

## Urothelial Cell DNA Adducts in Rubber Workers

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Workers employed in the rubber industry appear to have a significant excess cancer risk in a variety of sites, including cancer of the urinary bladder. In this cross-sectional study, we investigated the occurrence of DNA adducts in exfoliated bladder cells of currently exposed, nonsmoking rubber workers ( $n = 52$ ) and their relationship with occupational exposure estimates and acetylation phenotype (NAT2). Four DNA adducts were identified, with the proportion of positive samples (e.g., DNA samples with quantifiable levels of a specific DNA adduct) ranging from 3.8 to 79%. The highest proportion of positive samples and the highest relative adduct labeling levels were in workers involved in the production functions "mixing" and "curing," areas with potential for substantial exposure to a wide range of chemical compounds used

in rubber manufacturing ( $P < 0.05$  for adducts 2 and/or 3, compared to all other departments). No statistically significant relationships were found between identified DNA adducts and urinary mutagenicity or personal inhalable and dermal exposure estimates. Interestingly, subjects with a fast NAT2 acetylation phenotype tended to have higher levels of DNA adducts. This study suggests that rubber workers engaged in mixing and curing may be exposed to compounds that can form DNA adducts in urothelial cells. Larger studies among rubber workers should be conducted to study in more detail the potential carcinogenicity of exposures encountered in these work areas. *Environ. Mol. Mutagen.* 39:306–313, 2002. Published 2002 Wiley-Liss, Inc.†

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### INTRODUCTION

Epidemiological studies of workers employed in the rubber industry have indicated a significant excess cancer risk in a variety of sites (e.g., lung and urinary bladder cancer and leukemia) [IARC, 1982; Kogevinas et al., 1998]. Urinary bladder cancer in this industry has been attributed traditionally to exposure to antioxidants such as Nonox "S" that contained free  $\beta$ -naphthylamine as a contaminant [Case and Hosker, 1954]. The use of this antioxidant and similar compounds contaminated with  $\beta$ -naphthylamine was subsequently discontinued in the 1950s. However, several recent epidemiological studies have found a moderate excess risk of urinary bladder cancer among workers with supposedly no concomitant exposure to  $\beta$ -naphthylamine [Straif et al., 1998; Straughan and Sorahan, 2000]. Straif et al. [1998] reported a significant excess of bladder cancer (SMR 214, 95% CI 107–384) among workers hired after the phasing out of  $\beta$ -naphthylamine, whereas Straughan and Sorahan [2000] reported a nonsignificant excess in incident bladder cancer cases (SRR 207, 95% CI 76–450). Further evidence of an excess risk of bladder cancer among rubber workers

can be found in a Canadian proportionate mortality study [Choi and Nethercott, 1991]. Among workers that never used  $\beta$ -naphthylamine, benzidine, 4-aminobiphenyl, or 4-nitrobiphenyl, an excess of bladder cancer was found (SPMR 128). These observations placed in doubt the exclusive link between  $\beta$ -naphthylamine exposure and urinary bladder cancer in the rubber industry. Interestingly, an excess of urinary bladder cancer has also been reported in two cohort studies of workers manufacturing chemicals for the rubber industry; these workers were not exposed to either

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benzidine or  $\beta$ -naphthylamine [Ward et al., 1996; Sorahan et al., 2000]. In addition, exposure to carbon black, used in large quantities in the rubber industry, has been linked to bladder cancer as well [Hodgson and Jones, 1985; Puntoni et al., 2001].

Workers in the rubber industry are exposed to a wide range of potentially carcinogenic compounds [IARC, 1982]. The chemical composition and toxic properties of the complex exposure mixture is, to a large extent, unknown. Chemically nonspecific exposure- and biomarkers are often applied in these exposure conditions [Perera et al., 1990]. Examples of such markers are the *S. typhimurium* mutagenicity assay and the  $^{32}\text{P}$ -postlabeling method for bulky carcinogen-DNA adducts. In several studies in the rubber industry, exposure to mutagenic compounds was established with the *S. typhimurium* mutagenicity assay either by measuring the mutagenicity of airborne particulates and fumes or by measuring the mutagenicity in the urine of workers [Falck et al., 1980; Sorsa et al., 1982; Baranski et al., 1989; Bos et al., 1989; Fracasso et al., 1999; Vermeulen et al., 2000a]. Although the  $^{32}\text{P}$ -postlabeling method has been used in a large number of studies on occupational exposures to detect carcinogen-DNA adducts [Beach and Gupta, 1992], it has seldom been applied in the rubber industry. In the study of Schoket et al. [1999] aromatic DNA adducts in peripheral blood lymphocytes of workers in a vulcanizing plant were quantified; however, DNA adduct levels did not differ from those of the control subjects. DNA adducts in exfoliated bladder cells are, however, biological indicators that reflect exposure to carcinogens at the target organ, the level of binding DNA, and the individual differences in carcinogen biotransformation, cell turnover, and DNA repair rates [Perera et al., 1990]. Identification of DNA adducts in exfoliated bladder cells could therefore be a useful indicator to elucidate the mechanism of action of the complex exposure mixture and to identify the potential health hazard associated with exposures in this particular industry.

In the present study, we evaluated the relationships among inhalable and dermal exposure, ambient mutagenic exposure levels, urinary mutagenicity, and urothelial cell DNA adduct levels in a population of rubber workers selected from a large cross-industry survey in The Netherlands. In addition, the influence of the NAT2 biotransformation phenotype on DNA adduct levels was investigated.

## MATERIALS AND METHODS

### Cross-Sectional Study

The field phase of this study was conducted from January 1997 through July 1997 among male subjects working in the rubber industry in The Netherlands [Vermeulen et al., 2001]. Subjects ( $n = 224$ ) were employed in nine companies (three rubber tire, five general rubber goods, and one retreading company) and were randomly selected based on their production function (e.g., compounding and mixing, pretreating, molding, curing,

finishing, shipping, engineering service, and laboratory). Fieldwork was carried out within 1 week per company and comprised a medical survey, ambient and personal exposure measurements, and collection of biological samples. Informed consent was obtained after the study was explained to the subjects.

After completion of the field phase of the survey and preliminary analysis, each subject was grouped into one of two exposure categories for dermal exposure based on the median levels of mutagenic activity detected on likely skin contact surfaces [high ( $\geq 25$  rev/cm<sup>2</sup>), low ( $< 25$  rev/cm<sup>2</sup>)] and into two airborne mutagenic exposure categories [high ( $\geq 210$  rev/m<sup>3</sup>), low ( $< 210$  rev/m<sup>3</sup>)], resulting in four a priori exposure groups. The resulting exposure groups can be characterized as follows:

1. Low total suspended particulate matter (TSPM) mutagenicity and low surface mutagenic exposure levels (LL)
2. High TSPM mutagenicity and low surface mutagenic exposure levels (HL)
3. Low TSPM mutagenicity and high surface mutagenic exposure levels (LH)
4. High TSPM mutagenicity and high surface mutagenic exposure levels (HH).

No subjects matched the a priori categorization of low TSPM mutagenicity and high surface mutagenic exposure levels (LH), leaving three exposure groups (LL, HL, and HH). Twenty nonsmokers were randomly selected from each of these three remaining exposure groups, resulting in a study population of 60 subjects, of which 56 subjects (93.3%) had retrievable 24-hr urine samples.

Subjects were administered a questionnaire as part of the initial survey to collect information regarding age, medical history, and alcohol and roasted meat consumption. Environmental tobacco smoke (ETS) intake was evaluated by assessing urinary cotinine levels by high performance liquid chromatography according to the method of Barlow et al. [1987], using the modifications described by Parviainen and Barlow [1988]. Cotinine levels were expressed as  $\mu\text{g/g}$  creatinine.

### Occupational Exposure Measures

Ambient exposure measurements were collected as described previously [Vermeulen et al., 2000a]. Briefly, TSPM exposure was measured on random selected days (Tuesday–Thursday) using a high-volume sampler [ter Kuile, 1984]. On average, 1.8 (range 1 to 3) samples were collected per department within a company; samples within a department were subsequently pooled for mutagenicity analysis. Surface contamination was estimated by obtaining wipe samples of likely contact surfaces. Samples were taken by a modification of the OSHA wipe-sampling procedure [Robbins, 1979]. Personal inhalable particulate exposure was measured on three consecutive days (Tuesday, Wednesday, and Thursday) by means of a PAS6 sampling head mounted near the breathing zone of the worker [Kenny et al., 1997]. Personal dermal exposure to cyclohexane soluble matter (CSM) was measured with a dermal pad sampler worn at the lower part of the wrist of the hand of preference on the same days as the personal inhalable exposure measurements were taken [Durham and Wolff, 1962; Kromhout et al., 1994].

CSM contents of inhalable and dermal exposure samples were determined by means of the NIOSH PCAM 217 method [NIOSH, 1977]. In short, samples were placed in cyclohexane (Merck, Darmstadt, Germany) and sonicated for 20 min. The suspension was filtered through a glass intertube G4 (Schott, Germany) and collected in a preweighed 10-ml vial. After evaporation of cyclohexane under nitrogen and subsequent 2-hr drying at 40°C, the residue was weighed by means of a microbalance. No chemical analysis of the extract was performed.

## Mutagenicity Assays

TSPM and surface contamination samples were extracted consecutively with cyclohexane, dichloromethane, and methanol (all from Merck) as described previously [Vermeulen et al., 2000a]. After evaporation of organic solvents under nitrogen at 40°C, the residue was dissolved in 2.5 ml of dimethylsulfoxide (DMSO; Merck). Spot urine samples were collected on Wednesday and Thursday after work had ended. Volumes, corresponding to 0.5 mmol of creatinine of the Wednesday (10–510 ml) and Thursday (35–610 ml) urine sample were pooled for each subject before mutagenicity analysis. Pooled urine samples, corresponding to a volume of 1 mmol of creatinine (70–1120 ml), were neutralized to pH 7.0, extracted with XAD-2 resin (6-cm<sup>3</sup> bed volume), and eluted with 10 ml of acetone. After evaporation under nitrogen at 40°C, the residue was dissolved in 2.5 ml of DMSO [Bos et al., 1989].

TSPM, surface contamination, and urine extracts were assayed for mutagenic activity with *S. typhimurium* strain YG1041 using an S9-mix containing aroclor 1254–induced rat liver homogenate [Maron and Ames, 1983; Hagiwara et al., 1993] [mutagenicity of references: spontaneous, 144 ± 19 rev/plate; positive control (2-aminopyrene, 0.1 µg/plate), 2584 ± 232 rev/plate]. DMSO extracts were assayed at five different dose levels in triplicate. For determination of the mutagenic activity, the mean number of revertants was calculated for each dose level and subsequently a dose–response curve was constructed. The slope of the linear component was used as an estimate of mutagenic potency [Krewski et al., 1992]. Mutagenicity of TSPM and surface contamination was expressed as revertants/m<sup>3</sup> and as revertants/cm<sup>2</sup>, respectively. Urinary mutagenic activity was expressed as revertants/g creatinine.

## Urothelial Cell DNA Adduct Analysis

Twenty-four-hour urine samples were collected from Monday (after first morning void) to Tuesday (including first morning void). Samples were kept cold (±4°C) during collection and made 20% in glycerol (v/v) to minimize cell loss resulting from lysis after freezing. Consequently, samples were stored at –30°C until analysis.

Frozen samples were thawed and well mixed. A 500-ml aliquot of the urine samples was filtered through sequential 500- and 250-µm sieves, after which exfoliated urothelial cells were isolated by vacuum filtration using 5-µm nylon filters (Osmonics, Minnetonka, MN). Cells were washed from the filters with a phosphate-buffered saline solution (PBS, pH 7.4) and washed several times by resuspension in the same buffer followed by centrifugation at 800 rpm for 15 min in a refrigerated centrifuge. DNA was isolated from the cell pellets by a solvent-extraction technique and quantitated by normal nucleotide analysis with the <sup>32</sup>P-postlabeling method as described by Talaska et al. [1990]. <sup>32</sup>P-Postlabeling of the exfoliated urothelial DNA samples was performed under conditions of radiolabeled adenosine 5'-triphosphate (ATP) excess [Talaska et al., 1987]. The necessary excess of ATP was provided by the addition of 150 µCi to each sample. Carcinogen-DNA adduct levels were calculated by determining the relative adduct labeling (RAL), which was defined as: counts per minute (cpm) in adduct spots/cpm in normal nucleotides. In the absence of a particular adduct spot in the individual map, the DNA adduct level was designated zero RAL level.

<sup>32</sup>P-Postlabeling analyses were performed with blind-coded samples and each sample was analyzed in duplicate, at least. Samples that had insufficient amounts of DNA (arbitrary cutoff point, 20 × 10<sup>6</sup> nucleotides) were reanalyzed by increasing the amount of DNA sample to be tested or by reisolation of larger volumes (>500 ml) of the same 24-hr urine sample (n = 19).

## Acetylation Status

An aliquot of the Wednesday postshift urine sample was taken and analyzed for caffeine and its metabolites, according to the procedure

described by Grant et al. [1984]. Designation of the acetylator (NAT2) phenotype was based on the ratio between 5-acetylamino-6-formylamino-3-methyluracil (AFMU) and 1-methylxanthine (1×). Results showed a clear bimodality of N-acetylation capacity with a ratio cutoff point for fast- vs. slow-acetylation status of 1.7.

## Calculations and Statistical Analysis

Surface mutagenic contamination was calculated by averaging the mutagenic activity levels of relevant contact surfaces for each subject. In these calculations, surface wipe samples were weighed equally regardless of the frequency and duration of contact with the particular surface. Mean ambient TSPM mutagenicity was calculated based on the pooled TSPM samples according to the work areas in which the subject was employed. Cotinine-corrected pooled weekday urinary mutagenicity levels were calculated based on the algorithms found for the relation between urinary cotinine and urinary mutagenicity levels described previously [Vermeulen et al., 2000b].

Mean personal inhalable particulate, inhalable CSM, and dermal CSM exposure were estimated using the James–Stein estimator ( $B_g$ ) to maximize accuracy and precision of the exposure estimates [James and Stein, 1961; Efron and Morris, 1972; Seixas and Sheppard, 1996]. In this computation, a weighted mean of the individual and group mean exposure is calculated, based on a weighting factor derived from the between- and within-worker exposure variability. This weighting factor determines the optimal combination of the group and individual means by production function.

RAL levels were not normally distributed. Therefore, two-tailed non-parametric tests were used throughout the study. Spearman correlation coefficients were used to investigate the relationship between identified DNA adducts and several exposure indices. Differences in DNA adduct levels between exposure groups were tested with the Wilcoxon rank-sum test. Trends in the proportion of positive samples by phenotype or production function were investigated using the Cochran–Armitage test for trend. Because of the small number of positive DNA adduct samples, exact *P*-values were calculated [Rothman and Greenland, 1998]. All statistical analyses were performed using SAS version 12 software [SAS Institute, 1990].

## RESULTS

Sufficient DNA ( $\geq 20 \times 10^6$  nucleotides) for <sup>32</sup>P-postlabeling analysis was obtained from 52 of 56 available 24-hr urine samples (93%). There was a wide range in the quantity of radiolabeled nucleotides (20–256 × 10<sup>6</sup>), as would be expected, given that the amount of DNA in each sample was not known before the analysis. An excess of radiolabel was seen in the normal nucleotide maps for each sample, indicating that sufficient [<sup>32</sup>P]ATP was available to label all nucleotides in the samples.

During the analyses, 11 different possible DNA adducts were observed (numbered in temporal order of detection). Adducts 5, 7, and 8 were later identified as unadducted deoxyguanosine nucleotides. Adducts 4 (number of positive samples = 1), 6 (n = 2), 9 (n = 5), and 10 (n = 2) were detected in samples that had DNA amounts lower than the a priori minimum level of 20 × 10<sup>6</sup> normal nucleotides. Repeated analysis of the same samples, however, with increased DNA concentrations did not consistently demonstrate the previously mentioned adducts and these DNA adducts were therefore not included in the analyses (data not

**TABLE I. Spearman Correlation Coefficients (and *P*-Values) Between Identified DNA Adducts and Mean Relative Adduct Labeling (RAL × 10<sup>7</sup>) Among Rubber Workers (n = 52)**

Adduct number	Adduct number			
	1	2	3	11
2	0.23 (0.11)			
3	0.52 (0.0001) <sup>a</sup>	0.25 (0.08)		
11	NC <sup>b</sup>	NC	NC	
N <sup>c</sup>	41	13	29	2
Mean (SD) <sup>d</sup>	2.85 (2.99)	5.77 (5.17)	3.68 (3.42)	12.55 (5.30)

<sup>a</sup>Correlation coefficient significant at the 0.05 level.

<sup>b</sup>Not calculated due to the small number of subjects with DNA adduct 11 (n = 2).

<sup>c</sup>Number of subjects with a particular DNA adduct.

<sup>d</sup>Mean RAL and SD of the positive DNA samples for a particular DNA adduct.

**TABLE II. Median and Mean Relative Adduct Labeling Levels (RAL × 10<sup>7</sup>)<sup>a</sup> and Proportion of Positive Samples Stratified by the a priori Defined Exposure Groups**

Exposure group <sup>b</sup>	N	Adduct 1 (n = 41)			Adduct 2 (n = 13)			Adduct 3 (n = 29)			Adduct 11 (n = 2)		
		Median	Mean	% <sup>c</sup>									
LL	18	1.15	2.58	83.3	0.00	0.26	22.2	0.40	2.15	55.6	0.00	0.49	5.6
HL	18	1.38	1.32	72.2	0.00	0.74	27.8	0.08	1.42	50.0	—	—	0.0
HH	16	1.98	2.19	81.3	0.00	2.49	25.0	1.15	1.57	62.5	0.00	1.02	6.3

<sup>a</sup>In the absence of a particular adduct spot in the individual map, the DNA adduct level was designated zero RAL level.

<sup>b</sup>A priori defined exposure groups: [LL], low TSPM mutagenicity and low surface mutagenic exposure levels; [HL], high TSPM mutagenicity and low surface mutagenic exposure levels; [HH], high TSPM mutagenicity and high surface mutagenic exposure levels.

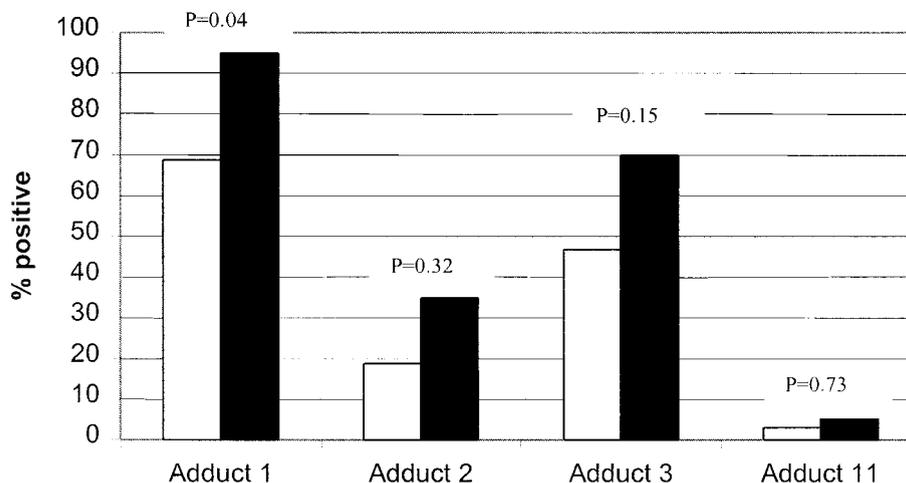
<sup>c</sup>Percentage of positive samples of DNA from exfoliated bladder cells.

shown). For four DNA adducts reliable results were found. Of the 52 samples, 46 (88%) had a consistently positive or negative result in duplicate test runs and 6 samples had a consistent result for three out of four runs. In Table I, Spearman correlation coefficients and mean RAL levels (RAL × 10<sup>7</sup>) for these four adducts (1, 2, 3, and 11) are shown.

In Table II, the median and mean RAL levels and the proportion of positive samples for DNA adducts by the a priori defined exposure groups are presented. No significant differences in RAL or the proportion of positive samples were observed between the three a priori defined exposure groups, indicating no relation between external mutagenic exposure levels and identified DNA adducts. In addition, Spearman correlation coefficients between the subject's urinary mutagenicity level (both cotinine and noncotinine corrected) and RAL levels of the four major DNA adducts were low (range 0.04–0.17) and statistically nonsignificant.

A consistent, moderate increase in the proportion of positive samples for all four identified DNA adducts was observed for subjects with a fast N-acetylation status (Fig. 1). The observed difference was statistically significant for adduct 1 (*P* = 0.04) but nonsignificant for the other adducts, likely because of the limited number of positive samples. Grouping the individuals according to the production function in which they were employed revealed that workers involved in "mixing" had the highest proportion of positive

samples for DNA adducts followed by the production function "curing." The production functions "mixing" and "curing" were investigated further because these production functions differ substantially from each other and the remaining production functions regarding chemical nature and level of exposure [IARC, 1982]. At least in our study, estimated mean personal inhalable dust levels were significantly higher in mixing than in the other production functions (Wilcoxon, *P* = 0.035) and rubber fume exposure quantified as CSM exposure was significantly higher in curing than in the other production functions (Wilcoxon, *P* = 0.0006). DNA adduct 11 was not included in these analyses because the number of positive samples (n = 2) was too small to enable any further statistical analyses. Stratified analysis by production function showed a significant difference in RAL levels and proportion of positive samples between the mixing and other production functions for both adduct 2 (*P* = 0.03 and *P* = 0.06, respectively) and adduct 3 (*P* = 0.01 and *P* = 0.02, respectively) (Table III). For the production function curing, a statistically significant difference in RAL level and proportion of positive samples was found only for adduct 3 (*P* = 0.03 and *P* = 0.06, respectively). Further stratification by both production function and NAT2 phenotype produced statistically significant trends both in the proportion of positive samples and median RAL for adducts 2 and 3, with higher adduct levels within the production functions mixing and curing com-



**Fig. 1.** Proportion of positive samples of DNA from exfoliated urothelial cells by N-acetyltransferase activity. □, slow N-acetylation phenotype (n = 32); ■, fast N-acetylation phenotype (n = 20). Fisher's exact *P*-values given for the difference in proportion of positive samples between slow and fast acetylators.

**TABLE III.** Median and Mean Relative Adduct Labeling Levels (RAL × 10<sup>7</sup>)<sup>a</sup> and Proportion of Positive Samples Stratified by Production Functions "Mixing" and "Curing"

Production function	N	Adduct 1 (n = 41)			Adduct 2 (n = 13)			Adduct 3 (n = 29)		
		Median	Mean	% <sup>b</sup>	Median	Mean	%	Median	Mean	%
Mixing	5	1.4	2.3	100	1.4 <sup>*c</sup>	5.0	60.0 <sup>d**</sup>	4.1 <sup>*c</sup>	3.8	100 <sup>d*</sup>
Curing	15	2.6	2.0	73.3	0	1.4	33.3	1.9 <sup>*c</sup>	2.4	73.3 <sup>d**</sup>
Other	32	1.1	2.0	81.3	0	0.4	15.6	0	1.1	40.6

<sup>a</sup>In the absence of a particular adduct spot in the individual map, the DNA adduct level was designated zero RAL level.

<sup>b</sup>Percentage of positive samples of DNA from exfoliated bladder cells.

<sup>c</sup>Relative adduct levels in the production functions "mixing" and "curing" vs. the "other" production functions were evaluated by the Wilcoxon rank sum test.

<sup>d</sup>Fisher exact *P*-values for difference in proportion of positive samples between the production functions "mixing" and "curing" vs. the "other" production functions.

\**P* < 0.05; \*\**P* < 0.10.

pared to the other production functions and with increased adduct levels among fast acetylators for both high exposed subjects (e.g., mixing and curing) and low exposed subjects (e.g., other production functions) (Table IV).

## DISCUSSION

We studied urothelial carcinogen-DNA adduct levels in a population of nonsmoking occupationally exposed rubber workers. These workers were potentially exposed to a complex mixture of chemicals with largely unknown composition and toxic properties. To study the genotoxic properties of this exposure mixture, the <sup>32</sup>P-postlabeling method with TLC detection was used to identify bulky DNA adducts.

Subjects were selected from a large cross-industry survey based on ambient mutagenic TSPM and mutagenic surface contamination and history of nonsmoking. The rationale for this selection was to increase the contrast in both inhalable and potential dermal mutagenic exposure conditions. Earlier studies had demonstrated genotoxic exposure on the basis of

urinary mutagenicity and mutagenicity of ambient air samples [Falck et al., 1980; Sorsa et al., 1982; Baranski et al., 1989; Bos et al., 1989; Fracasso et al., 1999; Vermeulen et al., 2000a]. In addition, the exposure group with low TSPM mutagenicity and low surface mutagenic exposure levels was intended to be used as an internal comparison group. Usually, an internal control group is desirable as long as there is a wide range of clearly identifiable exposures of interest within a workplace because selection bias and confounding are likely to be less of a problem than could be encountered when using an external comparison group [Checkoway et al., 1989]. However, external mutagenic exposure estimates and urinary mutagenicity turned out not to be related to the observed DNA adducts. Therefore the a priori grouping scheme based on total dose measures and the use of an internal comparison group were of limited value.

Four DNA adducts (adduct 1, 2, 3, and 11) were detected with prevalence rates of 79, 25, 56, and 3.8%, respectively. RAL levels, however, were low. Adducts 1 and 3 were

**TABLE IV. Trends in Median and Mean Relative Adduct Labeling Levels (RAL  $\times 10^7$ )<sup>a</sup> and Proportion of Positive Samples Stratified by Production Functions “Mixing” and “Curing” and *N*-acetylation Status (NAT2)**

NAT2	Production function	N	Adduct 1 (n = 41)			Adduct 2 (n = 13)			Adduct 3 (n = 29)		
			Median	Mean	% <sup>b</sup>	Median	Mean	%	Median	Mean	%
Fast	Mixing <sup>c</sup>	1	1.0	1.0	100	5.6	5.6	100	4.1	4.1	100
Slow	Mixing	4	2.8	2.7	100	0.7	4.8	50.0	3.6	3.7	100
Fast	Other <sup>d</sup>	13	1.7	1.9	92.3	0	0.6	23.1	0.2	2.4	53.8
Slow	Other <sup>d</sup>	19	0.8	2.1	68.4	0	0.2	10.5	0	0.2	31.6
			<i>P</i> = 0.062 <sup>e</sup>			<i>P</i> = 0.020			<i>P</i> = 0.009		
Fast	Curing	6	2.9	3.2	100	1.4	1.8	50.0	3.3	3.9	100
Slow	Curing	9	0.6	1.2	55.6	0	1.3	22.2	1.1	1.3	55.6
Fast	Other <sup>f</sup>	13	1.7	1.9	92.3	0	0.6	23.1	0.2	2.4	53.9
Slow	Other <sup>f</sup>	19	0.8	2.1	68.4	0	0.2	10.5	0	0.2	31.6
			<i>P</i> = 0.520			<i>P</i> = 0.066			<i>P</i> = 0.008		

<sup>a</sup>In the absence of a particular adduct spot in the individual map, the DNA adduct level was designated zero RAL level.

<sup>b</sup>Proportion of positive samples of DNA from exfoliated bladder cells.

<sup>c</sup>The subgroup “mixing” and fast-acetylation status comprises one subject. The presented mean and median values for this subgroup are therefore the actual RAL value for that particular adduct.

<sup>d</sup>Excluding subjects employed in the production function “curing”.

<sup>e</sup>Exact *P*-value for trend (Cochran–Armitage test for trend) for percentage of positive samples of DNA from exfoliated bladder cells.

<sup>f</sup>Excluding subjects employed in the production function “mixing.”

moderately correlated, suggesting that these adducts might reflect metabolites of the same compound or of compounds of similar exposure paths. Stratification by production function showed statistically significant differences in RAL levels and the proportion of positive samples, with the production functions mixing and curing showing the highest DNA adduct levels and proportion of positive samples. This trend was most pronounced for adducts 2, 3, and 11. The relationship between mixing and curing and adduct 1 was less clear and it can be argued that this particular DNA adduct might not be occupationally related. The high prevalence of adducts 2 and 3 (25 and 56%, respectively) and the observation that the adducts were found for all production functions suggested that the responsible exposures are common and widespread in the rubber industry, but occur more frequently and at higher levels in the mixing and curing departments. Preferential labeling of the DNA adducts, however, could have influenced the RAL levels and the proportion of positive samples of the four detected DNA adducts. Therefore, differences in RAL levels and proportion of positive samples between the four DNA adducts cannot be explained in terms of relative biological importance of these particular DNA adducts.

Occupational exposure to chemical compounds in the rubber industry essentially arises by the handling of bulk raw materials and chemical additives and results from the generation of rubber fumes and gases because of high process temperatures during activities like curing. The highest exposure levels to specific chemicals are likely to occur in the mixing department where most handling, weighing, and mixing of raw materials takes place and in the curing department where the highest process temperatures are reached (e.g., generation of rubber fumes and gases). In-

deed, significantly higher inhalable dust levels were found in this study within the mixing department, and for curing, significantly higher inhalable CSM exposure concentrations (e.g., measure for exposure to rubber fumes and gases) were observed. However, these exposure measures are chemically nonspecific and do not necessarily reflect variability in chemical-specific exposure levels. When these chemically nonspecific exposure measures are used across production functions and companies, misclassification attributed to differences in chemical composition of the complex mixture could obscure relationships with DNA adducts. The number of workers in a specific production function within a specific company, however, was too small to study the relationship between personal external exposure measures and the observed DNA adducts for each department/company separately.

Given that no real unexposed control group was present in this study and no strong unambiguous associations were found with occupational exposure estimates, the observed increase in DNA adduct levels for the production functions “mixing” and “curing” have to be interpreted with caution. Although exposure to nonoccupational (concomitant) genotoxic compounds cannot be ruled out, it seems unlikely that these would have resulted in the consistent DNA adduct patterns observed for the several production functions, particularly in a group of nonsmokers living in geographically different parts of The Netherlands. Note that no relation was found with possible confounding exposure proxies like urinary cotinine levels or alcohol and roasted meat consumption. Preferential labeling and measurement error, which can be considerable for bulky adduct analysis, is by and large considered to be nondifferential [Vineis and Veglia, 2001], which means that if existent, this would have atten-

uated the described relations rather than have resulted in false positive associations. Interestingly, the observed differences in DNA adduct levels by production functions were comparable to the distribution of the urinary bladder cancer risk in the past. Urinary bladder cancer risk was then observed in various work areas but mostly in the compounding and mixing departments [Parkes et al., 1982; Sorahan et al., 1986], which suggests that the observed DNA adducts in this study might be related to exposure to one or more constituents of raw rubber chemicals, carbon black, or process oils used within the rubber industry. Only structural characterization of the detected DNA adducts, however, could have given more insight into the chemical origin of these DNA adducts.

The observed DNA adducts levels tended to be moderately increased for subjects with a fast N-acetylation phenotype. Although N-acetylation is normally considered as a detoxification step in arylamine biotransformation, it has been shown that the fate of N-acetylated arylamines, such as benzidine may be different [Zenser et al., 1996]. Hence, fast acetylation has been shown to be marginally associated with an increased risk of urinary bladder cancer in workers exposed to benzidine [Hayes et al., 1993]. The participating rubber companies did not use benzidine; however, several other arylamines were widely used as antidegradants such as, for example, several derivatives of *p*-phenylenediamine. These arylamines are generally considered to be nonmutagenic or, at most, weak mutagens [Crebelli et al., 1984], which could explain the lack of correlation between urinary mutagenicity and observed DNA adduct levels. Moreover, these two biomarkers integrate exposure over different periods, urinary mutagenicity being more responsive to daily exposure variations and adduct levels integrating exposure over urothelial cell life span (~3 weeks), which could contribute to the apparent absence of a correlation between these two nonselective biomarkers.

In conclusion, this study showed low, but significantly increased levels of urothelial DNA adducts in certain groups of rubber workers. The study was too small to explore in depth possible relationships between external occupational exposure estimates and detected DNA adducts within the identified high risk production functions "mixing" and "curing." Larger studies among rubber workers employed in these production functions with inclusion of an unexposed control group should be conducted to study in more detail the potential carcinogenicity of the exposures encountered in these work areas and the possible role of N-acetylation status. Because DNA adduct formation is an important step in events leading from carcinogen exposure to disease, the results of our study warrant further investigation, especially as the results tend to corroborate the recent epidemiological findings of a moderate excess risk of urinary bladder cancer among rubber workers.

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