

## Biological monitoring of polycyclic aromatic hydrocarbons

### Metabolites in urine

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JONGENEELLEN FJ, BOS RP, ANZION RBM, THEUWS JLG, HENDERSON PT. Biological monitoring of polycyclic aromatic hydrocarbons: Metabolites in urine. *Scand J Work Environ Health* 12 (1986) 137–143. Assays of urinary mutagenicity, urinary 3-hydroxy-benzo(a)pyrene, and urinary 1-hydroxypyrene were used to study their suitability in estimating exposure to polycyclic aromatic hydrocarbons (PAH) in coal tar products. Rats exposed to coal tar solutions applied on the dorsal skin excreted mutagens, 3-hydroxy-benzo(a)pyrene, and 1-hydroxypyrene dose dependently in their urine. The correlation between the three parameters was high. Five dermatologic patients undergoing topical coal tar treatment excreted low concentrations of 3-hydroxy-benzo(a)pyrene and high concentrations of 1-hydroxypyrene. A significant correlation between the excretion of the two metabolites was found. The smoking workers of a coal tar distillation plant showed a significantly enhanced urinary mutagenicity compared with their nonsmoking colleagues, but an increase due to occupational exposure was not found. However, the concentration of 1-hydroxypyrene in the urine of these workers highly exceeded the upper 95 percentile of a reference population. The urinary excretion of 1-hydroxypyrene of smoking referents was not significantly increased compared with that of nonsmoking referents. The data suggest that urinary 1-hydroxypyrene is a sensitive and specific marker for the assessment of occupational exposure to PAH.

**Key terms:** benzo(a)pyrene, coal tar, dermatologic patients, mutagens, pyrene, workers.

The assessment of exposure in the workplace becomes very complicated when workers are exposed to a mixture of compounds with similar physical and chemical properties. The polycyclic aromatic hydrocarbons (PAH) are an important class of compounds occurring in tarry products. They include several carcinogens of varying potency (6). The measurement of each component is impractical to perform on a routine basis. A pragmatic approach to the problem of exposure assessment demands the use of an index of the class of compounds, ie, a marker.

The organic part of airborne particulates, characterized by the benzene-soluble matter (BSM) is an index which is widely used in the monitoring of PAH in the work environment (7). Lindstedt & Sollenberg (12) reviewed the occurrence of PAH in the occupational environment, referring to airborne benzo(a)pyrene measurements as a single marker.

Air monitoring of PAH only quantifies the respiratory intake (external dose). PAH may be absorbed not only in the lungs, but also in the gastrointestinal tract and through the skin (10). The monitoring of PAH or their metabolites in body fluids reflects the total uptake (internal dose). Biological monitoring of PAH may thus be useful in order to make an accu-

rate estimation of the individual dose. It can help industrial hygienists to identify highly exposed workers. Moreover, unbiased estimations of exposure to PAH are needed to increase the power of epidemiologic studies.

It is desirable to have a simple noninvasive test which can detect industrial exposure to PAH (15). This study reports the results of the application of the following three urine tests after exposure of man and rat to PAH: (i) the mutagenicity in urine extracts as an analytical detector of exposure (3, 4, 5, 11, 14, 18), (ii) determination of urinary 3-hydroxy-benzo(a)pyrene, a metabolite of benzo(a)pyrene (9), and (iii) urinary 1-hydroxypyrene as a marker of the internal dose of PAH (8). These methods were compared in reference to sensitivity and specificity.

### Materials, subjects and methods

#### Chemicals

1-Hydroxypyrene was synthesized by Janssen Chimica, Belgium. The identity was confirmed with gas chromatography-mass spectrometry and nuclear magnetic resonance. 3-Hydroxy-benzo(a)pyrene was obtained from the National Cancer Institute, Chemical Carcinogen Reference Standard Repository, United States. All other chemicals were of the highest purity obtainable.

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### *Topical application of coal tar to the skin of rats*

Special pathogen-free (SPF) male Wistar rats, weighing about 200 g, were housed individually in stainless steel metabolism cages designed for the separate collection of urine and feces. The rats had free access to water and food. Four groups of three rats were treated with a 0.1-ml solution of coal tar in xylene containing 0, 2.5, 12.6, and 53.0 mg of coal tar, respectively. The coal tar contained 23.2 mg of pyrene and 9.5 mg of benzo(a)pyrene per gram. The coal tar solution was administered to approximately 9 cm<sup>2</sup> of the clipped dorsal skin. After 24 h the treated back of the rats was cleaned with 90/10 % olive oil/light petroleum. Twenty-four-hour urine samples were collected for 5 d and stored at -18°C until required for analysis.

### *Dermatologic patients treated with a coal tar ointment*

Five female patients (patient A, B, C, D and E, mean age 25 years, range 20–29 years), suffering from eczematous dermatitis on the arms and legs, were treated during several days with an ointment containing 10 % pix lithanthracis dermatata (= coal tar). The pix lithanthracis dermatata used contained 16.7 mg of pyrene and 7.0 mg of benzo(a)pyrene per gram. During the treatment the ointment was removed daily with arachis oil, and a fresh dose of approximately 40 g of ointment was rubbed on to stay for the next 24 h. The patients volunteered to collect spot urine samples, one urine sample before the start of the application of the ointment and two samples per day (morning and evening) during the first 3 d of treatment. A pretreatment sample from patient A was not available. Patients A and B were nonsmokers; patients C, D, and E were smokers. The urine samples were stored at -18 °C.

### *Workers in a coal tar distillation plant*

The crude coal tar obtained from steel works was fractionally distilled in a closed system. Most of the distillate fractions were blended to meet product specifications. Products from this plant were: carbon black oil, creosote, electrode pitch, pitch, roofing material, coatings, fluxing oil (road binder), road tars, etc. Fourteen male workers from the "tar department" were employed in eight different jobs; they were foremen, assistant foremen, operators, a mechanic, and a cleaner. They were supposed to be highly exposed workers. The mean age of the four nonsmokers and 10 smokers was 24.2 (range 19–32) and 33.9 (range 21–46) years, respectively. All 10 smokers smoked more than 20 cigarettes per day. The personal sampling of PAH was performed during eight shifts. The method covered both gaseous and particulate PAH (17). Urine samples from the workers were collected on the first, the middle, and the last day of a 7-d work period. The participants were required to void at the beginning and end of the workshift. The work

period was preceded by a workfree period of 3 d. The urine samples were stored at -18 °C.

### *Referents*

Male referents (students and university personnel) were asked to collect spot urine samples. Age and smoking habits were recorded. The mean age of the 22 smokers was 30.4 (range 23–54) years; the mean age of the 24 nonsmokers was 31.4 (range 22–47) years. Ten men smoked 0–10 cigarettes, four men 10–20 cigarettes, and seven men smoked more than 20 cigarettes per day. One person smoked five cigars per day.

### *Analysis of urine samples*

**Mutagenicity in human urine.** The method for the determination of mutagenic metabolites in urine has been described by Yamasaki & Ames (22). We modified this method by using a volume of urine corresponding to an amount of 1.0 mmol of creatinine instead of urine concentrates of 100-ml portions. The urinary metabolites were concentrated with a column with a 4-cm<sup>3</sup> bed volume of Amberlite XAD-2. The adsorbed material was eluted with 10 ml of acetone. The eluate was evaporated to dryness, and the residue was dissolved in 0.4 ml of dimethylsulfoxide (DMSO). This solution (0.1 ml) was added per plate and assayed in triplicate for mutagenicity with the *Salmonella typhimurium* tester strain TA98 in the presence of S-9 mix and  $\beta$ -glucuronidase (150 U per plate). The solvent value was obtained with the use of 100 ml of aqua destilata (aqua dest, ie, distilled water) instead of urine. The human urinary mutagenicity values were expressed as follows:

$$R_T/R_S = \frac{\text{mean number of revertants found with urine}}{\text{mean number of revertants found with aqua dest}}$$

**Mutagenicity in rat urine.** A complete description of the mutagenicity assay of rat urine has been given elsewhere (9). The 24-h urine samples from the groups of three rats undergoing topical coal tar treatment were pooled. Two-thirds of the pooled 24-h samples were concentrated on a XAD-2 column. The 10-ml acetone eluate was evaporated, and the residue dissolved in 0.7 ml of DMSO. The amount of 0.1 ml per plate was assayed in triplicate for mutagenicity with the *S typhimurium* TA98 strain in the presence of S-9 mix and  $\beta$ -glucuronidase (150 U per plate). The mutagenicity of the rat urine was expressed in revertants per plate.

### *Urinary metabolites of polycyclic aromatic hydrocarbons*

The analytical method for the determination of 3-hydroxy-benzo(a)pyrene and 1-hydroxypyrene has been described elsewhere (8, 9). The analysis is based on enzymic hydrolysis, followed by a concentration

step, and the determination of the metabolite with a high-pressure liquid chromatograph (HPLC) equipped with a fluorescence spectrophotometer. The identity of the two PAH metabolites was confirmed in the mobile phase after HPLC separation through comparison of the fluorescence excitation and emission scans of the metabolites with that of the references. The detection limit (signal/noise > 4) for urinary 3-hydroxy-benzo(a)pyrene and 1-hydroxypyrene is 0.5 nmol/l for both metabolites.

The urinary creatinine was determined with an automated technique based on the reaction of creatinine to the alkaline picrate.

### Statistical methods

The statistical significance of the differences was tested with the nonparametric Mann-Whitney U test. Correlation was qualified with Spearman's rank correlation coefficient ( $r_s$ ). p-Values refer to two-tailed tests. Data are presented with either the arithmetic mean (AM) or the geometric mean (GM), the latter being more representative of a highly skewed distribution of data.

## Results

### Rats undergoing topical application of coal tar

The pooled rat urine samples were assayed for mutagenicity, 3-hydroxy-benzo(a)pyrene, and 1-hydroxypyrene. Table 1 shows the results of the analyses. With each of the three methods we were able to detect a dose-dependent uptake through the skin. It is obvious that the excretion of 1-hydroxypyrene was highly enhanced, even after low exposure levels. The association between the concentration of 1-hydroxypyrene and that of 3-hydroxy-benzo(a)pyrene was studied in urine samples from five postexposure days. The correlation was high, although the number of samples in which both metabolites were detected was limited ( $r_s = 0.94$ ,  $N = 9$ ,  $p < 0.005$ ). Regression analysis showed a linear relationship [slope  $5.3 \times 10^{-2}$ , 95 % confidence limits (95 % CL)  $4.4 \times 10^{-2}$  and  $6.2 \times 10^{-2}$ , intercept  $-0.26$ ,  $r = 0.98$ ,  $N = 9$ ]. The excretion of 1-hydroxypyrene was positively associated with and approximately 19 times higher than that of 3-hydroxy-benzo(a)pyrene.

Figure 1 shows the mutagenicity of the urine samples as a function of the amount of 1-hydroxypyrene in 24-h urine samples. Only those urine samples with higher mutagenicity values than the upper 95 percentile of the reference urine (= 50.2 revertants/plate) were taken into account. An association between 1-hydroxypyrene and mutagenic metabolites ( $r_s = 0.93$ ,  $N = 12$ ,  $p < 0.001$ ) was found. Regression analysis showed a logarithmic correlation as the best fit [mutagenicity =  $a + b \times \ln(1\text{-hydroxypyrene})$ , slope 25.1, 95 % CL 17.9 and 32.3, intercept 30,  $r = 0.93$ ].

### Eczema patients

The mutagenicity of the urine samples of the five patients with eczema could not be assayed because of the toxic properties of the urine extracts to the *S typhimurium* bacteria, even after dilution of the extracts.

3-Hydroxy-benzo(a)pyrene was detected in the urine samples, but the concentrations were not higher than  $0.40 \mu\text{mol/mol}$  of creatinine.

The excretion pattern of 1-hydroxypyrene in urine is given in figure 2. After the treatment was started, the concentration of 1-hydroxypyrene increased approximately 100 times. The enhanced level of the "pretreatment" samples of patients C, D, and E compared with the values of the referents might have been caused by an unknown treatment from days before.

Figure 3 shows the fitted linear regression line in a plot of the 1-hydroxypyrene concentration and that of 3-hydroxy-benzo(a)pyrene from the posttreatment urine samples (slope  $4.0 \times 10^{-4}$ , 95 % CL  $1.3 \times 10^{-4}$  and  $6.7 \times 10^{-4}$ , intercept 0.020,  $N = 13$ ). The level of the pyrene metabolite was about 2 500 times higher than that of the benzo(a)pyrene metabolite (figure 3). A significant association was found between the urinary level of 1-hydroxypyrene and 3-hydroxy-benzo(a)pyrene ( $r_s = 0.86$ ,  $p < 0.01$ ,  $N = 13$ ).

**Table 1.** Urinary excretion of mutagenic metabolites, 3-hydroxy-benzo(a)pyrene, and 1-hydroxypyrene after topical treatment of rats with solutions of coal tar in xylene.<sup>a</sup> [ND = not detectable (< 0.01 nmol/24 h)]

| Applied coal tar              | Mutagenic metabolites (revertants/plate) | 3-hydroxy-benzo(a)-pyrene (nmol) | 1-hydroxypyrene (nmol) |
|-------------------------------|------------------------------------------|----------------------------------|------------------------|
| <i>0- to 24-h excretion</i>   |                                          |                                  |                        |
| 0 mg/rat                      | 47                                       | ND                               | 0.14                   |
| 2.5 mg/rat                    | 90                                       | 0.03                             | 7.75                   |
| 12.6 mg/rat                   | 135                                      | 1.72                             | 26.3                   |
| 53.0 mg/rat                   | 146                                      | 2.99                             | 73.3                   |
| <i>24- to 48-h excretion</i>  |                                          |                                  |                        |
| 0 mg/rat                      | 49                                       | ND                               | 0.60                   |
| 2.5 mg/rat                    | 66                                       | ND                               | 3.57                   |
| 12.6 mg/rat                   | 101                                      | 0.54                             | 17.2                   |
| 53.0 mg/rat                   | 141                                      | 5.44                             | 99.9                   |
| <i>48- to 72-h excretion</i>  |                                          |                                  |                        |
| 0 mg/rat                      | 44                                       | ND                               | 1.15                   |
| 2.5 mg/rat                    | 44                                       | ND                               | 1.68                   |
| 12.6 mg/rat                   | 74                                       | 0.04                             | 7.28                   |
| 53.0 mg/rat                   | 109                                      | 1.21                             | 23.7                   |
| <i>72- to 96-h excretion</i>  |                                          |                                  |                        |
| 0 mg/rat                      | 44                                       | ND                               | 0.22                   |
| 2.5 mg/rat                    | 44                                       | ND                               | 0.98                   |
| 12.6 mg/rat                   | 52                                       | ND                               | 2.46                   |
| 53.0 mg/rat                   | 69                                       | 0.31                             | 12.75                  |
| <i>96- to 120-h excretion</i> |                                          |                                  |                        |
| 0 mg/rat                      | 44                                       | ND                               | 0.37                   |
| 2.5 mg/rat                    | 48                                       | ND                               | 0.55                   |
| 12.6 mg/rat                   | 55                                       | ND                               | 1.45                   |
| 53.0 mg/rat                   | 52                                       | 0.04                             | 4.88                   |

<sup>a</sup> Mean of three rats.

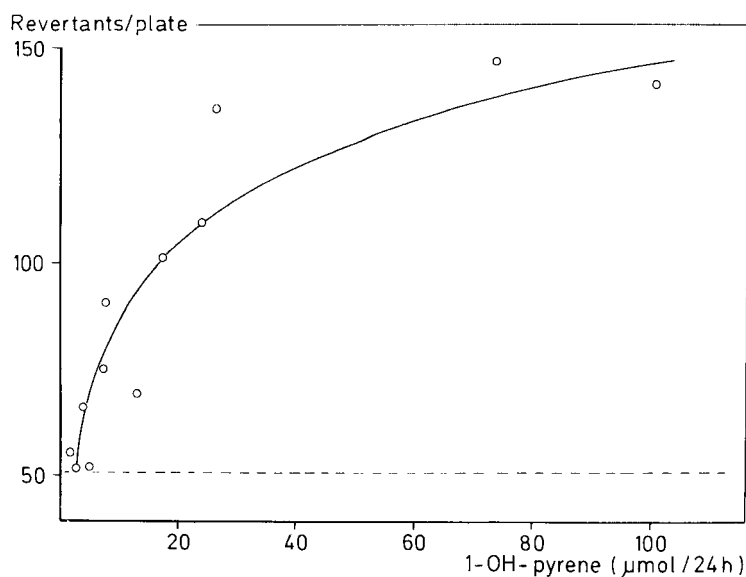


**Figure 1.** Correlation between 1-hydroxypyrene (1-OH-pyrene) and mutagenicity (revertants/plate) in 24-h urine samples from rats after topical application of coal tar solutions. Each point represents the mean of three rats ( $N = 12$ ,  $r_s = 0.93$ ,  $p < 0.001$ ,  $Y = 30 + 25.1 \times \ln X$ ).

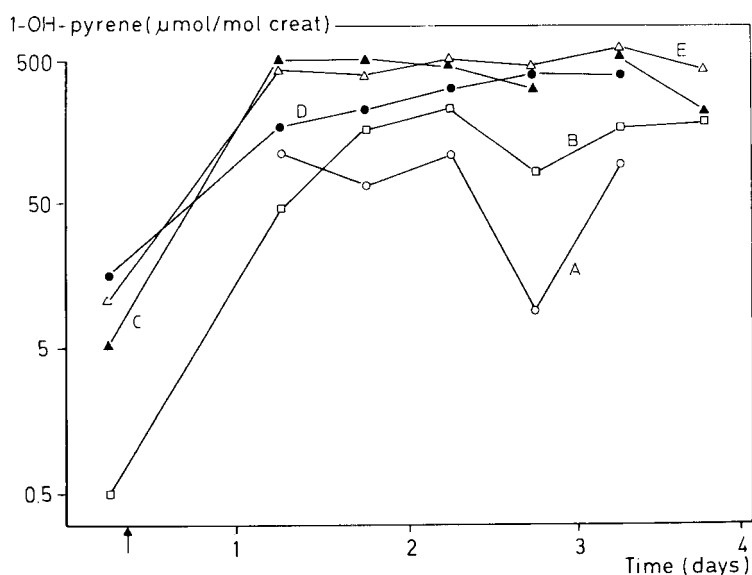


**Figure 2.** Semilogarithmic plot of the urinary excretion of 1-hydroxypyrene (1-OH-pyrene) for five patients undergoing topical coal tar treatment. The arrow indicates the start of the application. (creat = creatinine)

#### *Workers in a coal tar distillation plant*

The urine samples of the 14 men from the coal tar distillation plant were tested for mutagenicity ( $N = 69$ ). The smoking workers revealed significantly more mutagenic compounds and/or metabolites in their urine than did their nonsmoking colleagues [ $R_T/R_S$  (AM) = 4.8 and 1.8 and the 95 % confidence interval (95 % CI) 1.0–8.6 and 1.0–2.6, respectively, Mann-Whitney U test,  $p < 0.0001$ ]. No enhancement of urinary mutagenicity was observed, either in the course of the shift or in the course of the work period of 7 d.

The concentration of 3-hydroxy-benzo(a)pyrene in the urine of the workers was below the detection limit.

Only 10 urine samples from these workers were available for the determination of 1-hydroxypyrene. These urine samples were from four workers, namely, the operator of the pitch unit, the operator of the batch distillation, the operator of the pumping station, and the cleaner. Seven samples were collected before and three after the shift. The time-weighted average (TWA) exposure over 8 h for these four workers was  $< 2\text{--}280 \mu\text{g}/\text{m}^3$  for the sum of 11 PAH and  $< 2\text{--}96 \mu\text{g}/\text{m}^3$  for pyrene (17). The mean (GM) concentration of 1-hydroxypyrene in the urine from the four workers and 46 referents is shown in table 2. The enhanced level of 1-hydroxypyrene in the workers'

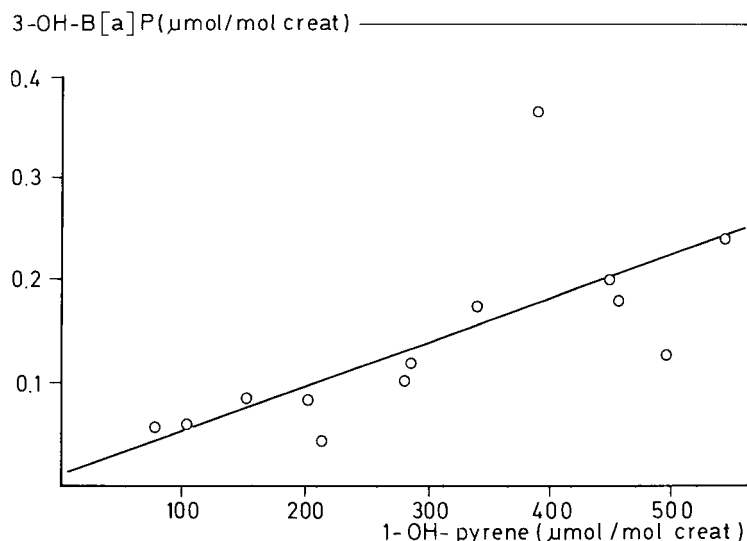


**Figure 3.** Correlation between 1-hydroxypyrene (1-OH-pyrene) and 3-hydroxy-benzo(a)pyrene (3-OH-B[a]P) in spot urine samples of patients undergoing topical coal tar treatment ( $N = 13$ ,  $r_s = 0.86$ ,  $p < 0.01$ ,  $Y = 0.020 + 4.0 \times 10^{-4} \times X$ ). (creat = creatinine)

**Table 2.** Exposure of employees in a coal tar distilling plant to polycyclic aromatic hydrocarbons (PAH).

| Function                     | Personal sampling <sup>a</sup> |                                |                             | Biological monitoring of 1-hydroxypyrene (μmol/mol creatinine) |                |                         |
|------------------------------|--------------------------------|--------------------------------|-----------------------------|----------------------------------------------------------------|----------------|-------------------------|
|                              | Number of samples              | Total PAH (μg/m <sup>3</sup> ) | Pyrene (μg/m <sup>3</sup> ) | Number of samples                                              | Geometric mean |                         |
| Operator, pitch unit         | 8                              | 26                             | 8.5                         | 2                                                              | 3.7            | (nonsmoker)             |
| Operator, batch distillation | 6                              | 14                             | 5.1                         | 2                                                              | 11.8           | (smoker)                |
| Operator, pumping station    | 5                              | 4.7                            | 1.4                         | 4                                                              | 4.0            | (nonsmoker)             |
| Cleaner                      | 4                              | 16                             | 5.2                         | 4                                                              | 4.6            | (smoker)                |
| Referents                    | 46                             | —                              | —                           | 46                                                             | 0.25           | (non-smokers + smokers) |

<sup>a</sup> Geometric mean of the time-weighted average exposure over 8 h as measured by Nossent & van de Ven (17).

urine in comparison with the referents' is obvious. Table 2 also shows the results of the personal monitoring of the four workers. The sum of the concentrations of 11 PAH, including pyrene, and the concentration of pyrene is presented. It demonstrates that pyrene was a major compound of the PAH mixture in this work environment.

### Referents

The concentration of urinary 3-hydroxy-benzo(a)pyrene was below the detection limit in the referents' urine. 1-Hydroxypyrene was identified in all the samples. The distribution of urinary 1-hydroxypyrene in the reference population appeared to be positively skewed. To make the data appropriate for analysis, a logarithmic transformation was applied. The samples revealed a mean (GM) concentration of 1-hydroxypyrene of 0.25 μmol/mol of creatinine (95 % CI 0.06—1.00). The concentration of 1-hydroxypyrene in the urine of the smokers (GM 0.27, 95 % CI 0.06—1.15) was not significantly increased compared with

that of nonsmokers (GM 0.23, 95 % CI 0.05—0.85, Mann-Whitney U test,  $p = 0.27$ ).

### Discussion

Recently, a new procedure for the estimation of exposure to PAH has been proposed (2). The procedure involves HPLC analysis after chemical reduction of the urinary PAH metabolites to their parent PAH. As yet, the proposed chemical reduction procedure has not been validated with urine samples spiked with PAH metabolites. Therefore, we did not include this assay in the comparison of the methods.

The exposure of rats to topically applied coal tar can be assessed with the urinary mutagenicity assay (table 1). The mutagenicity of the urine samples of rats was found to correlate with the (logarithm of the) concentrations of 3-hydroxy-benzo(a)pyrene and that of 1-hydroxypyrene. A similar relationship was found after oral administration of benzo(a)pyrene to rats (9). Two volunteers excreted mutagenic urine after a single application of 5 % crude coal tar in petrolatum to the

trunk skin (the dose was not given) (21). Nevertheless, we did not detect an elevated level of mutagenicity in the urine of PAH-exposed workers, either in this study or in another (3). The applicability of this assay after occupational exposure to PAH seems to be limited. This conclusion is supported by work of other investigators, who failed to detect an enhanced excretion of mutagens in urine of workers exposed to PAH (4, 14, 18). However, Kriebel et al (11) reported a higher mutagenic activity in urine of nonsmoking coke plant workers than in that of nonsmoking referents. Among nonsmoking employees of an aluminum plant, a slight but significantly higher percentage of workers exposed to coal tar pitch volatiles showed higher urinary mutagenicity than nonexposed workers (5). The evaluation of these conflicting data is difficult, because the mentioned studies did not report on simultaneous ambient monitoring, except the study of Clonfero et al (4) and the present report. It seems that the excretion of urinary mutagenic PAH metabolites after occupational PAH exposure is too low to detect the presence of these compounds in urine. Moreover, smoking is a major determinant in urinary mutagen activity, and this factor makes the assay less suitable for monitoring occupational exposure to PAH.

3-Hydroxy-benzo(a)pyrene was detected in human urine after the application of high (therapeutical) doses of a 10 % coal tar ointment. After topical treatment of rats with an equal (corrected for body weight) dose of coal tar, the urinary excretion of this benzo(a)pyrene metabolite was much higher than that of the patients. The benzo(a)pyrene-hydroxylating activity of human liver *in vitro* has been found to be much lower than that of rodent liver (16, 19). In spite of a high analytical sensitivity, 3-hydroxy-benzo(a)pyrene was not detectable in human urine after occupational exposure to coal tar products. For biomonitoring occupational exposure, the determination of urinary 3-hydroxy-benzo(a)pyrene therefore seems to be less suitable.

Pyrene is a predominant compound in environmental PAH exposure (6). The urinary excretion of 1-hydroxypyrene of dermatologic patients was highly enhanced after the application of a coal tar ointment. Three workers of a creosote impregnation plant showed enhanced levels of 1-hydroxypyrene in urine over a period of 10 d (8). Even after a TWA exposure to PAH of  $< 2\text{--}280\text{ }\mu\text{g}/\text{m}^3$  in the work environment of the coal tar distillation plant, the urinary 1-hydroxypyrene concentration highly exceeded the upper 95 percentile of the referents. These examples illustrate the high sensitivity of the test.

Mainstream smoke of cigarettes contains 5–27  $\mu\text{g}$  of pyrene per 100 cigarettes (6). Smoking 10 cigarettes a day results in a pyrene intake of 0.5–2.5  $\mu\text{g}/\text{d}$ . On the assumption that only a minor part of the pyrene metabolites, like benzo(a)pyrene metabolites (13, 20), will be excreted in urine, it is plausible that the maximum elevation of urinary 1-hydroxypyrene due to smoking is 0.2–1.1 nmol/d. Since the excretion of

creatinine in the urine of males is 16 mmol/24 h (1), the increase caused by smoking 10 cigarettes daily is estimated to be 0.01–0.07  $\mu\text{mol}/\text{mol}$  of creatinine. This estimated enhancement is in accordance with the measured difference between smoking and non-smoking referents. Smoking habits appear to be a minor confounder when exposure is assessed through the determination of the concentration of 1-hydroxypyrene in urine.

A significant correlation between the urinary 1-hydroxypyrene and the urinary 3-hydroxy-benzo(a)pyrene was found after exposure of patients and rats. Apparently, the kinetics of pyrene elimination from the body is comparable with that of benzo(a)pyrene. A significant correlation between 1-hydroxypyrene and mutagenicity was also shown in urine of rats treated with coal tar. It is very likely that most of the mutagenicity on strain TA98, a strain very sensitive for frameshift mutagens, originates from the PAH in coal tar. Therefore, the occurrence of elevated levels of 1-hydroxypyrene in urine not only signals exposure to pyrene but most probably also to other PAH. Since exposure to pyrene always occurs in PAH mixtures, the 1-hydroxypyrene assay is not only suitable for the assessment of exposure to pyrene, but it might also be used as a marker for the assessment of exposure to PAH. However, the representativeness of 1-hydroxypyrene in respect to biological potency has to be studied and verified for different PAH exposures.

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