Urinary screening for potentially genotoxic exposures in a chemical industry

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ABSTRACT Mutagenic activity, measured by the bacterial fluctuation assay and thioether concentration in urine from workers at a chemical plant producing pharmaceuticals and explosives, was determined before and after exposure. Of 12 groups only those exposed to trinitrotoluene (n = 14) showed a significant increase in mutagenic activity using *Salmonella typhimurium* TA 98 without any exogenous metabolic system. The same strain responded only weakly when the S-9 mix was used; with *Escherichia coli* WP2 uvrA no effect of exposure was observed. Urinary thioether concentration was higher among smokers than among non-smokers, but occupational exposure had no effect. Urinary mutagenicity testing may be a useful tool for screening potentially genotoxic exposures in complex chemical environments.

Exposure to genotoxic substances is thought to be associated with environmentally induced cancers. In workplaces where several potentially genotoxic substances are handled there is a need for methods to estimate biologically relevant exposures. During the past decade several such methods have been developed and systems using bacterial strains to detect mutagens are widely used to test single chemicals. These tests, however, have also been used to estimate the total content of mutagens excreted in the urine of exposed workers.¹ Occupational groups exposed to anaesthetic gases,^{2,3} cytotoxic drugs,⁴ epichlorohydrin,⁵ air pollutants in foundries,⁶ coke plants,⁷ and the carbon electrode industry⁸ have been studied. Various enzyme treatments have often been combined with the test system in order to activate metabolically conjugated substances and premutagens.

An alternative to the conventional plate incorporation assay (Ames test) is the more sensitive bacterial fluctuation test developed by Green *et al.*⁹ Among occupationally exposed groups, rubber workers¹⁰ and hospital personnel handling cytostatic drugs¹¹ have shown increased mutagenic activity in the urine compared with unexposed controls. Furthermore, the average mutagenic activity decreased when the exposure was lowered.¹²

Many genotoxic substances bind to macromolecules such as cellular proteins or nucleic acids and are often metabolised and conjugated with glutathione before excretion in the urine as thioethers such as mercapturic acid. Therefore, thioether concentration in the urine has been used as an estimate of exposure to potentially genotoxic substances among smokers,^{13,14} rubber workers,¹⁵ workers exposed to carbon disulphide and methyl chloride,¹⁶ incinerator workers,¹⁷ and nurses handling cytotoxic drugs.¹⁸

The study presented in the present paper was initiated in an attempt to identify groups of workers with an uptake of potentially genotoxic substances in a chemical plant producing a large variety of pharmaceuticals and explosives. An earlier epidemiological study of the plant had shown an increased risk of gastric cancer among those employed before 1968 (Andersson *et al.*, to be published). It was not possible, however, to relate this finding to any specific work process or exposure in that retrospective study.

Urine from 12 different groups of workers at the plant were assayed for mutagenicity and thioether concentration in samples taken after an exposed shift and compared with samples after a long vacation.

Study groups and urine sampling

All the 119 workers in the chemical process departments at the plant were asked to take part in the

study and only four refused. The participants were asked to deliver three urine samples, one after four weeks vacation (unexposed sample), one before a workshift after several days work (preshift sample), and one after the same shift (exposed sample). Thereby each individual could be used as his/her own control by comparing the mutagenic activity in the exposed sample with the unexposed and the thioether concentration in the exposed sample with the preshift sample. A total of 115 unexposed samples were originally collected, but owing to replacements only 97 exposed samples were obtained from 80 men and 17 women. Three of these were not able to give a preshift sample.

On both days of sampling, each worker completed a questionnaire concerning smoking habits and alcohol consumption, diet, health, and medication. Special attention was paid to the 48 hours preceding the sampling.

Four weeks of vacation had preceded most of the unexposed samples but, in addition, 16 samples delivered after only at least four days leave from work were also accepted and regarded as unexposed. As the exposure was generally considered to be highest during the morning shifts compared with evening shifts, all the exposed samples were collected in the middle or at the end of a morning shift. The unexposed and preshift samples were collected before the worker entered the work area. All samples were collected in plastic bottles and divided into several portions before freezing to -20°C , at which temperature they were transported to the laboratory and kept until analysed. According to earlier experience such preservation has no effects on the urinary mutagenic activity.¹⁹

Exposure

A careful characterisation of the exposure at each of the participating production units was performed. Detailed lists of raw materials and products handled were made as were descriptions of each step in the production process. Earlier data of air concentrations of important chemicals were completed by new measurements where this was considered necessary to achieve an appropriate estimate of the ambient concentrations. Some of these data are shown in table 1 with the 12 exposure categories. (A more detailed exposure specification is available on request from GA.)

The main chemical processes take place in closed systems and hence highest air concentrations of chemicals occur mainly at the workplaces where the final products are dried and packed, and to a certain extent where raw materials are added to the process machines. All deviations from the normal routine, such as process disturbances, leakages, or other inter-

ruptions, were noted during the exposed shift by the occupational hygienist who followed up the workers.

Methods and exclusions

BACTERIAL MUTAGENICITY ASSAY

The urine samples were concentrated on XAD-2 resin as described by Yamasaki and Ames.²⁰ The bacterial fluctuation test was performed according to Falck²¹ using indicator strain *Salmonella typhimurium* TA 98 for frame shift mutagen detection and *Escherichia coli* WP2 uvrA for detection of base pair substitution mutagens. Metabolic activation was accomplished by adding rat liver homogenate (S-9 mix), prepared routinely from clophen A 50 induced Wistar rats.²¹ The B-glucuronidase activity of S-9 mix is approximately equivalent to 20 IV B-glucuronidase of *Helix pomatia*,²¹ and therefore no additional enzyme treatment was used.

The mutagenic activity of the urine was calculated as follows:

$$\text{Mutagenic activity} = \frac{\text{Revertants in sample} - \text{revertants in solvent control}}{\text{Creatinine (mmol) in urine sample}}$$

The mutagenic activity was calculated separately for each of three to five dilutions of the concentrated sample. The dilution giving the highest mutagenic activity was used for the calculation of the results.

THIOETHER ANALYSIS

The thioether content of the urine samples was analysed according to the method described by van Doorn *et al*.¹³ with certain modifications.¹⁴ Each sample was divided into two portions which were analysed separately, and the mean value ($\mu\text{mol SH/l}$) of the two was included in the results after creatinine correction (mmol/l ; analysed according to Clark and Thompson²²) giving a final value in $\mu\text{mol SH/mmol creatinine}$.

EXCLUSIONS

Very dilute urine has a low creatinine concentration. In calculating the mutagenic activity and the thioether concentration, correcting the values for a low creatinine content may carry a risk of overestimating the true excretion. Therefore we chose to exclude all samples with a creatinine concentration below 3.0 mmol/l (the low fifth percentile of the total material). Eight samples from eight individuals were thereby excluded.

Individuals with liver disease may have an altered or reduced metabolic capacity that could influence the biotransformation of genotoxic substances and the results.²³ Individuals with a significant increase (above the 97.5:th percentile value) in liver enzymes

Table 1 Numbers of workers in each exposure group, substances produced, and maximum average air concentration measured in the workroom

Group	No of workers	Production	Maximum air concentration (mg/m ³)
A	14	Trinitrotoluene	0.29
B	6	o-, m-, p-Nitrotoluene	2.0 (O-NT)
C	7	p-Nitrobenzoic acid	5.4
D	8	Dinitrobenzoic acid	0.6*
E	3	p-Nitrobenzoylchloride	—
F	12	Aminobenzoic acid	3.2*
G	5	Hexaminetetranitrate	—
H	4	Mesoinositolhexanicotinate	2.6 (Pyridine)
I	3	5-Nitroisophthalic acid	1.1*
J	7	Polyacrylates	29.1 (Methylacrylate)
K	5	Regaining acids (HCl, HNO ₃)	—
L	7	Other†	—

*Concentration of total organic dust.

†Repair and maintenance work (five workers) and production of mepivacaine (two workers).

(SGOT, SGPT, SALP, or SGT) were excluded from the results, ten in all.

Mutagenic activity cannot be measured when the sample is toxic to the indicator bacteria even at the lowest concentration. Such samples were excluded from the analyses of that particular test system. Ten individuals were lost for evaluation in the test using *S typhimurium* TA 98 with S-9-mix, seven with *S typhimurium* TA 98 without S-9-mix, and 11 with *E coli* WP2 uvrA.

Seventy five individuals were finally tested for urine mutagenicity with *S typhimurium* TA 98 without metabolic activation, 78 with *S typhimurium* TA 98 without metabolic activation, 72 with *E coli* WP2 uvrA with metabolic activation, 81 with analysis of thioether concentration in unexposed and exposed samples, and 77 analysed for thioether concentration in preshift and exposed samples. The excluded individuals were evenly distributed across the 12 groups.

STATISTICAL METHODS

For each of the 12 exposure groups the mean and standard deviations of the mutagenic activity and thioether concentrations were calculated. The difference between exposed and unexposed samples was tested for statistical significance using Student's *t* test for paired observations and between different exposure groups with Student's *t* test for independent samples.²⁴ In those cases where the distributions strongly deviated from normal Wilcoxon's signed rank test or Wilcoxon's rank sum test was used (the result of Student's *t* test is given for comparison). The 5% level has been applied with two tailed calculations ($p < 0.05$).

Results

TOTAL STUDY POPULATION

In the total study population the mean mutagenic activity in exposed urine samples was significantly

increased (fig 1 and table 2; $p < 0.01$) compared with unexposed samples tested with *S typhimurium* TA 98 without exogenous metabolic system (S-9 mix). No differences between the exposed and unexposed samples were observed with the two other test systems used (fig 1a & c), nor was the mean thioether concentration increased in the exposed samples compared with the preshift or unexposed samples.

URINE MUTAGENICITY IN THE SEPARATE EXPOSURE GROUPS

Only two of the 12 exposure groups showed an increased mutagenic activity in the exposed samples compared with the unexposed (table 3) in the test system using *S typhimurium* TA 98 with metabolic activation—namely, the TNT (A) and hexaminetetranitrate (G) groups. The differences did not, however, reach statistical significance. One individual in the hexaminetetranitrate group, who is a smoker, had a high mutagenic activity in the exposed sample (3510 rev/mmol creat) and therefore accounts for most of the observed increase of the group mean figure. There was a statistically significant ($p < 0.05$) difference in mean mutagenic activity between the exposed samples in the TNT (A) group compared with the exposed samples in the other groups (B-L).

Those who smoked at least one cigarette during the 24 hours preceeding the urine sampling had higher average mutagenic activity than non-smokers (table 4). Only non-smokers exposed to TNT had an increased mutagenic activity after exposure ($p < 0.05$).

The increase in mutagenic activity after exposure detected in the test system with *S typhimurium* TA 98 without metabolic activation is almost totally explained by the increase in the group exposed to TNT (table 3). In this group the difference between exposed and non-exposed samples is statistically highly significant ($p < 0.01$), and the highest urine mutagenicity is found among the most highly exposed

Fig 1 Distribution of mutagenic activity (numbers of revertants/mmol creatinine) in individual unexposed and exposed urine samples from all workers, tested with *Salmonella typhimurium* TA 98 with (A) and without (B) metabolic activation and *Escherichia coli* WP2 uvrA with metabolic activation (C).

workers, those who work in the washing, drying, and packing departments. No difference between smokers and non-smokers is seen with this test system (table 4).

Only one individual exposed to TNT had a higher mutagenic activity in the unexposed sample compared with the exposed (fig 2). This was a male smoker (10–20 cig/day) who had also consumed alcohol (30 cl) the day before the exposed sampling.

No indication of base pair substitution mutagens from the work environment was found using the test system with *E coli* WP2 uvrA with metabolic activation in any of the exposure subgroups. The highest mean value and the largest difference between the exposed and unexposed samples was found in the group of workers regaining acids ($\bar{x}$ 99; SD 149; $\bar{d}$ 86). The TNT group showed low urine mutagenicity with this test system ($\bar{x}$ 45; SD 37; $\bar{d}$ 18).

THIOETHER CONCENTRATIONS

None of the exposure subgroups showed any significant increase in mean thioether concentration after exposure compared with preshift or unexposed samples. A male smoker who, at the time of sampling, was taking antibiotics and expectorants for an infection in the upper respiratory tract had an extremely high value in the preshift sample (49.2 μ mol SH/mmol creat). Smokers had higher thioether concentration on average than non-smokers (table 5).

Table 2 Mutagenic activity (rev/mmol creat), thioether ($\mu\text{mol SH}/\text{mmol creat}$) and creatinine (mmol) concentration in urine samples from workers included in the study

Test	No of workers†	Unexposed samples‡	Preshift samples‡	Exposed samples‡
TA 98 + S-9	75	406 ± 464	—	396 ± 529
TA 98 - S-9	78	157 ± 309	—	473 ± 844*
WP2 uvrA + S-9	72	29 ± 60	—	36 ± 63
Thioethers	77	4.0 ± 2.4	4.5 ± 5.8	4.0 ± 2.2
Creatinine	78	13.4 ± 6.8	17.2 ± 6.6	12.6 ± 5.0

* $p < 0.01$ (difference between exposed and unexposed samples; Wilcoxon's signed rank test and Student's t test).

†Only the number of workers with a result in both (mutagenic activity) or all three (thioether and creatinine concentration) samples are presented for each test system.

‡Mean ± standard deviation.

Table 3 Mutagenic activity in urine for each exposure group analyzed with *S typhimurium* TA 98 with and without metabolic activation (S-9 mix)

Group	+ S-9			- S-9		
	No	Unexposed	Exposed	No	Unexposed	Exposed
A	13	467 ± 480	732 ± 524	14	233 ± 539	1784 ± 1293*
B	6	205 ± 194	305 ± 328	6	36 ± 40	180 ± 213
C	5	241 ± 181	291 ± 231	6	119 ± 152	295 ± 408
D	7	768 ± 718	375 ± 461	7	137 ± 184	306 ± 184
E	3	221 ± 351	123 ± 213	3	21 ± 22	75 ± 13
F	11	303 ± 353	211 ± 241	11	315 ± 446	85 ± 107
G	5	410 ± 340	971 ± 1465	5	144 ± 186	260 ± 309
H	3	514 ± 665	164 ± 216	4	82 ± 114	17 ± 22
I	3	248 ± 354	228 ± 175	3	81 ± 100	289 ± 478
J	7	305 ± 209	289 ± 311	7	215 ± 224	190 ± 323
K	5	301 ± 422	328 ± 300	5	107 ± 105	130 ± 222
L	7	651 ± 761	266 ± 351	7	49 ± 70	226 ± 226
B-L†	62	393 ± 464	325 ± 506	64	140 ± 234	187 ± 248

* $p < 0.01$ (difference between exposed and unexposed samples; Wilcoxon's signed rank test and Student's t test).

†All non-TNT-exposed.

Table 4 Mutagenic activity in urine for smokers (at least one cigarette within 24 hours of the sampling) and non-smokers with *S typhimurium* TA 98 with and without metabolic activation (S-9 mix)

Test	Group	Smokers			Non-smokers		
		No	Unexposed	Exposed	No	Unexposed	Exposed
+ S-9	All	41	522 ± 525	491 ± 603	32	276 ± 343	296 ± 411
	A	7	626 ± 545	750 ± 438	6	283 ± 348	711 ± 655*
	B-L	34	501 ± 526**	437 ± 623	26	274 ± 349	200 ± 270
- S-9	All	40	205 ± 359	450 ± 648	34	116 ± 252	547 ± 1069
	A	7	418 ± 739	1412 ± 979	7	49 ± 56	2156 ± 1530
	B-L	33	159 ± 206	246 ± 293	27	134 ± 280	130 ± 181

* $p < 0.05$ (difference between non-smokers in group A and B-L; Wilcoxon's rank sum test— $p < 0.01$ with Student's t test).

** $p < 0.05$ (difference between smokers and non-smokers; Wilcoxon's rank sum test and Student's t test).

Discussion

An increase in the excretion of genotoxic metabolites (premutagens or mutagens or both) in the urine after occupational exposure was detected only in the subgroups of workers handling TNT. 2,4,6-Trinitrotoluene is produced at the plant and other TNT-isomers are formed in small amounts as contaminants. 2,4,6-TNT has earlier been shown to be a frame shift mutagen in the Ames assay without metabolic activation²⁵ and waste water from TNT producing plants contains several mutagenic com-

ponents.^{26,27} TNT-metabolites such as 2,6-dinitro-4-aminotoluene and 2,4-dinitro-6-aminotoluene have not shown mutagenicity. Transformation of TNT in oxidative systems may occur in addition to metabolism by reduction, although this has not yet been found in man. It is not clear to what extent TNT is excreted unmetabolised in the free or conjugated form through the kidneys.²⁸ Nevertheless, in this study we were able with a gas chromatographic technique to detect TNT in the urine of the workers with the highest mutagenic activity.

Falck has estimated the limit value for a

Fig 2 Mutagenic activity (number of revertants/mmol creatinine) in individual unexposed and exposed urine samples from TNT-workers ($n = 14$) tested with *Salmonella typhimurium* TA 98 without metabolic activation.

significantly increased mutagenic activity in the urine from several hundred non-exposed, non-smoking individuals to be about 600 rev/mmol creat in *S typhimurium* TA 98 and 300 in *E coli* WP2 uvrA.²¹ Only the TNT group had a mean value above either of these limits (discounting the effect of smoking detectable with *S typhimurium* TA 98 using S-9 mix). No sign was found of an increased uptake of base pair substitution mutagens during exposure. The increase in the frame shift mutagen indicating system without metabolic activation was, however, clear in the TNT group. There was also a tendency for increased mutagenic activity when metabolic activation was used, although this was not statistically significant.

Exposure to other mutagenic or premutagenic substances such as dimethylsulphate and acrylonitrile may occur in some of the other production units. Several explanations for the absence of an effect in samples from workers in those units are possible.

- (1) No biological uptake due to low exposure.
- (2) The exposure is too low for the sensitivity of the assay.
- (3) Too few individuals exposed to achieve a significant effect in the group analyses.
- (4) The urine metabolites are not mutagenic/premutagenic or cannot be activated.
- (5) The bacterial strains used were not sensitive to the mutagenicity of the chemicals or their metabolites.

Since the handling of such substances is subject to rigorous safety regulations, the first two explanations seem the most plausible. Dinitrotoluenes, which are not produced in the plant but occur in small amounts as contaminants, are less potent mutagens than the trinitrotoluenes.²⁶ Mononitrotoluenes which are produced at the plant have little, if any, mutagenic potency and toluene itself is non-mutagenic in bacterial test systems.²⁶ No data on the mutagenicity of the urine metabolites of the nitrotoluenes are available.

VALIDITY OF THE RESULTS

The individual variation in the urinary mutagenic activity has been found to be relatively wide in most studies.²⁹ Genetic factors, health and background factors such as diet, medication, smoking, alcohol consumption, and other chemical exposures, may account for this. Smoking, as a source of urinary mutagenicity, has been confirmed by several researchers,²⁹ and the effect is usually seen on bacterial strains sensitive to frameshift mutagens. This effect was also seen in the present study. We have not, however, in comparing the questionnaire data on the different exposure subgroups found any other background factors that can explain the main findings in the group analysis. The proportion of smoking individuals is not greater in the TNT group than in the others. The non-smokers in the TNT group showed an increased mutagenic activity in *S typhimurium* TA 98 with metabolic activation, although the difference between the exposed and unexposed samples did not

Table 5 Thioether concentration ($\mu\text{mol SH}/\text{mmol creatinine}$) in urine from smokers and non-smokers

Group	Smokers			Non-smokers		
	No	Unexposed	Exposed	No	Unexposed	Exposed
All	42	4.2 ± 1.7	4.8 ± 2.1	35	3.6 ± 2.7	$3.1 \pm 1.9^*$
A	7	4.4 ± 1.6	5.7 ± 2.4	7	3.8 ± 2.7	2.4 ± 0.5
B-L	35	4.1 ± 1.8	4.7 ± 2.0	28	3.6 ± 2.8	3.4 ± 2.0

* $p < 0.001$ (difference between smokers and non-smokers; Student's t test).

reach statistical significance (possibly due to the small number of individuals or variation in the group). Compared with the other non-smokers their exposed values are clearly increased.

As shown above, many of the samples were toxic to the indicator bacteria. This is a general problem with the use of urine for biological testing, and may be due to toxic metabolites at high concentrations or the samples might have been contaminated by toxic substances during preparation. Nevertheless, the numbers of toxic samples are relatively evenly distributed among the exposure subgroups and not associated with any specific exposure. TNT is considered to inhibit the growth of *S typhimurium* at concentrations above 10 µg/ml²⁵ but none of the exposed samples in the TNT group was toxic to the bacteria.

In the prestudy design it was considered important to exclude people with liver disease or disturbed liver function since that may influence their ability to metabolise the genotoxic substances to which they are exposed. TNT is toxic to the liver and such exposure might have caused an increase in the activity of the hepatic enzymes.²⁸ Only one individual exposed to TNT was excluded for this reason, however, and no other exposure subgroup was severely reduced.

The sensitivity of the bacterial fluctuation assay when using histidine dependent strains has been discussed as some researchers have found significant amounts of histidine in samples after concentration on XAD-2 resin.³⁰ This could cause the growth of non-mutant bacteria leading to a false positive test result. The amount of histidine was checked in the urine concentrates prepared, however, and not found high enough to permit growth of non-revertant bacteria in the assay system used.³¹ Moreover, it is not likely that such an effect would be associated with any specific exposure.

The indicator bacteria *S typhimurium* TA 98 has some nitroreductase activity. In a recent study by Whong and Edwards TNT and some other nitroaromatic explosives were tested for mutagenicity using a nitroreductase deficient *Salmonella* strain (TA 100 NRD).³² In the Ames's plate-incorporation assay TNT showed little, if any, mutagenic effect with this indicator strain. Their conclusion was that "reduced metabolites, possibly hydroxyl amines, are the proximal mutagenic intermediate." It has also been shown that many other nitroarenes depending on nitroreductase activity for mutagenicity are potent animal carcinogens.^{33 34} An explanation for this may be the presence of gastrointestinal nitro reduction in vivo or possibly an alternative metabolism (ring hydroxylation for example) in mammalian cells.³⁵ Consequently, the increase of urine mutagenicity in the TNT workers might not only be a sign of exposure and biological uptake but also an indication of an

increased genotoxic risk. At present, however, it is not possible to relate the earlier epidemiological finding of an increased risk of gastric cancer among people who worked at the plant in earlier decades to TNT or any other specific exposures.

THIOETHER ANALYSIS

Certain foodstuffs—for example, white cabbage and horse radish—give a drastic increase in thioether concentration in the urine after ingestion.³⁶ Only a few individuals in this study showed such an increase, and only in the unexposed or preshift samples. No statistically significant differences were noted, neither between the exposed and unexposed samples nor between the exposed and preshift samples for any of the exposure groups. The explanation might be that the method is too insensitive to detect the contribution of thioethers originating from the work environment exposures, even though the method is sensitive enough to detect a constant difference between smokers and non-smokers.

During our later interlaboratory cooperation for modification of the thioether method some critical steps in the analysis have been identified. Thawing and mixing of the sample must be done in a careful and standardised way. The number of extractions could be increased for maximal yield and the time between neutralisation and colour reaction must be short to eliminate the risk of oxidation. Possibly, deficiencies in our earlier analysis techniques have left existing differences in thioether concentrations unrevealed.

EXPOSURE

Each exposure group consisted of workers producing or handling, or both, the same main substances. In most cases the groups worked in separate buildings. Dust exposure occurred mainly when raw materials were filled or when the dried product was packed. TNT is mainly absorbed through the skin and usually to a lesser extent through the lungs or the gastrointestinal tract.³⁷ The measured air concentrations in the production units are therefore not necessarily representative of the biologically relevant exposure. Skin contamination, however, is most likely to occur where the dust concentration is high. Therefore the exposure related increase in the mutagenic activity of the workers in those departments supports a causal relation between exposure to TNT and the increased urinary mutagenic activity. The average TNT concentration in the air has been well below the recommended ACGIH value of 0.5 mg/m³. In Finland the TLV is 1.5 mg/m³; there is no Swedish standard.

There were no indications that any of the other chemicals had made a significant contribution to the mutagenicity in the samples exposed to TNT. Work-

ers on the same shift and area have somewhat specialised duties that may result in considerable differences in exposure. Occasional high exposures may take place when production is interrupted accidentally and when repair or cleaning work is performed. The industrial hygienists, the production leaders, and the workers agreed, however, that exposures during the shifts under study were normal.

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