/or transport of ultimate carcinogenic metabolites (Skipper 1996).

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### *Hb adducts as markers of metabolism*

*CYPIA2.* Before investigating human populations, the metabolic influences on the levels of 4-ABP adducts were studied by Hammons *et al.* (1991). Since hepatic CYP1A2 has been regarded as the predominant enzyme catalysing the *N*-oxidation of 4-ABP and other carcinogenic arylamines, a study was undertaken to assess whether or not 4-ABP–Hb adduct formation, which arises putatively from circulating *N*-hydroxy-4-ABP and its subsequent reactions with erythrocyte Hb, can be used as a reliable measure of hepatic *N*-oxidation *in vivo*. The ability to inhibit adduct formation in the rat was examined in male Charles River CD rats initially treated with different doses (10, 30 and 100 mg kg$^{-1}$) of 2-ethynylnaphthalene (a selective mechanism-based inactivator of CYP1A2 activity *in vitro*) given intraperitoneally 1 h prior to dosing with 4-ABP (0.5 and 5 mg kg$^{-1}$ intraperitoneally). 2-Ethynylnaphthalene caused a dose-dependent decrease in the amount of 4-ABP–Hb adduct formed, with values of 16–29%, 28% and 67–69%, respectively, at the three doses. Pretreatment of animals with isosafrole, a known CYP1A2 inducer and high affinity ligand, also resulted in a decrease (92%) in the amount of adduct formed. Inhibitors of other monooxygenases were, however, much less effective in inhibiting formation of the adduct. The results of this study demonstrate a strong association between CYP1A2 activity and Hb adduct levels. Additionally, they indicate that the CYP1A2-catalysed reaction is the predominant pathway for the *N*-oxidation of 4-ABP *in vivo*.

*NAT2.* The effect of NAT2 on the levels of Hb adducts was studied by Feng *et al.* (1994) using [$^{3}$H]2-AF in homozygous rapid and slow acetylator hamsters. 2-AF–Hb adduct levels increased in a dose-dependent manner ($r = 0.95$, $p = 0.0001$) and were consistently higher in slow versus rapid acetylator congenic hamsters. The magnitude of the acetylator genotype-dependent difference was a function of dose: 2-AF–Hb adduct levels were 1.5-fold higher in slow acetylator congenic hamsters following a 60 mg kg$^{-1}$ 2-AF dose, but 2-fold higher following a 100 mg kg$^{-1}$ 2-AF dose. These results show a specific and significant role for NAT2 acetylator genotype in the formation of arylamine–Hb adducts. Similar results were obtained by Flammang *et al.* (1992a). A single high dose of 2-AF (60 mg kg$^{-1}$ intraperitoneally) was administered to male and female homozygous rapid acetylator hamsters and homozygous slow acetylator hamsters. The levels of 2-AF–Hb adducts, evaluated by GC-MS, were significantly higher in the homozygous

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slow acetylators than in homozygous rapid acetylators. DNA adducts were investigated in the same animals. In liver and urinary bladder, only a non-acetylated DNA adduct, which co-chromatographed with authentic *N*-(deoxyguanosin-8-yl)-2-AF, was detected using a $^{32}$P-postlabelling assay. The average levels of hepatic 2-AF–DNA adducts were similar between male and female rapid and slow acetylators. However, DNA adducts in the urinary bladder at 18 h were about four-fold lower than in the liver, and were significantly greater in homozygous rapid acetylators than in their homozygous slow counterparts ($p < 0.1$). These experiments suggest that acetylator genotype affects an individual's capacity to form arylamine–Hb adducts and arylamine–DNA adducts in a tumour target organ, such as hamster urinary bladder. However, difference in the level of DNA adduct between slow and fast acetylators is small. A similar effect was seen in mice dosed with 4-ABP; again in this case the difference in DNA adduct levels in the target organ between slow and fast acetylator mice was only 10–20% (Flammang *et al.* 1992b).

Larger differences were seen in slow acetylating and fast acetylating mice given 4-CA (0.2 mmol kg$^{-1}$) and/or DcBz (0.2 mmol kg$^{-1}$) (Zwirner-Baier and Neumann 1998). In fast acetylators, 4-CA–Hb adducts and DcBz-Hb adducts were reduced by a factor of 20 and 9, respectively.

*Oltipraz and N-acetylcysteine.* Izzotti *et al.* (2001) showed a decrease in 4-ABP–Hb adducts in rats exposed to smoke after treatment with the chemo-preventive agents Oltipraz and *N*-acetylcysteine. Giving both together had more than an additive effect.

Although these studies indicate a correlation between metabolic changes (enzyme polymorphism, enzyme inducer) and the amount of Hb adducts, it should be remembered that most of them were performed with 4-ABP, which binds to Hb at a level of 10% of the dose. The same is true for 4-CA, which binds at a level of 8%. For other compounds such as 2-AF, which binds at a level of less than 1% of the dose, the differences are not so clear. Further studies showing the use of Hb adducts as possible marker of metabolism will be discussed in the human biomonitoring and DNA sections below.

### *Hb adducts of arylamines after exposure to nitroarenes*

A few laboratories have investigated Hb adducts of arylamines after dosing animals with nitroarenes. The results are summarized in table 1. In general, lower hydrolysable Hb adduct levels were found in rats given nitroarenes than in rats dosed with equimolar amounts of the corresponding arylamines, except for nitrobenzene, 2-CNB, 3-CNB and 4-fluoronitrobenzene (Sabbioni 1994a, b, Sabbioni and Sepai 1995). The structure–activity relationships of nitroarenes and arylamines are very similar. The highest levels of Hb binding were found for 4-bromonitrobenzene and 4-CNB. The lowest binding was found with nitrobenzenes with electron-donating substituents such as 4-methylnitrobenzene. Seven nitroarenes (2,4-dichloronitrobenzene, 2,4-dimethylnitrobenzene, 2,6-dimethylnitrobenzene, 3,4-dimethylnitrobenzene, 2,3,4,5,6-pentachloronitrobenzene, 2,4,6-trimethylnitrobenzene and 1-NP) did not form Hb adducts, which on

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base treatment release the corresponding arylamine (e.g. 2,4-DCA release after administration of 2,4-dichloronitrobenzene).

At the Chinese Academy of Science in Beijing, TNT has been a major topic of research for several years. Liu *et al.* (1992) investigated blood protein binding of TNT in rats. When a single dose of [$^{14}$C]TNT was administered intraperitoneally to rats at 1, 10 or 50 mg kg$^{-1}$, covalently bound radioactivity was detected in globin and plasma proteins. The extent of covalent binding was dose dependent and was highest in plasma proteins up to 4 h after dosing. Levels of globin adducts declined slower than plasma protein adducts. At a dose of 50 mg kg$^{-1}$, globin covalent adduct levels peaked (182 pmol mg$^{-1}$ protein) at 1 h after dosing and subsequently decreased to approximately 50 pmol mg$^{-1}$ protein between days 1 and 8. Four hours after dosing, about 0.4% of the dose was bound to Hb, of which about 48% was susceptible to dilute acid hydrolysis. 2-Amino-4,6-DNT and 4-amino-2,6-DNT were the major products recovered by solvent extraction. *In vitro* incubation of [$^{14}$C]TNT with blood yielded the same hydrolysable adducts with Hb and plasma proteins.

Hb binding of TNT in female Wistar rats was also investigated by Neumann's group (Zwirner-Baier *et al.* 1994). In contrast to the results of Liu *et al.* (1992), only 4-amino-2,6-DNT was found as an adduct in rats given TNT by gavage and sacrificed after 24 h.

In female Wistar rats given 2,6-DNT, Hb adducts of 2-amino-6-nitrotoluene and 2,6-DAT in a ratio of 5:1 were found (Zwirner-Baier *et al.* 1994). After dosing with rats 2,4-DNT, only 4-amino-2-nitrotoluene was detected. In rats given 4-amino-2-nitrotoluene, only 4-amino-2-nitrotoluene adducts were obtained.

The Hb binding of five nitroarenes (tables 1 and 2), i.e. nitrobenzene, 4-NBP, 1-NP, 2-nitronaphthalene and 2-nitrofluorene (2-NF), and their corresponding amines administered orally to male Sprague-Dawley rats was determined by HPLC with UV and fluorescence detection (Suzuki *et al.* 1989). The rats were sacrificed after 48 h. Hb was hydrolysed in 1 M HCl (for 2 h in suspension at room temperature). Hb binding of the nitroarenes, except for nitrobenzene, was significantly lower than that of the corresponding amines. In rats treated with antibiotics, the covalent Hb binding of nitrobenzene, 4-NBP and 2-NF decreased by factors of 7, 30, and 2, respectively. The reduction of 2-nitronaphthalene was not affected by antibiotic treatment.

The role of the intestinal microflora in the metabolic activation of nitroarenes was also studied by Scheepers *et al.* (1994). Female Wistar rats received a dose of 1 mmol kg$^{-1}$ 2-NF in sunflower oil by gavage. Another group received the same dose of 2-AF. A third group of animals was used as controls. Germ-free rats, germ-free rats with a rat microflora, and germ-free rats with a human microflora were treated. In germ-free rats, no Hb adducts were present. In rats with human microflora and rats with rat microflora, the adduct levels were 24 and 50 pmol g$^{-1}$ Hb, respectively. DNA adducts in the liver, lung and white blood cells were only present in rats with microflora. Hb adducts were determined by GC-MS (Scheepers *et al.* 1993), and DNA adducts were measured using the $^{32}$P-postlabelling technique.

### *Hb adducts of arylamines in humans*

In the first studies by Tannenbaum's group (Bryant *et al.* 1987), the Hb adducts of 4-ABP in humans were correlated to the dose (smoker, non-smoker

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blond or black tobacco). The results of these studies made a great impact in the scientific community of molecular epidemiology. Several research groups with different specialities started to collaborate with Tannenbaum's group. In these extended collaborative studies, phase I enzymes (CYP1A2), phase II enzymes (NAT2, GST), DNA adduct levels in urothelial epithelial cells, disease outcome (bladder cancer) and smoker status were compared with the Hb adduct levels of 4-ABP.

### *Environmental exposure to arylamines*

*Hb adducts of 4-ABP in smokers and non-smokers.* Bryant *et al.* (1987) developed a method for the analysis of 4-ABP covalently bound as the sulphinamide to the $\beta$-93 cysteine (Ringe *et al.* 1988) of human Hb. Mild basic hydrolysis (0.1 M NaOH) of Hb to release the parent amine was followed by extraction with hexane, derivatization with PFPA, and GC-MS with detection by NCI. This method gives reproducible results on multiple blood samples taken from individuals over 48 h. The precision for 4-ABP was 10%, but was 20% for the monocyclic arylamines. The method was used for the determination of 4-ABP–Hb adducts in smokers ($n = 19$) and non-smokers ($n = 26$) from New York City. The mean value for smokers was 154 pg 4-ABP $g^{-1}$ Hb, compared with 28 pg $g^{-1}$ Hb for non-smokers, with no overlap of adduct levels between the two groups. Studies on quitting smokers showed that adduct levels declined over a period of 6–8 weeks to non-smoker levels. The finding of 4-ABP adducts in all non-smokers is consistent with low level ubiquitous contamination of air, food or water. In other animals sampled, rats and dogs had measurable adduct levels, but monkeys and fish did not. The same authors (Bryant *et al.* 1988) found Hb adducts of 15 arylamines in non-smokers ($n = 25$) and smokers of blond tobacco (40 subjects) or black tobacco (18 subjects) cigarettes in Turin, Italy. The subjects were all males aged 55 years or less, and were representative of the population previously examined in a case control study of bladder cancer. 4-ABP adduct levels were significantly different in the three groups, and the differences were approximately proportional to the relative risk of each group. Adjustment for age and cigarette consumption did not materially influence the differences. A significant correlation between adduct levels and cigarette consumption was also observed for all smokers as well as for smokers of blond tobacco. Other amines for which significant differences between smokers and non-smokers were observed were 3-ABP, 2-NA, 2-MA, 3-MA, 2,4-DMA and 2-EA. Some of these amines are human bladder carcinogens, and their occurrence in blood as Hb adducts is evidence for their metabolic activation. A further study published by Tannenbaum's group (Skipper *et al.* 1988) demonstrated that 3- and 4-ABP adducts are highly associated with cigarette smoking. The adduct levels of four studies were compared. The first group was from New York (Bryant *et al.* 1987), the second from Turin (Bryant *et al.* 1988), the third one from several stop-smoking clinics ($n = 20$, 17 and 21) in Boston, and the fourth from non-smokers in the Boston area selected from different sources ($n = 23$). Minor differences in the mean adduct levels of several amines were observed in populations from different geographic areas.

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The stability of Hb adducts in humans was studied in smokers ($n = 34$) enrolled in a withdrawal programme (Maclure *et al.* 1990). 4-ABP–Hb adducts declined from a mean SD of $120 \pm 7\,\text{pg g}^{-1}$ Hb at the start to $82 \pm 6\,\text{pg g}^{-1}$ after 3 weeks and to $34 \pm 5\,\text{pg g}^{-1}$ in the 15 ex-smokers who had not resumed smoking after 2 months. 4-ABP–Hb adducts declined faster than expected from the assumption that the human erythrocyte has a lifespan of 120 days. The percent decline over the first week was calculated to be 40% compared with an expected 32%. By 9–11 weeks, it was 90% compared with an expected decline of 79%. Assuming $33\,\text{pg g}^{-1}$ as the final level in ex-smokers, the observed decline over the first 3 weeks was most compatible with an erythrocyte lifespan of 80 days, while the decline over 9–11 days would correspond to a lifespan of about 60 days. Assuming $23\,\text{pg g}^{-1}$ as the final level, the observed declines at 3 and 9–11 weeks were most compatible with a lifespan of 95 and 100 days, respectively. However, the shorter lifespan could be a result of a faster elimination of damaged erythrocytes in smokers or possibly of a slight instability of the adduct (see pages 363–364).

Hb adducts of 4-ABP have been measured in a case control study of lung cancer (53 lung cancer cases with 23 smokers, 56 controls with 18 smokers) (Weston *et al.* 1991). Data obtained for lung cancer cases were compared with those obtained for controls consisting of patients with either chronic obstructive pulmonary disease or non-pulmonary cancers. Both simple and multivariate analyses found a positive association between 4-ABP–Hb adducts (41 smokers, 68 non-smokers) and the quantity of tobacco smoked as determined by either urine cotinine or questionnaire data. No association was found between 4-ABP–Hb adducts and cancer diagnosis, and adduct levels were not related to overall tobacco use, i.e. the total pack years of smoking. Whereas 4-ABP–Hb adduct levels reflected recent tobacco smoking, they were not correlated with lung cancer risk. In a similar study, Hb adducts of 4-ABP were measured in 13 smoking controls and in 13 patients with transitional cell bladder carcinoma (del Santo *et al.* 1991). Quantification was performed by GC-MS and NCI using 4-ABP-$d_9$ as an internal standard. Smoking was quantified by measuring the urinary excretion of cotinine. Thirteen cases and controls were paired for urinary cotinine levels. Bladder carcinoma patients had slightly higher levels of 4-ABP–Hb adducts than controls (mean SD values of $103 \pm 47$ versus $65 \pm 44$ $\text{pg g}^{-1}$ Hb). This difference was significant using a *t*-test for paired samples ($p = 0.04$) and non-parametric Kruskal–Wallis rank analysis ($p = 0.033$). These results provide evidence for a biochemical basis for the observed association between cigarette smoking and bladder cancer.

Sex differences in adduct formation were noticed in a study involving 1514patients with bladder cancer and 1514 individually matched population control subjects from Los Angeles, California, USA (Castelao *et al.* 2001). The slopes of the linear regression lines of the 3- and 4-ABP–Hb adducts by cigarettes per day were statistically significantly steeper in women than in men ($p$ values for sex differences were $< 0.001$ and 0.006, respectively). Consistent with the sex difference in adduct levels, the risk of bladder cancer in women who smoked was statistically significantly higher than that in men who smoked comparable numbers of cigarettes ($p = 0.016$ for sex–lifetime smoking interaction).

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*Maternal–fetal exchange of 4-ABP.* The maternal–fetal exchange of 4-ABP was studied in smoking ($n = 14$) and non-smoking ($n = 38$) pregnant women (Coghlin *et al.* 1991). Levels of 4-ABP–Hb adducts were measured in maternal–fetal paired blood samples obtained from women during labour and delivery. 4-ABP–Hb adducts were detected in all maternal and fetal blood samples. Levels of such adducts were significantly higher ($p < 0.001$) in maternal and fetal blood samples from smokers. The mean ($\pm$ SD) 4-ABP–Hb adduct level was $92 \pm 54$ pg g$^{-1}$ Hb in blood samples from the fetuses of smokers, and $17 \pm 13$ pg g$^{-1}$ Hb in blood samples from fetuses of non-smokers. The mean maternal 4-ABP–Hb adduct level was $183 \pm 108$ pg g$^{-1}$ Hb in smokers and $22 \pm 8$ pg g$^{-1}$ Hb in non-smokers. Fetal carcinogen adduct levels were consistently lower than maternal levels: the mean maternal to fetal ratio was $2.4 \pm 1.1$ in smokers and $1.9 \pm 0.98$ in non-smokers. Fetal 4-ABP–Hb adduct levels were strongly associated with maternal 4-ABP–Hb adduct levels (correlation coefficient $r = 0.71$, $p = 0.002$) when paired samples from smoking mothers were analysed. Myers *et al.* (1996) obtained similar results. The number of cigarettes smoked per day by each of the women in their study was assessed via questionnaire and by measurement using immunoassay of serum and urine cotinine in maternal and fetal blood samples. Maternal and fetal blood samples were classified as coming from non-smokers ($n = 74$), individuals smoking less than one pack of cigarettes per day ($n = 16$), individuals smoking one pack of cigarettes per day ($n = 19$), individuals smoking one to two packs of cigarettes per day ($n = 19$), and individuals smoking more than two packs of cigarettes per day ($n = 20$). Both maternal and fetal blood samples were obtained at the time of delivery. Background levels of 4-ABP–Hb adducts were detected in maternal non-smokers ($18.3 \pm 12.7$ pg 4-ABP g$^{-1}$ Hb) and in the corresponding fetal samples ($8.9 \pm 5.8$ pg g$^{-1}$ Hb). Increasing levels of 4-ABP–Hb adducts were found as the smoking status of the women increased, ranging from $144 \pm 22.2$ ($< 1$ pack/day) to $633 \pm 87.9$ ($> 2$ packs/day). A corresponding increase in the presence of fetal 4-ABP–Hb adducts was also detected, ranging from $74 \pm 17.8$ ($< 1$ pack/day) to $319 \pm 50.5$ ($> 2$ packs/day).

An additional study on the maternal–fetal exchange of 4-ABP was performed by Myers' group. In this case, the adducts levels were measured by GC-MS and by HPLC with UV detection (Pinorini-Godly and Myers 1996). Background levels of 4-ABP–Hb adducts were detected in 21 maternal non-smokers ($29.6 \pm 16.2$ pg 4-ABP g$^{-1}$ Hb on GC-MS; $23.7 \pm 13.5$ pg g$^{-1}$ on HPLC) and their fetuses ($14.0 \pm 6.5$ pg g$^{-1}$ on GC-MS; $10.0 \pm 4.6$ pg g$^{-1}$ on HPLC). Elevated levels of 4-ABP–Hb adducts were found in 21 maternal smokers ($488 \pm 174$ pg g$^{-1}$ on GC-MS; $423 \pm 54$ pg g$^{-1}$ on HPLC) as well as in the corresponding fetal blood samples ($244 \pm 91$ pg g$^{-1}$ on GC-MS; $197 \pm 77$ pg g$^{-1}$ on HPLC). These studies demonstrate that 4-ABP, or its active metabolite, *N*-hydroxy-4-ABP, crosses the human placenta and binds to fetal Hb in concentrations that are significantly higher in smokers than in non-smokers.

*Hb adducts of 4-ABP and environmental tobacco smoke.* The successful distinction of smokers from non-smokers by measuring 4-ABP adducts with Hb has lead scientists to investigate the effect of environmental tobacco smoke (ETS) on the uptake of 4-ABP.

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Hammond *et al.* (1993) investigated the relationship between exposure to ETS and levels of 4-ABP–Hb adducts in non-smoking pregnant women and in smoking pregnant women. A questionnaire on smoking and exposure to ETS was administered to 15 pregnant women who smoked cigarettes and 40 who did not smoke. Exposure was quantified for 1 week with a personal diary and by air sampling nicotine monitor worn by each woman. Aliquots of maternal blood and cord blood collected during delivery were analysed for 4-ABP–Hb adducts by GC-MS with NCI. The mean adduct level in smokers (184 pg 4-ABP $g^{-1}$ Hb) was significantly higher than that in non-smokers (22 pg $g^{-1}$). Among non-smokers, the levels of 4-ABP adducts increased significantly with increasing ETS level ($p = 0.009$). Those in the lowest exposure category ($< 0.5\,\mu g\,m^{-3}$ weekly average nicotine) had median 4-ABP–Hb adduct levels of 15 pg $g^{-1}$, while those in the highest exposure category ($\geq 2.0\,\mu g\,m^{-3}$) had median levels of 26 pg $g^{-1}$. Non-smokers in this study had a median adduct level of 20 pg $g^{-1}$, and smokers had a median level of 143 pg $g^{-1}$. In a larger study (Tang *et al.* 1999), the effect of exposure to ETS was analysed in 109 Hispanic and African-American preschool children (1–6 years of age). Cotinine in urine, 4-ABP–Hb adducts and sister chromatid exchanges (SCEs) were measured as biomarkers. 4-ABP–Hb adducts were significantly higher ($p > 0.05$) in the ETS-exposed children compared with the unexposed children; SCEs were marginally higher ($p = 0.076$). ETS-exposed children had significantly higher 4-ABP–Hb adduct levels than unexposed children. Therefore, non-smokers may receive a non-trivial dose of carcinogens from ETS proportional to their exposure.

*Hb adducts of 4-ABP and individual susceptibilities.* The efforts of Tannenbaum's group in studying the role of *N*-acetylation phenotype have joined by the Italian epidemiologist Vineis and the arylamine specialist Kadlubar from the US Food and Drug Administration's National Center for Toxicological Research (NCTR) and Bartsch from the IARC in Lyon. In 97 healthy volunteers (Bartsch *et al.* 1990, Vineis *et al.* 1990), the relationship between the type (air- or flue-cured) and number of cigarettes smoked and different biomarkers relevant to the risk of bladder cancer, including the levels of 4-ABP–Hb adduct, the *N*-acetylation phenotype (63 slow acetylators) and the urinary mutagenicity in *Salmonella typhimurium* TA98, were investigated. Levels of the 4-ABP–Hb adduct were higher in smokers ($n = 16$) of black tobacco (air-cured) than in smokers ($n = 31$) of blond tobacco (flue-cured), confirming earlier studies. In addition, 'slow' acetylators had higher levels of the 4-ABP–Hb adduct for the same type and quantity of cigarettes smoked. Urinary mutagenicity was associated with the quantity of cigarettes but not with the acetylation phenotype. Convex dose–response relationships were found between the amount smoked and the 4-ABP–Hb adduct levels or urinary mutagenicity. The same effect was seen with 4-ABP–Hb adducts measured in 55 smokers by Dallinga *et al.* (1998). A levelling-off of the relationship between the number of cigarettes smoked and the relative risk of bladder cancer has also been reported from several case control and cohort studies (Vineis *et al.* 2000). In 15 non-smokers who reported exposure to ETS, 4-ABP–Hb adduct levels, unlike urinary mutagenicity, were found to be a non-specific exposure indicator.

With the progress of genotyping of polymorphic enzymes involved in the metabolism of arylamines, researchers were able to compare the genotype of

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NAT2 and NAT1 with the levels of ABP–Hb adducts. Hispanic white residents of Los Angeles County ($n = 403$) were assessed (Probst-Hensch *et al.* 2000) for their NAT2 acetylator phenotype, NAT1*10 acetylator genotype, and 3- and 4-ABP–Hb adduct levels. Eighty two subjects were current tobacco smokers of varying intensities. Tobacco smokers had significantly higher mean 3- and 4-ABP–Hb adduct levels relative to non-smokers. The levels increased with increased amounts smoked per day ($p < 0.0001$). With adjustment for NAT1 genotype and race, the smoking-adjusted geometric mean level of 3-ABP–Hb adducts in NAT2 slow acetylators was 47% higher than that in NAT2 rapid acetylators ($p = 0.01$). The comparable value for 4-ABP–Hb adducts was 17% ($p = 0.02$). In contrast, no association between NAT1*10 genotype and 3- or 4-ABP–Hb adduct levels was observed after adjustment for NAT2 phenotype, smoking and race.

With regard to CYP1A2, Hammons *et al.* (1991) have shown an association between 4-ABP–Hb adducts and CYP1A2 activity in rats. Therefore, higher 4-ABP–Hb adduct levels are expected in humans with high CYP1A2 activity. Landi *et al.* (1996) studied Hb adduct levels and the expression of CYP1A2 in 97 healthy male volunteers. CYP1A2-dependent *N*-oxidation activity was measured using a molar ratio of urinary caffeine metabolites [(paraxanthine + 1,7-dimethyluric acid)/caffeine] obtained between the fourth and fifth hour after drinking a standardized cup of coffee. *N*-Oxidation activity was induced by blond-tobacco smoke, meat consumption the meal before the test, or more than four cups of coffee a day. The regular use of medication appeared to be associated with a decrease in *N*-oxidation levels. Age and alcohol consumption was not related to CYP1A2 activity. A polymorphic distribution of the CYP1A2 and NAT2 (determined by the ratio of the caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil and 1-methylxanthine) phenotypes was examined in relation to susceptibility to 4-ABP–Hb adduct formation. Rapid oxidizers and subjects with the combined slow acetylator-rapid oxidizer phenotype showed the highest 4-ABP–Hb adduct levels at a low smoking dose. Blond-tobacco smokers ($n = 22$) exhibited higher adduct levels compared with black-tobacco smokers ($n = 23$), after adjustment for the quantity of cigarettes smoked. This was confirmed by later studies (Dallinga *et al.* 1998, Godschalk *et al.* 2001). At the highest levels of smoking exposure, no major differences in 4-ABP–Hb adduct levels were found among the different combinations of CYP1A2 and NAT2 phenotypes.

Yu *et al.* (1995) studied 151 subjects (age > 30 years) from different racial groups in Los Angeles, California. Non-Hispanic white (white), black and Asian males had comparable smoking habits but different risks of bladder cancer (31 out of 100 000 in whites, 16 out of 100 000 in blacks, and 13 out of 100 000 in Chinese and Japanese). The GSTM1 genotype, acetylator phenotype, levels of 3- and 4-ABP–Hb adducts and smoking status were determined. Whites (27%) had the highest prevalence of the highest risk profile (slow acetylator, GSTM1 null), followed by blacks (15%) and Asians (2.7%); the difference was statistically significant ($p = 0.006$). Whites also had less than one-half the prevalence of the 'protective' profile (rapid acetylator, GSTM1 non-null) relative to blacks and Asians (23% versus 57%, $p = 0.0001$). Regardless of race and level of cigarette smoking, mean levels of 3- and 4-ABP–Hb adducts were higher in subjects possessing the higher risk GSTM1/acetylator profile. The mean level of 4-ABP–Hb adduct (adjusted for race, cigarette smoking and acetylator phenotype) was significantly higher in subjects possessing the GSTM1 null versus the GSTM1

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non-null genotype (46.5 versus 36.0 pg g$^{-1}$ Hb; $p = 0.037$). The comparable difference in mean levels of 3-ABP–Hb adduct was borderline significant (1.6 versus 1.1 pg g$^{-1}$ Hb; $p = 0.07$). These results suggest that GSTM1 is involved in the detoxification of 3- and 4-ABP and may contribute to the racial variation in bladder cancer incidence among white, black and Asian males.

Dallinga *et al.* (1998) studied a group of 55 smokers and four non-smokers and found no influence of GSTM1 and NAT2 polymorphisms on Hb adduct formation. The Hb adduct levels increased with the number of cigarettes smoked, but at a cigarette consumption of >30 cigarettes/day, a saturation effect was observed. Hb adducts in 15 smokers redetermined after a 6 month interval correlated ($r = 0.78$) with the first determination. The DNA adduct levels in lymphocytes were also measured in the same subjects (see DNA section). In another study, Hb adducts of 4-ABP and NAT2, NAT1, GSTM1 and GSTT1 genotypes were measured in 67 smokers (Godschalk *et al.* 2001). No overall effects of these genotypes were observed on 4-ABP–Hb adduct levels. However, in subjects smoking less than 25 cigarettes/day, 4-ABP–Hb adduct levels were higher in NAT2 slow acetylators (0.23 ± 0.10 ng g$^{-1}$ Hb) compared with fast acetylators (0.15 ± 0.07 ng g$^{-1}$, $p = 0.03$).

*Hb adducts of arylamines metabolically released from lignocaine.* Following administration of lignocaine, all patients had high levels of 2,6-DMA–Hb adducts. Differences of adduct levels in patients treated with lignocaine (70–3760 mg) ranged from 93–636 ng g$^{-1}$ Hb (Bryant *et al.* 1994). These data indicate that *N*-hydroxy-2,6-DMA is a metabolite of lignocaine in humans. This demonstrates that Hb adducts of arylamines can result from exposure to compounds that metabolically release arylamines.

*Hb adducts of musk xylene.* Musk xylene (1-tert-butyl-3,5-dimethyl-2,4,6-trinitrobenzene) metabolites bound to Hb were found in blood samples from 10 human volunteers not knowingly exposed to musk xylene (Riedel *et al.* 1999). The bound metabolites were liberated from Hb as amines by alkaline hydrolysis. 1-Tert-butyl-3,5-dimethyl-4-amino-2,6-DNB and, after chemical derivatization (100 µl PFPA at 50°C for 30 min), the corresponding *N*-perfluoropropyl amide were identified by GC-MS using electron impact ionization and NCI in all the samples. The amounts of 1-tert-butyl-3,5-dimethyl-4-amino-2,6-DNB bound to Hb in the human blood samples ranged from 3.47 ng (13 pmol) to 12.29 ng (46 pmol) per g Hb. These levels are very high in comparison to adducts of 4-ABP in smokers, which are about 180 pg (1.06 pmol) per g Hb.

### *Occupational exposure to arylamines*

Not many studies have described the analysis of Hb adducts in workers. Workers at Bayer in Leverkusen, Germany, are routinely monitored by Lewalter (Lewalter and Korallus 1985). Most of the results are not published in peer-reviewed journals (summarized in Lewalter and Neumann 1996). Urine metabolites and Hb adducts were measured in workers exposed to the following arylamines: aniline, 4-ABP, benzidine, 4-CA, 4-chloro-2-methylaniline, 2,4-DAT, DcBz, 2,4-DMA, 3,3′-dimethyl-4,4′-MDA, 2-MA, MDA, MOCA, 2-NA

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and 1,3-phenylenediamine. For these arylamines Lewalter mentions the upper limits measured and the maximum values allowed at Bayer. No such information is available from other companies. Hb adducts of 3,4-DCA have been reported in workers exposed to diuron (Lewalter and Korallus 1986). The formation of such adducts from the metabolic release of arylamines in pesticides was confirmed in animal experiments (Sabbioni and Neumann 1990b) and in a pilot study with workers exposed to propanil, a pesticide synthesized from 3,4-DCA (Pastorelli *et al.* 1998).

*Workers exposed to 2-MA and aniline.* A significant cluster of bladder cancer excess was discovered among workers employed at a chemical manufacturing facility in Niagara Falls, New York (Ward *et al.* 1996). This excess was primarily confined to the 708 workers who had ever been employed in the rubber chemicals manufacturing area of the plant, where the arylamines aniline and 2-MA have historically been used. A total of 73 workers (46 exposed and 27 unexposed) were investigated for urine metabolites and Hb adducts of aniline and 2-MA. Personal air-sampling data were also collected for 28 of the workers. Personal air sample measurements showed that airborne concentrations of aniline and 2-MA were well within the limits allowed in the workplace by the OSHA. Urine samples were collected pre-shift and post-shift, and stored at $-70^{\circ}$C. Acetanilide and *N*-acetyl-2-methylaniline, metabolites of aniline and 2-MA present in the urine, were converted to the parent compounds by applying base hydrolysis (Brown *et al.* 1995). Urinary aniline and 2-MA levels were substantially higher among exposed workers than among unexposed control subjects. The most striking difference was for post-shift urinary 2-MA levels, which averaged ($\pm$SD) $2.8 \pm 1.4\,\mu g\,l^{-1}$ in unexposed subjects and $98.7 \pm 119.4\,\mu g\,l^{-1}$ in exposed subjects ($p = 0.0001$). For the measurement of Hb adducts of aniline, 2-MA and 4-ABP, precipitated Hb was dissolved in NaOH in the presence of the corresponding deuterated compounds as internal standards, and the hydrolysate was extracted with hexane, derivatized with PFPA, and analysed by GC-MS with NCI (Sabbioni and Beyerbach 1995). Average aniline–Hb (17.4 versus $3.1\,ng\,g^{-1}$ Hb) and 2-MA–Hb (40.8 versus $3.5\,ng\,g^{-1}$ Hb) adduct levels were also significantly higher ($p = 0.0001$) among exposed workers than among unexposed control subjects. Average levels of adducts to 4-ABP, a potential contaminant of process chemicals, were not significantly different ($p = 0.48$), although three exposed workers had 4-ABP levels above the range in unexposed workers. The adduct data suggest that, among the current workers, 2-MA exposure exceeds aniline exposure, and that 4-ABP exposure, if it occurs at all, is not widespread. These data support the conclusion that occupational exposure to 2-MA is the most likely causal agent of the bladder cancer excess observed among workers in the rubber chemicals department of the plant under study, although exposures to aniline and 4-ABP cannot be ruled out. The conclusions drawn by this study were the subject of several controversial comments (Ward 1997). The criticism concentrated on the fact that at the time of the genetic lesions by chemicals, probably 20 years ago, other arylamines (e.g. 4-ABP, 2-NA) of known human carcinogenic potency might have been present. Ward (1997) replied that 2-MA was also the main

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chemical used in the chemical factory 20 years ago. This example shows the difficulties inherent in understanding the cause of a disease that develops 20 years after exposure. In addition, the number of cases encountered after exposure to weak carcinogens are only spotted in a large group of workers. In the study described above there were seven cases of bladder cancer in 752 workers, whereas only 1.2 cases would be expected for that region.

*Workers exposed to 4-CA and aniline.* Hb adduct levels of arylamines were determined in a group of workers by HPLC with electrochemical detection (Riffelmann *et al.* 1995). The arylamines in urine were determined with GC and electron capture detection. The acetylator status was determined by comparing the ratio of acetanilide/aniline or 4-chloro-acetanilide/4-CA in urine. In the case of fast acetylators, more than 50% of the arylamines in urine were acetylated. Such a classification should be compared to the NAT2-phenotyping procedure with caffeine, since the acetylation ratio of arylamines is dose dependent (Sagelsdorff *et al.* 1996, Bailey *et al.* 1990). Low levels of aniline, 4-MA, 2-NA, 4-chloro-2-methylaniline and 2,4-DAT were found in urine of 16 controls. Higher levels were found in the workers, especially for 4-CA and aniline. In fast acetylators 4-CA (aniline) was bound at a level of $663 \pm 160\,\mathrm{ng\,l^{-1}}$ blood ($805 \pm 1282\,\mathrm{ng\,l^{-1}}$) compared with $1443 \pm 441\,\mathrm{ng\,l^{-1}}$ ($876 \pm 476\,\mathrm{ng\,l^{-1}}$) in slow acetylators.

*Workers exposed to 2,4-DFA.* Hb binding of 2,4-DFA was compared in rats and workers (Boogard *et al.* 1994a). 2,4-DFA was administered orally at doses of 0–7.5 $\mu\mathrm{mol\,kg^{-1}\,day^{-1}}$ to 10 male and 10 female Fischer 344 rats for 10 consecutive days (two rats of each gender in each dose group). A linear relationship between dose and adduct concentration was observed. At the two lowest doses (0.078 and 0.775 $\mu\mathrm{mol\,kg^{-1}\,day^{-1}}$) no metHb was observed, but adducts (40 and 300 $\mathrm{pmol\,g^{-1}}$ Hb in males; 80 and 450 $\mathrm{pmol\,g^{-1}}$ Hb in females) could easily be measured. At these doses, the mean adduct levels were in the same range as found in human studies. In two studies, involving 20 and 16 workers potentially exposed to low concentrations of 2,4-DFA, median concentrations of 10 pmol [1.29 ng] $\mathrm{g^{-1}}$ Hb (range 1–83 $\mathrm{pmol\,g^{-1}}$ Hb) and 20 $\mathrm{pmol\,g^{-1}}$ Hb (range 4–322 $\mathrm{pmol\,g^{-1}}$ Hb), respectively, were found. Since in the animal experiment 2,4-DFA (Sabbioni 1992) and aniline (Albrecht and Neumann 1985) bind to a similar extent, Boogard *et al.* (1994a) proposed using the same BAT value for 2,4-DFA as that set for aniline (7.2 $\mathrm{nmol\,g^{-1}}$ Hb). The BAT value is based on the relationship between methaemoglobinaemia and adduct formation (Lewalter 1986). In both studies of workers exposed to 2,4-DFA, the adduct levels were well below this tentative BAT value.

*Workers exposed to 3-chloro-4-fluoroaniline.* In two studies involving 75 and 72 workers (Boogard *et al.* 1994b), potential exposure to 3-chloro-4-fluoroaniline (CFA) was biologically monitored by determination of its main urinary metabolite, 2-amino-4-chloro-5-fluorophenol sulphate (CFA-S). As this method allows only the detection of recent exposure, analysis of CFA adducts bound to Hb was investigated as a method to allow biological monitoring of exposure to CFA over longer periods. The median CFA-S concentration in

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67 samples from the first study was 0.14 μmol $g^{-1}$ creatinine, and 0.21 μmol $g^{-1}$ creatinine in 201 samples from the second study. The median Hb adduct concentration in 75 samples from the first study was 1.31 ng (9 pmol) CFA $g^{-1}$ Hb (range 0.73–93.1 ng $g^{-1}$) and 1.75 ng (12 pmol) $g^{-1}$ Hb (range 0.44–3.5 ng $g^{-1}$) in 46 samples from the second study. Urinary CFA-S and Hb adducts correlated well in samples collected shortly after incidental exposures. However, in 25% of the operators, no CFA-S was detected during routine biological monitoring, while Hb adduct analysis showed clear evidence of exposure. This indicates that intermittent exposure to CFA is more reliably monitored by determination of Hb adducts of CFA than by assessment of urinary CFA-S.

*Workers exposed to MDA or MDI.* Air levels, urine metabolites and Hb adducts of workers exposed to MDA or MDI in a large German company were investigated by Schütze *et al.* (1995) using GC-MS with NCI (Sabbioni and Beyerbach 2000). The air levels of MDA and MDI were below detection limits. However, adduct and metabolite levels were detected in a high percentage of the samples. Hb adducts of MDA (64–2614 fmol [13–518 pg] $g^{-1}$ Hb) were found in 97% and AcMDA (299–2234 fmol $g^{-1}$ Hb) in 65% of the MDA workers. Hb adducts of MDA (57–219 fmol $g^{-1}$ Hb) were found in 38% of the MDI workers. Urine collected at the same time as the blood samples from these MDA and MDI workers was extracted at alkaline pH with and without preceding acid treatment. MDA (0.13–2.76 nmol [26–547 ng $l^{-1}$]) and AcMDA (0.73–23.46 nmol $l^{-1}$] were found in the urine of 97% and 82%, respectively, of the MDA workers. MDA (7.1–139.2 pmol $l^{-1}$) and AcMDA (96–3000 pmol $l^{-1}$) were found in 96% and 83%, respectively, of the MDI workers. In order to release MDA and AcMDA from possible conjugates, urine was treated under strong acidic conditions. Following this procedure, higher levels of MDA were found than the sum of MDA and AcMDA after base extraction alone. MDA (0.42–187.72 nmol [0.08–37.2 μg] $l^{-1}$) was present in 96% of the MDA workers, and in 96% of the MDI workers (0.66–10.18 nmol $l^{-1}$). The same biomarkers were measured in another group of MDI workers. Hb adducts of MDA (70–710 fmol $g^{-1}$ Hb] were found in all workers, and AcMDA was detected in one worker (Sepai *et al.* 1995b). The urine levels were up to four-fold higher compared with the former study. The presence of MDI adducts or other isocyanate metabolites were not investigated. By measuring urine metabolites alone, the exposure of some workers was strongly under- or overestimated compared with measurement of the level of Hb adducts (Schütze *et al.* 1995, Sepai *et al.* 1995b).

*Workers exposed to MOCA.* Hb adducts, urine and plasma levels of MOCA were determined in a pilot study with five workers (Vaughan and Kenyon 1996). MOCA was quantified as a PFPA-derivative using GC-MS with NCI or GC with electron capture detection. DcBz was the internal standard. The following concentrations were found: Hb adducts, 0.19–11.6 ng $g^{-1}$ Hb; plasma alkaline hydrolysate, 0.013–5.9 μg $l^{-1}$; urine alkaline hydrolysate, 1.2–638 μg $l^{-1}$. Urine and plasma were hydrolysed at 95°C; Hb was hydrolysed at room temperature.

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*Workers exposed to TNT.* Another study involved the biomonitoring of workers employed in a Chinese TNT factory (Liu *et al.* 1995, Sabbioni *et al.* 1996). The factory controls were fire fighters, white-collar workers, security guards and the director. The Medical Department of the factory collected the blood. The Hb was hydrolysed with NaOH, extracted with dichloromethane and analysed by GC-MS with NCI. The 4-amino-2,6-DNT levels of the workers ranged up to 522 ng $g^{-1}$ Hb. The highest levels were found in the screening and loading group. For 2-amino-4,6-DNT, the highest level was 14.7 ng $g^{-1}$ Hb. Hb adducts of TNT were found in all the factory controls. This demonstrates that there is general contamination of the factory, since the Hb of German laboratory workers was free of 2-amino-4,6-DNT and 4-amino-2,6-DNT (Sabbioni *et al.* 1996). The Hb adducts determined in this study were compared with the air levels and skin levels. The air and skin concentrations were measured in the same workplace, but at a different time point to the blood collection. The adduct levels in exposed workers were more related to skin contamination than to air concentration, indicating that skin contamination is the main source of the internal dose.

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### *The use of rat Hb binding indices to estimate environmental exposure of humans*

Chronic exposure leads to accumulation of Hb adducts (Ehrenberg *et al.* 1974). If Hb binding data are available from animal experiments, it is possible to estimate the daily dose from the measured Hb adduct levels. However, it is necessary to make the following assumptions

(1) The adduct levels result from steady-state exposures ($A_c$): $A_c = 0.5 \times A \times T_{er}$, where $A$ is the average daily increment per total Hb and $T_{er}$ is the lifetime of an erythrocyte. Thus, to calculate the single dose, the adduct level has to be divided by 60 (Tannenbaum *et al.* 1986).
(2) The modified Hb has the same lifespan as unmodified Hb and the adducts are stable to repair mechanisms.
(3) The pharmacokinetics of the xenobiotic compound is comparable in rats and humans.

Using this formula, Tannenbaum *et al.* (1986) calculated the daily exposure of smokers to 4-ABP (17–38 ng). The percentage of the 4-ABP dose bound to Hb in rats was very similar to the percentage of the dose bound to Hb in smokers.

Taking 2-MA found in a worker as an example (Ward *et al.* 1996), the amount of adduct associated with Hb can be divided by 0.00059, which is the proportion of an administered dose found associated with the Hb in rats (Birner and Neumann 1988, Sabbioni 1992), to calculate the exposure. Thus, for a 70 kg individual with 200 ng 2-MA $g^{-1}$ Hb, the exposure dose is 68 µg 2-MA $kg^{-1}$ $day^{-1}$. As a rough risk estimate, these daily doses can be compared to the $TD_{50}$ (daily dose which yields tumours in 50% of rodents) values in rats (Gold *et al.* 1993).

Repeating the same calculations for the adducts of the other amines found in non-smokers (Bryant *et al.* 1988), humans appear to be exposed to very high levels of amines (Sabbioni 1992), with estimated exposure levels (Hb adduct level given in parentheses) as follows: 2-Na, 3.38 µg (11 pg $g^{-1}$ Hb); 2-MA, 4.46 µg (188 pg $g^{-1}$); 3-MA, 22.1 µg (1141 pg $g^{-1}$); 4-MA, 4.64 µg (209 pg $g^{-1}$); 2,4-DMA, 1.69 µg (40 pg $g^{-1}$); 2,5-DMA, 0.652 µg (50 pg $g^{-1}$); 2,6-DMA, 23.1 µg

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(264 pg g$^{-1}$); 3,4-DMA, 6.35 µg (47 pg g$^{-1}$); 3,5-DMA, 5.05 µg (931 pg g$^{-1}$); 2-EA, 0.056 µg (3 pg g$^{-1}$); 3-EA, 0.764 µg (102 pg g$^{-1}$); 4-EA, 0.688 µg (92 pg g$^{-1}$); 4-ABP, 0.014 µg (51 pg g$^{-1}$). The exposures vary from 0.014 to 23.1 µg day$^{-1}$ for a 70 kg man. These figures are based on the assumption that all amines have the same dose relationship in humans and in rats, and that the lifetime of the Hb adducts in rats or humans are not altered. Some amines might react with the two additional cysteine groups present in the $\alpha$-chain of the Hb of rats but not in mice or humans. Maples *et al.* (1989) deduced from electron spin resonance measurements that the additional cysteine groups in the $\alpha$-chain react with nitrosobenzene. Two to ten times lower Hb binding in mice than in rats was found for five arylamines (Birner and Neumann 1988).

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*Experimental procedures for the analysis of human Hb adducts and quality control*

In general the determination of arylamines in biological matrices has been performed according to the following procedures: (1) isolation of Hb by precipitation or dialysis; (2) hydrolysis in 0.1 M NaOH in the presence of internal standards; (3) extraction with organic solvents or reversed phase cartridges; (4) derivatization with a perfluorinated anhydride; (5) quantification by GC-MS with NCI against a calibration curve obtained from PFPA derivatives in organic solvents or from a calibration curve obtained from spiked Hb samples; and (6) determination of accuracy and precision of the method. These important and critical steps will be discussed below.

Hb is isolated by precipitation (Albrecht and Neumann 1985, Sabbioni 1992, Lewalter and Gries 2000) or by dialysis of lysed erythrocytes (Bryant *et al.* 1993, Skipper and Stillwell 1994). In both cases blood is centrifuged to generate packed red cells and, after removal of the serum, the erythrocytes are washed three times with 0.9% saline. The major concern for both procedures is the removal of unbound arylamines. Both approaches performed well in experiments with arylamine-spiked Hb, demonstrating the elimination of non-covalently bound material in the work-up. For both methods it is advisable to perform extractions of Hb at pH 7.4 to check if unbound material is present.

All the procedures described by the group of Skipper and Tannenbaum (Bryant *et al.* 1987, 1993, Skipper and Stillwell 1994) begin with 10 ml of blood; this corresponds to about 1.5 g of Hb. The dialysates are made basic (final concentration 0.1 M NaOH) and left at room temperature for 3–4 h. Extraction with hexane is followed by drying with $Na_2SO_4$/$MgSO_4$, addition of trimethylamine and PFPA (2 µl) at room temperature, and evaporation to dryness. The residue is taken up in 20 µl of hexane, and one quarter is analysed by GC-MS with NCI. The analytes were quantified against a calibration line obtained from standard solutions of PFPA derivatives. The precision for 4-ABP was $\pm$10%, but $\pm$20% for the monocyclic arylamines. The procedure has been modified over the years. Originally, 4′-fluoro-4-ABP (1 ng) was used as the internal standard for the quantification of 4-ABP, 1-NA and 2-NA, and aniline-$d_5$ for the quantification of the monocyclic arylamines (Bryant *et al.* 1987, 1988, Skipper *et al.* 1988, Maclure 1990). The determination of monocyclic arylamines was dropped after the first studies. Stillwell *et al.* (1987) proposed the use of 2-amino-5-fluoronaphthalene (0.5 ng) as an additional internal standard, and dichloromethane as the extraction solvent instead of hexane. In 1993 a standard operation procedure for

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the determination of Hb adducts was presented in great detail (Bryant *et al*. 1993). Additional internal standards were used: 4-ABP-$d_9$ (1 ng), 2NA-$d_7$ (0.1 ng) and 2-MA-$d_7$ (1 ng). The rest of the procedure corresponds to the earlier method published by Bryant *et al*. (1987). In 1991, the same research group presented a very interesting modification of the method (Coghlin *et al*. 1991, Skipper and Stillwell 1994). A solution of Hb previously adducted with *N*-hydroxy-4-ABP-$d_9$ and containing 150 pg of hydrolysable amine was introduced as a new internal standard. Using this new standard, the precision of the method could be improved from $\pm$10% to $\pm$4%. The following studies were performed according to this protocol; Hammond *et al*. (1993), 55 subjects; Yu *et al*. (1995), 151 subjects; Landi *et al*. (1996), 97 subjects; Tang *et al*. (1999), 109 subjects; Probst-Hensch *et al*. (2000), 403 subjects; Castelao *et al*. (2001), 1325 subjects.

Another method used for routine analysis has been published by Lewalter and Gries (2000). Hb is precipitated from the lysed erythrocytes. In contrast to the method published by Albrecht and Neumann (1985), the precipitated Hb is washed with large volumes of cold water (250 ml) in order to eliminate possible glutathione adducts that might be present. In addition, the precipitated Hb is washed with a sequence of organic solvents on a filter. The dried Hb (200 mg) is hydrolysed in 0.1 M NaOH in the presence of the deuterated internal standards (2-MA-$d_7$, 4-ABP-$d_9$ and DcBz-$d_6$) and extracted on a C18 reversed phase cartridge. After the cartridge is washed with water and dried, the analytes are eluted with chloroform, dried over sodium sulphate and derivatized with HFBA (2 µl). Evaporation to about 50 µl and addition of toluene (250 µl) was followed by washing with phosphate buffer. The organic phase was evaporated to 50 µl, and a 1 µl sample is analysed by GC-MS and NCI. Calibration graphs are plotted with calibration standards prepared using bovine Hb for quantitative evaluation. The calibration standards are treated in the same manner as the Hb samples to be investigated. The detection limit of the method (detected in a high resolution sector field mass spectrometer) is $< 7$ pg g$^{-1}$ Hb. The accuracy and precision of the method have been determined from Hb solutions spiked with different amounts of the analytes: aniline, 2-, 3-, 4-MA, *ortho* anisidine, 4-CA, 1-, 2-NA, 4-ABP, Bz, MDA, and DcBz.

A method for the analysis of over 30 monocyclic arylamines was developed by Sabbioni and Beyerbach (1995). The syntheses of several deuterated arylamines, which can be used as internal standards, are described in their paper. Precipitated Hb (200 mg) is hydrolysed in 0.1 M NaOH (4 ml) in the presence of the internal deuterated standards. The hydrolysate is extracted after 1 h at room temperature with hexane (6 ml). The organic phase is then dried over sodium sulphate, derivatized with PFPA (2 µl), evaporated to dryness, taken up in hexane (15 µl) and analysed by GC-MS with NCI. The method has been used for animal experiments and for workers exposed to arylamines (Ward *et al*. 1996). For the Hb adduct analysis of polycyclic arylamines, diamino bicyclic compounds and mono *N*-acetylated diamino bicyclic compounds, another method has been published recently (Sabbioni and Beyerbach 2000). The synthesis of several deuterated arylamines is described. The hydrolysis of Hb (200 mg) is performed in 0.1 M NaOH (4 ml) in the presence of deuterated internal standards. The hydrolysate is extracted with dichloromethane, dried with sodium sulphate, derivatized with HFBA (2 µl), taken up in ethyl acetate (15 µl) and analysed by GC-MS with NCI. In order to avoid the loss of the acetyl group in *N'*-acetyl-

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MDA or AcBz, a large amount of 4-MA is present in the work-up; in addition, HFBA must be eliminated after the derivatization (by washing with buffer solutions or the addition of methanol). The method was used for the analysis of workers exposed to MDA, MDI and benzidine (Schütze *et al*. 1995, Sepai *et al*. 1995a, b, Beyerbach *et al*. unpublished data). In both methods quantification was performed against a calibration curve obtained from Hb solutions spiked with a constant amount of the deuterated internal standards and a varying amount of the arylamines to be analysed. For the analysis of Hb adducts of polycyclic aromatic amines (2-AF, 1-aminopyrene, 6-aminochrysene and 9-aminophenanthrene) in bus drivers, Zwirner-Baier and Neumann (1999) precipitated Hb as described earlier (Albrecht and Neumann 1985). Hydrolysis of Hb (200 mg) in the presence of 1-AF as an internal standard was followed by extraction on reversed phase C18 cartridges on a polymeric base. The analytes were eluted with ethyl acetate, dried over $MgSO_4$ and derivatized with a large amount of PFPA (100 µl). After evaporation to dryness the residue was taken up in 100 µl of ethyl acetate, and 2 µl were analysed using column injection, GC-MS and NCI. The authors determined the recoveries of the compounds, which varied by up to a factor of 2. The authors repeated the recovery experiment every day and corrected the values of the measured samples according to these recoveries. The recoveries were good and consistent for compounds (2-AF) similar to the internal standard (1-AF). However, for compounds such as 9-aminophenanthrene, 3-aminofluoranthene, 1-aminopyrene and 6-aminochrysene the variance in the recovery was large. Such effects might be reduced by using the corresponding deuterated polyaromatic compounds as the internal standards. The differences in the values obtained in two independent hydrolyses were reported to be up to 50%.

To date, the method of Skipper and Stillwell (1994) has been used for testing the largest number of environmentally exposed people. This laboratory has about 20 years' experience in the analysis of such samples. Moreover, Dr Skipper has mostly performed the analyses himself. This allows a comparability of the data over the years. In addition to the quality control procedures proposed by Skipper (Bryant *et al*. 1993, Skipper and Stillwell 1994), we suggest that accuracy and precision should be determined using spiked Hb samples (with at least two concentrations). Testing the precision of the assay on a human sample of unknown content could just show the variance of an artefact. We also recommend testing the linearity of the method from Hb samples spiked with different concentrations of the analytes. As a further quality control, we recommend using alternative methods for the identification of the compounds. In all the methods described above the compounds are quantified by GC-MS with NCI. To confirm the structure of the analytes, pooled samples could be analysed using the following methods: GC-MS with electron impact ionization, HPLC with UV detection (Pinorini-Godly and Myers 1996), HPLC with electrochemical detection (Birner *et al*. 1990, Sabbioni and Neumann 1990a, Sabbioni and Schütze 1998, Zwirner-Baier *et al*. 1994, 1998), and HPLC with fluorescence detection (Cheever *et al*. 1992, Suzuki *et al*. 1989). In addition derivatizing agents other than PFPA might be considered. Another useful approach would be to perform an interlaboratory comparison for a few samples. After measuring Hb adducts in humans, it is advisable to perform other exposure assessments (urine, air monitoring, food monitoring) or to estimate and rationalize the daily exposure. A further way to

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avoid artefacts is to determine only Hb adducts of arylamines, which are known to form hydrolysable Hb adducts in animal experiments.

In the future the methods should be automated in order to perform analyses of larger collectives and to reduce variations in the procedures, For example, the analysis of Hb and albumin adducts has been automated in the laboratory of Lewalter (Bayer Leverkusen, Germany) (personal communication). Hb is obtained from lysed erythrocytes by cation exchange chromatography. The purified Hb solution is then made basic and extracted on a reversed phase C18 cartridge. The method has been fully automated. The major advantages of this procedure are the high throughput of samples and the increased purity of Hb compared with other methods.

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## DNA adducts

DNA adducts of arylamines can be formed through several metabolic pathways, including *N*-hydroxyarylamines, *N*-hydroxyarylamides, *O*-glucuronides and a variety of reactive arylamine esters such as *N*-sulphonyloxy, *N*-acetoxy and *N*-prolyoxy derivatives (reviewed by Beland and Kadlubar 1990). The predominant site of attachment on DNA is guanine. The major adduct involves the C8 position of guanine attaching to the carcinogen's nitrogen. The mechanistic aspects of this adduct formation are still controversial (Humphreys *et al*. 1992, Kennedy *et al*. 1997, McClelland *et al*. 1999). It is generally accepted that bioactivation goes through *N*-oxidation and in most cases *O*-esterification. These esters have been assumed to react via an SN1 (substitution nucleophilic unimolecular) pathway, with a nitrenium ion as the intermediate, which reacts with guanine (Novak and Kennedy 1998, McClelland *et al*. 1998). However, C8 is not regarded as the normal position of electrophilic addition in guanine, and it has been proposed that the C8 adduct is the final product after initial N7 adduct formation (Humphrey *et al*. 1992). However, kinetic and spectroscopic analyses fit best with a C8 adduct intermediate (McClelland *et al*. 1999).

### *DNA adduct synthesis*

The synthesis of DNA adducts with arylamines has been reviewed elsewhere (Beland and Kadlubar 1990, Kadlubar 1994) and is summarized in table 3. We will consider here the products not included in the former reviews. *In vitro* reactions of deoxyguanosine (dG) with *N*-acyloxyarylamines, *N*-sulphonyloxyarylamides and *N*-acyloxyarylamides yielded C8 guanine adducts, and in some cases N2 dG adducts. The yields for compounds with relatively stable nitrenium are higher: yields for dG-C8–4-ABP are higher than for dG-C8–4-CA. The same reactions can be performed with DNA. In this case reaction products other than dG-C8 adducts can be isolated. Interestingly, the products resulting from the reaction of *N*-acetyl-*N*-(2-fluorenyl)nitrenium with dG and denatured DNA yield only the C8 adduct, whereas reactions with double-stranded DNA yield 5–20% adducts with the N2 of guanine (Novak and Kennedy 1998). Another method that has been often used is the formation of the nitrenium intermediate after laser or light irradiation of the corresponding azides (McClelland *et al*. 1999, Branco *et al*. 1999). The released nitrenium is trapped *in situ* by dG or DNA. In most cases the reaction products have to be purified by HPLC.

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Table 3. DNA adducts of arylamines found *in vivo*.

| Compound | DNA adducts | Species | References |
|---|---|---|---|
| *N*-Acetyl-2-aminofluorene | C8-, N2-dG | R, M | Kriek 1972, Westra *et al*. 1976, Poirier and Beland 1992 |
| 2-Aminofluorene | C8-dG | R | Culp *et al*. 1993, Huitfeldt *et al*. 1994 |
| *N*-Methyl-4-aminoazobenzene | C8-, N2-dG, N6-dA | R, M | Lin *et al*. 1975, Beland *et al*. 1980, Tarpley *et al*. 1980, Tullis *et al*. 1981, 1987 |
| 4-Aminoazobenzene | C8-dG | M | Delclos *et al*. 1984 |
| *N*-Acetylbenzidine | C8-dG | R, M, H, hamster | Martin *et al*. 1982, Yamazoe *et al*. 1988, Rothman *et al*. 1996a,b, 1997, Kennelly *et al*. 1984 |
| Benzidine | C8-dG | D | Yamazoe *et al*. 1988 |
| *N*-Acetyl-4-ABP | C8-, N2-dG | R | Kriek 1971, Kriek and Westra 1971, Kadlubar *et al*. 1982 |
| 4-ABP | C8-dG | R, M, D, H, | Poirier *et al*. 1995, van de Poll *et al*. 1990, Talaska *et al*. 1990, 1991a,b |
| 3,2-Dimethyl-4-ABP | C8-, N2-dG | R | Westra *et al*. 1985 |
| N-Acetyl-4-fluoro-4-ABP | C8-dG | R | Kriek and Hengeveld 1978 |
| 4-Fluoro-4-ABP | C8-dG | R | van de Poll *et al*. 1989 |
| *N*-Acetyl-2-aminophenanthrene | C8-, N2-dG | R | Gupta *et al*. 1989 |
| 2-Naphthylamine | C8-, N2-dG, N6-dA | R, D | Kadlubar *et al*. 1980, Yamazoe *et al*. 1985, Dooley *et al*. 1984 |
| 1-Naphthylamine | O6-dG | R | Kadlubar *et al*. 1978 |
| 4,4-Methylenebis(2-chloroaniline) | C8-dA | R, H | Segerbäck and Kadlubar 1992, Silk *et al*. 1989, Kaderlik *et al*. 1993 |
| 1-Nitropyrene | C8-dG | R, M | Stanton *et al*. 1985 |

D, dog; H, human; M, mouse; R, rat.

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DNA adducts of monocyclic arylamines (2-, 3- and 4-MA, 2,3-, 2,4-, 2,5-, 3,4- and 3,5-DMA, 2-, 3- and 4-EA) have been synthesized by reacting *N*-acetoxy-arylamines with dG, nucleotides and DNA (Marques *et al.* 1996, 1997). In addition, the nucleotide adducts with 3-P-dG for postlabelling analysis were synthesized. The predominant products from reactions with dG and the nucleotides were *N*-(deoxyguanosin-8-yl)arylamines. HPLC and spectroscopic analyses of the modified DNA indicated the same adducts. In the same year Beyerbach *et al.* (1996) synthesized dG-C8 adducts of 2-MA, 2-CA, 4-CA, 2,4-DMA and 2,6-DMA, and the corresponding 3′-P-dG adducts for the quantification of DNA adducts by the $^{32}$P-postlabelling technique. Using the same methods, the DNA adducts of MDA – *N*′-acetyl-*N*-(deoxyguanosin-8-yl)-MDA and *N*-(deoxyguanosin-8-yl)-MDA – and their corresponding 3-monophosphate derivatives, were synthesized by Schütze *et al.* (1996).

Branco *et al.* (1999) presented a new synthetic pathway for the formation of dG–arylamine adducts involving the deoxygenation of nitroarenes and nitrosoarenes by triethyl phosphite in the presence of dG. This proved to be an effective method to synthesize dG-C8–arylamine (0.5–12% yield) and *N*-(2′-deoxyguanosin-N1-yl) arylamine (1.4–5.7% yield). The new adducts – 4-(2-deoxyguanosin-8-yl)-2-MA, 4-(2-deoxyguanosin-O6-yl)-2-MA, 6-(2-deoxyguanosin-N1-yl)-2-MA and 4-(2-deoxyguanosin-N1-yl)-2-MA – were obtained from 2-NT.

An unusual pattern of DNA adducts was seen with 2,6-DMA (Goncalves *et al.* 2001). In addition to dG-C8–2,6-DMA, two new adducts – 4-(deoxyguanosin-N2-yl)-2,6-DMA (dG-N2-2,6-DMA) and 4-(deoxyguanosin-O6-yl)-2,6-DMA (dG-O6-2,6-DMA) were isolated – from the reaction of *N*-acetoxy-2,6-DMA with deoxyguanosine. A similar reaction conducted with deoxyadenosine (dA) yielded 4-(deoxyadenosin-N6-yl)-2,6-dimethylaniline (dA-N6-2,6-DMA). All four adducts were detected in DNA reacted with *N*-acetoxy-2,6-DMA, with the relative yields being 46% for dA-N6-2,6-DMA, 22% for dG-N2-2,6-DMA, 20% for dG-O6-2,6-DMA, and 12% for dG-C8-2,6-DMA. This product profile contrasts markedly with the usual pattern of adducts obtained with arylamines, where C8-substituted deoxyguanosine products typically predominate.

Only a few syntheses of adenine adducts have been described. The Johnson's group (DeRiccardis *et al.* 1999) presented a general method for the synthesis of N2 and N6 carcinogenic amine adducts with dG and dA. The key step is a Buchwald–Hartwig coupling reaction between an appropriately protected derivative of dG or dA and an ortho-nitroaryl bromide or triflate. Subsequent reduction, acetylation and deprotection of the N2 adducts of dG and the N6 adducts of dA then gives the desired adducts. These syntheses yielded products of 4-ABP, 2-AF, aniline, 2-MA and 2-NA with overall yields >50%. In the same year Lakshman *et al.* (1999) successfully synthesized N6 adducts of adenine with 4-ABP, 2-AF, 3-cyanoaniline, 2-methoxyaniline and 4-MA.

In addition, the structure of oligonucleotides with 4-ABP (Cho *et al.* 1992), 2-AF (Cho *et al.* 1994, Mao *et al.* 1997, 1998a, b), 1-aminopyrene (Gu *et al.* 1999) and aniline (Shapiro *et al.* 1998) have been studied by nuclear magnetic resonance (reviewed in Patel *et al.* 1998).

### *DNA adducts of arylamines found* in vivo

DNA adducts found *in vivo* have been reviewed by Kadlubar (1994). In table 3, the major adducts found in rats, mice, dogs and humans are listed. The

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identification of arylamine–DNA adducts in experimental animals was usually performed with radiolabelled compounds. DNA was isolated, hydrolysed with enzymes and purified by HPLC. In the last 20 years, $^{32}$P-postlabelling, immunochemical assays, GC-MS and recently liquid chromatography with tandem mass spectroscopy (LC-MS-MS) have provided useful alternative approaches.

DNA adducts have been found whose structure could not be characterized. This is the case for animals given MDA, dinitrotoluenes and diaminotoluenes. The dG-C8 and 3-P-dG-C8 adducts of MDA have been synthesized (Schütze *et al.* 1996). The authors developed methods to identify these adducts of MDA in liver DNA using $^{32}$P-postlabelling, HPLC and GC-MS techniques. Liver DNA was obtained from rats treated with radiolabelled MDA (1.11 and 116.5 μmol kg$^{-1}$ body weight). The total radioactivity bound to the DNA corresponded to 0.06 and 2.7 adducts per $10^7$ nucleotides, respectively, giving covalent binding index (CBI) (i.e. μmol of adduct per mol of nucleotide)/mmol of compound per kg body weight) values of 1.05 and 2.3, respectively. This DNA-binding potency is in the range of weakly genotoxic compounds. Liver DNA was analysed for the presence of the synthesized adducts using HPLC analysis of nucleotides and purines after enzymic and acid hydrolysis, and by $^{32}$P-postlabelling after enzymic hydrolysis. The major adducts found *in vivo* did not correspond to the synthesized standards. It was possible to release MDA and MDA-$d_4$ from liver DNA of female Wistar rats dosed with MDA and/or MDA-$d_4$ using base hydrolysis overnight at 110°C, with CBI values of 0.82 and 1.0, respectively.

$^{32}$P-postlabelling was used to examine DNA adduct formation in male Fischer 344 rats exposed to the animal carcinogen 2,4-DAT (La and Froines 1994). 2,4-DAT produced three distinct DNA adducts. At the highest concentration examined (2046 μmol kg$^{-1}$), the level of the major adduct was 29.2 adducts per $10^7$ nucleotides. The yields for the two minor adducts were approximately one-tenth of that for the major adduct (La and Froines 1992a). Among the organs examined, DNA binding was highest in the liver (target organ), with levels approximately 10 times greater than that of the mammary gland (target organ) and up to 50 times greater than of the two non-target sites (kidney and lung). DNA binding by 2,4-DAT was also compared to that of its weakly carcinogenic analogue 2,4-DNT (La and Froines 1992b). The two compounds produced identical adduct patterns, suggesting that they share common metabolites and adducts, but adduct yields from 2,4-DNT were lower. The results suggest that the differences in carcinogenic potency between 2,4-DAT and 2,4-DNT, as well as the organotropic effects of 2,4-DAT, may be explained in part by quantitative differences in the extent of DNA adduct formation (La and Froines 1992a).

The same authors (La and Froines 1993) used $^{32}$P-postlabelling to compare DNA binding between the potent hepatocarcinogen 2,6-DNT and its non-carcinogenic analogue 2,6-DAT. Four adducts were detected following administration of 2,6-DNT, with a total adduct yield of 13.5 adducted nucleotides per $10^7$ nucleotides. Qualitatively identical adducts were also detected after treatment with the derivative 2-amino-6-nitrotoluene. Adduct yields from 2,6-DNT were 30 times greater than from 2-amino-6-nitrotoluene. No adducts were observed following treatment with 2,6-DAT. The differences between the two compounds in DNA binding were consistent with the differences in their carcinogenicity.

DNA adduct and Hb adduct levels were compared after dosing male Fischer 344 rats with 0–250 mg kg$^{-1}$ body weight of 2,4- or 2,6-DAT (Wilson *et al.* 1996)

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Hb adducts were determined using GC-MS, and DNA adducts using the $^{32}$P-postlabelling technique. Both DAT isomers formed Hb adducts, but only the 2,4-isomer yielded DNA adducts. Maximum DNA and Hb adduct levels were detected 24 h following administration. Both DNA and Hb binding increased in a dose-dependent manner.

### *DNA adducts of 4-ABP in exfoliated urothelial bladder cells*

For human biomonitoring studies, the following experiment with 4-ABP in dogs is very important (Talaska *et al.* 1990). Urine samples were collected from dogs treated with 4-ABP over a 2 week period and pooled according to an experimental plan that involved analysis of cumulative 48 or 72 h samples. The DNA of the exfoliated urothelial bladder cells was hydrolysed and postlabelled with $^{32}$P. Adducts observed on the resulting thin layer chromatograms were identical to those obtained from DNA modified *in vitro* with *N*-hydroxy-4-ABP and from dog bladder urothelial DNA isolated from the 4-ABP-dosed animals on termination of the experiment. Furthermore, a dose-related increase in 4-ABP–DNA adduct formation was demonstrated. Thus, it appears that the carcinogen–DNA adduct levels in the exfoliated bladder cells are reflective of the levels in the intact urothelium once steady-state levels have been achieved. To establish the identity of the major 4-ABP–urothelial DNA adduct in chronically treated dogs, the predominant $^{32}$P-postlabelled adduct was eluted from the thin layer chromatograms and coinjected onto an HPLC system with a synthetic 3′,5′-bisphosphate [$^{3}$H]*N*-(deoxyguanosin-8-yl)-4-ABP standard. Dual-label analysis of $^{3}$H and $^{32}$P indicated that both eluted from the column in the same fraction, which coincided with the UV absorbance peak of the synthetic marker.

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### *Method development for the determination of DNA adducts in human*

The levels of DNA adduction are typically in the range of 1 in $10^6$ to 1 in $10^9$ normal nucleotides (Garner 1998). Therefore, highly sensitive techniques are required for the analysis of small amounts of DNA (1–300 µg) that are available in human studies. The various methods used for DNA adduct determinations have been recently reviewed (Philipps *et al.* 2000). DNA adducts of arylamines have been determined using radiolabelled compounds, $^{32}$P-postlabelling, immunoassays, HPLC and electrochemical detection, and mass spectrometry. Immunoassays have been used for the determination of DNA adducts deriving from AAF or 4-ABP.

### *$^{32}$P-Postlabelling*

The $^{32}$P-postlabelling assay is the method most widely used for the analysis of DNA adducts (Talaska *et al.* 1992, Beach and Gupta 1992, Izzotti 1998, Philipps and Castagnaro 1999). This assay uses $\gamma$-$^{32}$P-labelled adenosine triphosphate to incorporate a highly radioactive reporter group into nucleotides After enzymatic hydrolysis of DNA, and the postlabelling procedure, the nucleotides are separated from normal nucleotides using thin layer chromatography or HPLC and visualized using radioautography or in-line scintillation counting, respectively. This procedure is usually the most sensitive method for the detection of adducted

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nucleotides. The major drawbacks are: (1) poor reproducibility; (2) difficult interpretation of the spots on the two-dimensional and four-dimensional thin layer chromatography; (3) that the use of an isotopically stable analogue as an internal standard is not possible; and (4) the large variability in the $^{32}$P-labelling efficiencies.

$^{32}$P-labelling efficiency varies among different adducts. Without synthetic standards the quantification of DNA adducts by $^{32}$P-postlabelling is achieved by relative adduct labelling, via comparison of the radioactivity incorporated into the adducts to that associated with the normal nucleotides. This approach assumes that normal and adducted nucleotide 3-phosphates are converted to 3′,5′-bisphosphates with similar efficiencies. The $^{32}$P-postlabelling efficiencies have been investigated for 3′-P-dG-C8–arylamine adducts (Mourato *et al.* 1999). The labelling efficiencies decreased in the following order: 3′-P-dG-C8–4-ABP $\gg$ 3′-P-dG-C8–4-MA > 3′-P-dG-C8–3,4-DMA > 3′-P-dG-C8–3-MA > 3′-P-dG-C8–2, 4-DMA. 3′-P-dG-C8–2-MA and 3′-P-dG-C8–2,3-DMA produced very poor and irreproducible labelling. Therefore, for quantification by $^{32}$P-postlabelling it is essential to have a synthetic standard of the compound to be determined.

Philipps and Castagnaro (1999) coordinated an interlaboratory comparison of 25 laboratories for the determination of dG adducts using the postlabelling procedure. Liver DNA from mice treated with [$^3$H]4-ABP was assayed together with synthetic DNA 3′-P-dG-C8–4-ABP in the different laboratories. The results varied by up to $\pm$93%. Adduct levels were found to be significantly lower using $^{32}$P-postlabelling than using $^3$H incorporation (see below). A recommended set of procedures was developed for detection, quantification, quality control and standardization.

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### *Liquid chromatography-mass spectrometry*

Recently, liquid chromatography-mass spectrometry (LC-MS) analyses were successfully conducted on the dG-C8 adduct of 4-ABP in rodents. Doerge *et al.* (1999) developed a quantitative isotope dilution method for the analysis of dG-C8–4-ABP using dG–4-ABP-$d_9$ as an internal standard. Column switching valves were used for on-line sample concentration and clean-up. The trapped sample was separated on a HPLC system and detected by electrospray ionization mass spectrometry (ESI-MS). The detection limit for dG-C8–4-ABP in DNA hydrolysates was 10 pg on-column. For a sample containing 100 µg DNA, this corresponds to 0.7 dG-C8–4-ABP adducts per $10^7$ normal nucleotides. The same authors compared different detection methods for the quantification of dG-C8–4-ABP adducts (Beland *et al.* 1999). A calf thymus DNA sample modified with [$^3$H]-*N*-hydroxy-4-ABP was synthesized to serve as a quantification standard. The adduct level was $62 \pm 0.8$ adducts per $10^8$ nucleotides on the basis of $^3$H content, and HPLC analyses following enzymatic hydrolysis to nucleosides indicated the presence of one major adduct, dG-C8–4-ABP. The modification levels determined with LC-MS, $^{32}$P-postlabelling and dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) were 19, 0.84 and 63 dG-C8–4-ABP per $10^8$ nucleotides, respectively. Liver DNA from mice treated with [$^3$H]4-ABP was quantified by $^{32}$P-postlabelling using synthetic 3′-P-dG-C8–4-ABP as the quantification standard. Levels of dG-C8–4-ABP were underestimated, while DELFIA, using a G-C8–4-ABP quantification standard, overestimated the adduct levels. The adduct

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levels detected by LC-MS reflected best those obtained from $^{3}H$ incorporation. When the $^{32}P$-postlabelling analyses and the DELFIA were conducted using DNA modified *in vitro* with dG-C8–4-ABP as a quantification standard, accurate estimations of the extent of *in vivo* formation of dG-C8–4-ABP were obtained.

The major drawback of LC-ESI-MS analyses are the ion suppression effects of a partly unknown mechanism (King *et al*. 2000, Lips *et al*. 2001, Soglia *et al*. 2001). This effect limits the amount of sample that can be analysed.

### GC-MS

Lin *et al*. (1994) established a method for the quantification of 4-ABP–DNA adducts by GC-MS. Aliquots of DNA (typically 100 μg ml$^{-1}$) were spiked with an internal standard, 4-ABP-$d_9$, and hydrolysed in 0.05 M NaOH at 130°C overnight. The released 4-ABP was extracted with hexane, derivatized with PFPA and analysed by GC-MS and NCI. The authors determined the recovery of 4-ABP and the linearity of the method with *in vitro* [$^{3}H$]*N*-hydroxy-4-ABP-modified DNA standards. The method was linear from 1 adduct per $10^4$ nucleotides to 1 adduct per $10^8$ adducts, and the yield of released 4-ABP was $59 \pm 7\%$ ($n = 9$). The method was further validated by comparison of the results with those obtained by the $^{32}P$-postlabelling method. There was excellent agreement ($r = 0.994$, $p < 0.001$) between the two methods for quantification of the adduct in eight samples of *Salmonella typhimurium* DNA treated with 4-ABP and rat liver S9, although the $^{32}P$-postlabelling method gave slightly higher values. The DNA adducts in 11 human lung and eight urinary bladder mucosa specimens were then determined by the GC-MS method. The adduct levels were found to be $< 0.32$ to 49.5 adducts per $10^8$ nucleotides in the lungs and $< 0.32$ to 3.94 adducts per $10^8$ nucleotides in the bladder samples. The same method has been used for the determination of MDA adducts in rats (Schütze *et al*. 1996). The method also worked with DNA spiked with dG adducts of 2-CA, 4-CA, 2-MA, 4-MA, 2,4-DMA and 2,6-DMA. The parent arylamines were released after hydrolysis with NaOH and quantified by GC-MS (Beyerbach *et al*. 1996). For the GC-MS method the DNA has to be free of RNA and proteins; otherwise the released 4-ABP might originate from adducts other than DNA adducts. In addition, the general contamination problems that have been noted for the Hb adduct analyses can arise.

### Immunoassays

Immunochemical determination of 4-ABP–DNA adducts have been performed using an enzyme-linked immunoassay (Roberts *et al*. 1988, 1991, Kadlubar *et al*. 1989, Culp *et al*. 1997). The method was validated in animal experiments and used for the determination of adducts in human lung and bladder epithelial DNA. Santella (al-Atrash *et al*. 1995, Santella 1999) developed a method for the quantification of 4-ABP–DNA adducts in cells by immunohistochemical methods (immunofluorescence and an immunoperoxidase method). The immunofluorescence method was validated by comparing the adduct levels measured in 4-ABP-treated mouse liver to those determined by the GC-MS method according to Lin *et al*. (1994). A good correlation ($r = 0.98$, $p = 0.0001$) was found between the relative fluorescence intensity and the adduct levels determined by GC-MS. On the basis of the adduct levels determined by GC-MS, the immunohistochemical

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method has a limit of sensitivity of approximately 1 adduct per $10^7$ nucleotides. The immunoperoxidase method was then used for the detection of adducts in exfoliated oral (Hsu *et al*. 1997, Romano *et al*. 1997) and bladder (Hsu *et al*. 1997) cells of smokers and non-smokers. Higher adduct levels were observed in smokers in both studies. The great advantage of this method is that it can be applied to paraffin blocks of tissue, which permits the analysis of DNA adducts in samples that are unsuitable for analysis by other methods. The major disadvantage of all immunoassays is the possible cross-reactivity with structurally similar compounds.

### *Determination of DNA adducts in human*

Carcinogen–DNA adducts are generally regarded as relevant biomarkers of carcinogen exposure, and their levels in target tissues have often been predictive of tumour incidence in experimental animals. Thus, human risk assessment procedures have utilized dose–response models that assume proportional relationships between carcinogen exposure and cancer susceptibility, even though wide inter-individual variations in human metabolic activating enzymes have now been clearly established. To evaluate these approaches, the relationship between carcinogen exposure, DNA adduct levels, metabolic activation phenotypes, and cancers of the urinary bladder, lung and larynx have been investigated.

To date, the DNA adducts of arylamines detected in humans are MOCA, 4-ABP and benzidine. Most studies have been performed with 4-ABP. At the beginning of the 1990s, all human DNA adduct determinations were performed by Glenn Talaska in cooperation with different scientists: Tannenbaum (Massachusetts Institute of Technology, Boston), Bartsch (IARC, Lyon), Kadlubar (NCTR, Jefferson, Arkansas) and Vineis (Turin, Italy). Immunoassays of 4-ABP–DNA adducts were developed by Groopman and Kadlubar (Roberts *et al*. 1988), and by Santella's group (Columbia University, New York). Van Schooten published his first results about the determination of 4-ABP–DNA adducts in 1998 using the immunohistochemistry technique of Santella. In the following sections the work of these principal investigators will be summarized.

### *DNA adducts of 4-ABP in the bladder*

A major obstacle to developing a human biomonitoring method for carcinogen–DNA adducts has been the problem of obtaining target tissue DNA samples by non-invasive means. A breakthrough experiment was performed by Talaska (Talaska *et al*. 1990). In a dog experiment (see page 395), Talaska showed the DNA adduct level in the target organ correlated well with the DNA adduct levels of the excreted urothelial epithelial cells. The lifetime of urothelial epithelial cells is 100 days. In 1991, Talaska reported the presence of DNA adducts of 4-ABP in the bladders of smokers (Talaska *et al*. 1991a, b, 1992, 1993). The adduct levels in exfoliated urothelial cells in cigarette smokers were related to the smoking history (packs per day, black or blond cured tobacco), urinary mutagenicity and the level of 4-ABP–Hb adducts. The association of four adduct measures with smoking was corroborated by significant correlations with the levels of 4-ABP–Hb adducts, the type and number of cigarettes smoked, and/or the urinary mutagenicity (Talaska *et al*. 1993). One adduct (specified as adduct 4) seemed chromatographically similar to 3′-P,5′-P-dG-C8–4-ABP. This adduct showed the strongest correlation

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with 4-ABP–Hb adduct levels. The same adduct 4 was found in biopsies of human bladder (Talaska *et al*. 1991a). These data suggest that non-invasive techniques can be applied to the study of carcinogen–DNA adducts in the target organ of humans at risk for urinary bladder cancer. This work has been summarized in table 3 and reviewed by Talaska *et al*. (1992) and Kadlubar (1994). Here we will focus on work on the determination of DNA adducts in humans published after 1994.

Talaska *et al*. (1994) determined the levels of DNA adducts (using $^{32}$P-postlabelling) in the biopsies of 20 bladder cancer patients and in the exfoliated bladder cells of 36 healthy volunteers. A dose–response relationship between smoking levels and adduct levels was present among both cancer cases and controls. Cancer cases and controls had similar adduct levels for the same level of smoking.

4-ABP–DNA adducts, 4-ABP–Hb adducts and urinary mutagenicity were measured in 47 healthy smokers and 50 non-smokers (Vineis *et al*. 1996). DNA adducts were determined by $^{32}$P -postlabelling in the exfoliated bladder cells of 39 healthy subjects. 4-ABP–Hb adducts were associated with smoking habits and with the consumption of black, air-cured tobacco. The levels of two DNA adducts (adducts 2 and 4) in urothelial cells were clearly associated with 4-ABP–Hb adducts, in all subjects and in smokers.

The immunoperoxidase method was developed for the detection and quantification of 4-ABP–DNA damage in single cells (Hsu *et al*. 1997). The method was initially tested on liver tissues of BALB/c mice treated with 4-ABP, and then applied to the detection of adducts in oral mucosa and exfoliated urothelial cells of smokers and non-smokers. Levels of 4-ABP–DNA in exfoliated urothelial cells were elevated in each of the 20 smokers (mean relative staining intensity measured as optical density multiplied by 1000, $517 \pm 137$) compared with age-, race- and sex-matched non-smokers ($313 \pm 79$). Significantly higher damage levels were also observed in the oral mucosa cells of smokers compared with non-smokers ($552 \pm 157$ versus $326 \pm 101$). A similar two- to three-fold range in relative staining was found in smokers and non-smokers for 4-ABP–DNA, suggesting the importance of individual differences in the capacity to metabolize carcinogens and/or to repair damaged DNA.

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### *DNA adducts of 4-ABP in the respiratory system*

In surgical samples of peripheral lung tissue from smokers and ex-smokers, Culp *et al*. (1997) utilized a sensitive immunochemical assay, in combination with $^{32}$P-postlabelling, to quantify the major 4-ABP–DNA adduct dG–C8-4-ABP. No differences in adduct levels were detected between 14 smokers and 11 ex-smokers by immunoassay. In contrast, the $^{32}$P-postlabelling method showed statistically significant differences between adduct levels in smokers and ex-smokers. However, a relatively high background of smoking-related adducts chromatograph near the major ABP adducts, which may compromise estimation of the level of dG–C8-4-ABP adducts in smokers. The levels measured by $^{32}$P-postlabelling were 20- to 60-fold lower than those measured by immunoassay. Since $^{32}$P-postlabelling may underestimate and immunochemical assays may overestimate adduct levels in the lung, two samples were also evaluated by GC-MS. The immunochemical and GC-MS data were concordant. This is surprising since in an earlier study a good

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correlation between GC-MS and $^{32}$P-postlabelling was shown in lung and bladder tissue (Lin *et al.* 1994). Since 4-ABP–DNA adduct levels in human lung did not correlate with smoking status as measured by immunoassay and GC-MS, the metabolic activation capacity of human lung microsomes and cytosols was examined to determine whether another exposure (e.g. 4-NBP) might be responsible for the adduct. Enzymatic studies with the lung tissue indicate that 4-ABP–DNA adducts in human lung can result from environmental exposure to 4-NBP.

Romano *et al.* (1997) examined 4-ABP–DNA adducts in the oral cells of 12 smokers and non-smokers using an immunohistochemical assay (immunoperoxidase method). Higher staining for 4-ABP–DNA was detected in the cells of smokers (optical density multiplied by 1000, $187 \pm 42$) versus non-smokers ($135 \pm 35$) ($p = 0.004$), with a two-fold range in relative staining for both groups, suggesting the relevance of individual differences in metabolizing carcinogens and/or repairing DNA damage. Besarati *et al.* (2000) studied the formation of 4-ABP–DNA adducts in induced sputum, a non-invasively obtainable matrix from the lower respiratory tract, of smokers ($n = 20$) and non-smokers ($n = 24$) utilizing a semi-quantitative immunohistochemical (immunoperoxidase) method. Smokers had significantly higher levels of 4-ABP–DNA adducts compared with non-smokers (optical density of immunohistochemical staining multiplied by 1000; $80 \pm 20$ versus $40 \pm 10$, $p = 0.001$), and the levels of adducts were related to current smoking indices (cigarettes per day: $r = 0.3$, $p = 0.04$).

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### *DNA adducts in lymphocytes*

In animal experiments, a linear relationship between DNA adducts in lymphocytes and DNA of the target organ has only been shown for MOCA. For a small number of benzidine workers a positive correlation between exfoliated urothelial bladder cells and lymphocyte DNA was demonstrated (see below). Smoking-related DNA adducts were measured in lymphocytes. In all publications (Dallinga *et al.* 1998, Peluso *et al.* 1998, 2000) the aromatic DNA adducts (polyaromatic hydrocarbon [PAH] adducts) found after nuclease P1 treatment of the DNA hydrolysates were used for the correlations with the other biomarkers. Enrichment of the DNA hydrolysate with butanol extraction containing the arylamine adducts and the PAH adducts was performed by Peluso *et al.* (1998, 2000). However, the specific dG-C8–4-ABP adduct was not quantified. Aromatic DNA adducts were measured in 162 bladder cancer patients and 106 hospital controls (Peluso *et al.* 1998, 2000). DNA adduct levels did not correlate with NAT2 and GSTM1. Dallinga *et al.* (1998) analysed 4-ABP–Hb adducts and aromatic DNA adducts in the lymphocytes of 55 smokers and four non-smokers. 4-ABP–Hb and aromatic DNA adducts correlated very poorly ($r = 0.26$, $n = 57$, $p = 0.04$).

### *DNA adducts of benzidine in workers*

Several epidemiological studies indicate that NAT2-related slow *N*-acetylation increases bladder cancer risk among workers exposed to arylamines, presumably because *N*-acetylation is important for the detoxification of these compounds (Cartwright *et al.* 1982). Previously, Hayes *et al.* (1993) showed that NAT2 polymorphisms did not influence bladder cancer risk among Chinese workers

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exposed exclusively to benzidine, suggesting that NAT2 *N*-acetylation is not a critical detoxifying pathway for this aromatic amine. In addition, Zenser *et al.* (1996) showed using human recombinant NAT1 and NAT2 and human liver slices that benzidine and AcBz are better substrates for NAT1 than for NAT2. Of the polymorphic NAT1 forms, a higher average acetylation ratio was observed in human liver slices possessing the NAT1*10 compared with the NAT1*4 allele. To evaluate the biological plausibility of these finding, Rothman (Rothman *et al.* 1996a, b, 1997, DeMarini *et al.* 1997, Zhou *et al.* 1997) carried out a cross-sectional study of 33 workers exposed to benzidine and benzidine dyes and 15 unexposed controls in Ahmedabad, India. The presence of benzidine-related DNA adducts in exfoliated urothelial cells and lymphocytes, the excretion pattern of benzidine metabolites, and the impact of NAT2 and NAT1 activity on these outcomes were investigated (Rothman *et al.* 1996a). Four DNA adducts were significantly elevated in exposed workers compared with controls; of these, the predominant adduct co-chromatographed with a synthetic *N*-(3′-phosphodeoxyguanosin-8-yl)-*N*′-acetylbenzidine (3′-P-dG-AcBz) standard and was the only adduct that was significantly associated with total benzidine urinary metabolites ($r = 0.68$, $p < 0.0001$). The predominant adduct formed was *N*-acetylated, supporting the concept that monofunctional acetylation is an activation, rather than a detoxification, step for benzidine. Thus, it is unlikely that interindividual variation in NAT2 function is relevant for benzidine-associated bladder carcinogenesis, because almost all benzidine-related metabolites measured in the urine of exposed workers were acetylated in both slow and rapid acetylators ($95 \pm 1.9\%$ versus $97 \pm 1.6\%$, respectively), and the NAT2 or NAT1 (NAT1*10 allele) activity did not affect the levels of any DNA adduct measured.

Rothman *et al.* (1996b) investigated whether people with the GSTM1 null genotype, who lack functional GSTM1, had higher levels of the dG-AcBz adduct in the exfoliated bladder epithelial cells. However, the GSTM1 genotype had no impact on urothelial cell DNA adduct and urinary mutagenicity levels in workers currently exposed to benzidine. Furthermore, *in vitro* experiments showed that human GSTM1 did not conjugate benzidine or its metabolites. This is in agreement with a case control study of bladder cancer among workers previously exposed to benzidine in China, which showed no overall increase in bladder cancer risk for the GSTM1 null genotype among 38 bladder cancer cases and 43 controls.

In the same group of workers, Rothman *et al.* (1997) studied the influence of urine pH on the proportion of urinary benzidine and AcBz present in the free, unconjugated state, and on exfoliated urothelial cell DNA adduct levels in 32 workers. Post-shift urine pH was inversely correlated with the proportions of benzidine ($r = -0.78$; $p < 0.0001$) and AcBz ($r = 0.67$; $p < 0.0001$) present as free compounds. Furthermore, the average of each subject's pre-and post-shift urine pH was negatively associated with the predominant urothelial DNA adduct ($p = 0.0037$, adjusted for urinary benzidine and metabolites), which has been shown to co-chromatograph with a 3′-P,5′-P-dG-AcBz adduct standard. Individuals with urine pH $< 6$ had 10-fold higher DNA adduct levels compared with subjects with urine pH $\geq 7$.

The relationship between urinary mutagenicity and the biomarkers described above were studied in 15 workers exposed to benzidine (high exposure), 15 workers exposed to benzidine dyes (low exposure) and 13 unexposed controls (DeMarini *et al.* 1997). Urinary organics were extracted by C18 reversed phase

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cartridges with methanol and evaluated for mutagenicity in the presence of S9 in the *Salmonella* strain YG1024, which is a frameshift strain that overproduces acetyltransferase. The results were compared with those for other biomarkers from the same urine samples (the sum of the urinary levels of benzidine + AcBz + DiAcBz) and a DNA adduct biomarker (3′-P-dG-C8-AcBz in exfoliated urothelial cells). The mean urinary mutagenicity (revertants per μmol creatinine) of the low exposure (benzidine dye) workers was $8.2 \pm 2.4$, which was significantly different from the mean of the controls ($2.8 \pm 10.7$, $p = 0.04$), as was the mean of the high exposure (benzidine) workers ($123.2 \pm 26.1$, $p < 0.0001$). Urinary mutagenicity showed strong, positive correlations with urinary metabolites ($r = 0.88$, $p < 0.0001$) and the level of the presumptive dG-C8-AcBz urothelial DNA adduct ($r = 0.59$, $p = 0.0006$).

In the same group of workers, Zhou *et al*. (1997) evaluated DNA adduct levels in peripheral white blood cells (WBCs) of a subset of 18 exposed workers and seven controls. DNA was analysed using $^{32}$P-postlabelling, along with *n*-butanol extraction. One adduct, which co-chromatographed with a synthetic 3′-P-dG-C8-AcBz standard, predominated in those samples with adducts present. The median level of this adduct in WBC DNA was $194.4 \times 10^{-9}$ (range $3.2–975 \times 10^{9}$) in exposed workers and $1.4 \times 10^{-9}$ (range 0.1–6. $4 \times 10^{-9}$) in the control subjects ($p = 0.0002$, Wilcoxon rank sum test). There was a striking correlation between WBC and exfoliated urothelial cell adduct levels (Pearson $r = 0.84$, $p < 0.001$) among exposed subjects. In addition, the sum of urinary benzidine, AcBz and DiAcBz correlated with the levels of this adduct in both tissues. This is the first study in humans to show a relationship for a specific carcinogen adduct in a surrogate tissue and in urothelial cells, the target for urinary bladder cancer.

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### *DNA adducts versus variation of metabolism by genotype or chemopreventive agents*

It has been shown in animal experiments that the formation of DNA and protein adducts by environmental pollutants is modulated by host polymorphisms in genes that encode metabolizing enzymes. Recently DNA adducts of 4-ABP in pancreas were studied, since epidemiological studies have suggested that arylamines (and nitroarenes) may be carcinogenic for human pancreas (Anderson *et al*. 1997, Thompson *et al*. 1999). Pancreatic tissues from 29 organ donors (13 smokers, 16 non-smokers) were examined for the presence of 4-ABP–DNA adducts. In eight of the 29 DNA samples, a major adduct was observed that was chromatographically identical to the predominant 4-ABP–DNA adduct dG-C8-4-ABP. The presence of 4-ABP–DNA adducts was strongly associated with the putative slow NAT1*4/*4 genotype, suggesting a role for this pathway in 4-ABP detoxification (Thompson *et al*. 1999). The rational for the formation of 4-ABP–DNA adducts in the pancreas was studied in pancreatic microsomes and cytosol (Anderson *et al*. 1997).

In eight smokers, Malaveille *et al*. (1998) studied the effect of chemopreventive effects of food components on the levels of dG-4-ABP in exfoliated urothelial cells and on the antimutagenic properties of 24 h urine. 4-ABP–DNA adducts was measured by $^{32}$P-postlabelling, and mutagenicity was measured in liquid incubation assays using 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as a representative mutagen for tobacco smoke exposure. The level of dG-C8-4-ABP in urothelial epithelial cells was inversely related to bacterial antimutagenicity

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($r = -0.81$, $p = 0.01$). The number of investigated subjects was very small. Further studies are needed to study the effects of chemopreventive agents on the formation of DNA and Hb adducts.

### *DNA adducts and biological effects*

Studies in mammalian and bacterial mutagenicity assays have shown that the dG-C8 adducts formed from the different arylamines are not equally efficient mutagens. The number of mutations induced per adduct as well as the type of mutation (base pair substitution versus deletion) can vary depending on the structure of the arylamine (Beland *et al*. 1983, Melchior *et al*. 1994, Delclos and Kadlubar 1997). The role in mutagenicity and carcinogenicity of minor adducts such as dG-N2 or dA-C8 is presently unknown. The minor adduct of 4-ABP with dA, dA-C8-4-ABP, has been found to be strongly mutagenic (Lasko *et al*. 1988). In mice administered chronic doses of AAF and 4-ABP, the relationship between DNA adduct formation and urinary bladder tumour induction has been studied (Poirier and Beland 1992). Tumour response in the liver correlated directly with DNA adduct formation. In contrast, in the bladder DNA adducts appeared to be necessary for tumour induction. However, tumours developed only at doses where bladder epithelial cell proliferation was induced.

Currently great efforts are being made to establish detailed mutational spectra for chemical carcinogens, especially in oncogenes (*ras* family) or tumour suppressor genes such as *p53* (Sorlie *et al*. 1998, Martone *et al*. 1998). Sorlie *et al*. (1998) analysed the pattern of point mutations from several tumour genes in 21 cases of bladder cancer in western European workers exposed to arylamines. Of the four genes analysed (*p53*, *p16(MTS1)*, *p21(WAF1)* and *H-ras*), only *p53* showed a high frequency of mutations (in eight out of 21 cases; 38%). Two mutations were found in *p16*, one in *H-ras* and none in *p21* exon 3. All mutations were at G:C base pairs, mostly at non-CpG residues. This spectrum of mutations is, however, identical to the spectrum of *p53* mutations detected in bladder cancers in the general population. In exposed workers, *p53* mutations were associated with tumour grade and with high occupational and tobacco exposure. These data suggest that the same carcinogens may be responsible for the development of bladder cancers in workers exposed to arylamines and in the general population.

Martone *et al*. (1998) analysed the biopsies of 45 patients with bladder cancer. Mutations in the *p53* gene were sought by direct sequencing, and 4-ABP–DNA adducts were measured by GC-MS and NCI. 4-ABP–DNA adducts were higher in smokers of air-cured tobacco and in current smokers, but had no relationship with the number of cigarettes smoked, and no association was found between 4-ABP adducts and GSTM1 or NAT2 genetic polymorphisms. Adducts were higher in more advanced histological grades of tumours. No pattern was evident for *p53* mutations.

The overexpression of *p53*, mutation in *p53*, cigarette smoking, DNA adducts of 4-ABP and recurrence of the disease were studied in Italian bladder cancer patients (Curigliano *et al*. 1996, Romano *et al*. 1999). The outcome of these two studies is contradictory, although the same methods were used. An immunoperoxidase method using a monoclonal antibody that recognizes 4-ABP–DNA adducts was applied to stored paraffin blocks of transurethral resection specimens of 46 patients with T1 bladder cancer (Curigliano *et al*. 1996). The mean relative

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staining intensity for 4-ABP–DNA adducts was significantly higher in current smokers (optical density multiplied by 1000, 275 ± 81, $n = 24$) compared with non-smokers (113 ± 71, $n = 22$) ($p < 0.0001$). There was a linear relationship between the mean levels of relative staining and the number of cigarettes smoked, with lower levels in the 1–19 cigarettes per day group (205 ± 30, $n = 5$) compared with the 20–40 (289 ± 40, $n = 7$) and the >40 cigarettes per day (351 ± 57, $n = 3$)($p < 0.001$) groups. Nuclear overexpression of *p53*, analysed by immunoperoxidase staining, was observed in 27 (59%) of the 45 stage T1 tumours analysed. There was a significant correlation between *p53* overexpression and recurrence of disease. Nuclear staining of *p53* was also correlated with smoking status, cigarettes smoked per day and 4-ABP–DNA adduct levels. Romano *et al.* (1999) investigated a larger number of bladder cancer patients (106) than in the former study. 4-ABP–DNA adducts was compared with *p53* overexpression and mutations. 4-ABP–DNA adduct levels were higher in smokers compared with non-smokers, with a borderline statistical value. This is in contrast to the former study (Curigliano *et al.* 1996), where significantly higher levels were found in smokers. There was no significant correlation between *p53* nuclear overexpression and 4-ABP–DNA adduct levels, smoking habit or disease recurrence. Point mutations in *p53* were found in 17 of the 106 cases (16%) and the mutational pattern was significantly associated both with higher grade and stage, but no correlation was found with disease recurrence. These contradictory results might be a result of cross-reactivities in the determination of 4-ABP–DNA adducts. In addition, it appears that *p53* expression/mutation might not be a prognostic factor in bladder cancer.

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### *DNA adduct levels of 4-ABP and risk assessment*

The levels of 4-ABP–DNA adducts obtained in animal carcinogenicity experiments were compared with the levels of 4-ABP adducts found in bladder cancer cases (Poirier and Beland 1992, Gaylor *et al.* 1992, Talaska *et al.* 1994). In mice treated with 4-ABP, the concentration of dG-C8-4ABP adducts associated with 50% tumour incidence was 150 pmol $mg^{-1}$ DNA, while the level in the human bladder has been estimated to be 0.81 pmol $mg^{-1}$ DNA, calculated from a smoking-related risk of 2.16% and a smoking-related adduct level of 0.036 pmol $mg^{-1}$ DNA (Poirier and Beland 1992). The daily dose in a smoker (20–40 cigarettes) has been estimated to be 0.64–1.28 ng of 4-ABP $kg^{-1}$ body weight. The observed level of smoking-related dG-C8-4-ABP in the DNA of human bladder epithelium, expressed as a function of daily 4-ABP intake (28–56 pmol dG-C8-4-ABP per mg DNA per μg of 4-ABP per kg body weight), is about 3500 times higher than similar data for mice, which suggests that humans may perform the biotransformation of 4-ABP more efficiently than mice. At a similar bladder tumour incidence, mouse bladder contained adduct concentrations that were much higher than those observed in human bladder. For example, at a 2.6% tumour incidence, mouse bladder contained an average of 55.5 pmol dG-C8-ABP $mg^{-1}$ DNA, while bladders from Caucasian male smokers contained an average of 0.036 pmol dG-C8-4-ABP $mg^{-1}$ DNA (1.2 adducts per $10^8$ nucleotides). The authors concluded that factors other than 4-ABP–DNA adducts, such as adducts of other carcinogens, the influence of promoters and the synergistic effects of all of these factors contribute substantially to smoking-related bladder cancer in humans (Poirier and Beland 1992).

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## Conclusions

Biomonitoring methods have been developed in animal experiments to measure urine metabolites, Hb adducts and DNA adducts of several nitroarenes and arylamines. The basic tools to perform molecular epidemiological studies are available for several compounds. Several studies have been were performed in humans, especially for 4-ABP. Protein adducts and DNA adducts were found. These have been shown to be potential marker of exposure, of metabolism and of dosimetry for the target dose. Correlations with early biological effects and disease have only been demonstrated in a few cases. For other arylamines, only small numbers of human subjects, mainly occupational exposures, have been analysed. Generally in occupational exposures the differences between exposed and unexposed subjects were clear and significant. In environmentally exposed collectives the levels were much lower and the differences between exposed subjects and controls were closer or in the range of measurement error. Although statistically there was a significant difference between the groups in such cases, it is important to analyse the same samples in different laboratories or using different methods. The work of Culp *et al.* 1997 was very important in this regard, since DNA adducts were determined with three different methods, resulting in different outcomes. In environmentally exposed humans the measurements are performed close to the limit of detection. It is a difficult task to maintain the quality of the data throughout the whole study. This might be a problem in laboratories without accreditation such as many academic institutions. Good guidelines for the quality control of molecular epidemiological studies has been published recently (Vineis 1997, Albertini *et al.* 2000), in particular for the determination of DNA (Philipps and Castagnaro 1999). Interlaboratory comparison of methods used for epidemiological studies is needed for the study of populations where small biomarker differences are expected. Therefore, it is very important that for all studies the methods are presented in great detail, in order to be reproducible. In the future methods should be fully automated in order to perform analyses of larger collectives and to reduce variations in the procedures.

For arylamines, 4-ABP has been the compound of major interest, mainly in relation to tobacco smoke. Unfortunately the interest of scientists involved for years in the study of arylamines and nitroarenes has now shifted to the investigation of the heterocylic arylamines. Thus, in the future not many new molecular epidemiological studies are expected for the compounds presented in this review, although the general population is exposed to many of these important industrial chemicals. There was a dramatic progress in the last 20 years of research in molecular epidemiology (Bartsch 2000), and hopefully some of the compounds reviewed here will be included in future studies.

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