Persistence of 7-(2-hydroxyethyl)guanine–DNA adducts in rats exposed to ethene by inhalation

Chunyan Zhao, Rajiv Kumar, Kolbjørn Zahlsen, Heidi Bager Sundmark, Kari Hemminki and Ingvar Eide

Quantification of 7-(2-hydroxyethyl)guanine (7-HEG) adduct in DNA of livers and lymphocytes of male Sprague–Dawley rats exposed to 300 ppm ethene by inhalation (12 h a day for three consecutive days) was performed to evaluate the potential of ethene to produce DNA adducts in these tissues. The persistence of 7-HEG in livers and lymphocytes was studied in rats sacrificed 0, 1, 5, and 20 days after the last exposure. DNA samples from control and treated animals were analysed for 7-HEG and 7-methylguanine (7-MG) adducts by thin-layer chromatography (TLC) combined with a high pressure liquid chromatography (HPLC) assay. After a 3-day exposure to ethene, 7-HEG accumulated to a similar extent in liver and lymphocytes, with the mean adduct level of 7.0 ± 0.7 adducts per 10^7 nucleotides in liver and 7.4 ± 0.7 adducts per 10^7 nucleotides in lymphocytes of rats sacrificed immediately after cessation of exposure. The approximate half-life of 7-HEG was 5 days in liver and 3 days in lymphocytes, which is consistent with the loss of adduct primarily by spontaneous depurination. In addition, the background levels of 7-HEG and 7-MG were determined in the liver and lymphocytes from the control rats.

Keywords: *ethene, DNA adducts, HPLC, 7-(2-hydroxyethyl)guanine.*

Abbreviations: *DMS, dimethylsulphate; ETO, ethylene oxide; 7-HEG, 7-(2-hydroxyethyl)guanine; HPLC, high pressure liquid chromatography; 7-MG, 7-methylguanine; TLC, thin-layer chromatography.*

Introduction

Ethene is one of the most important chemicals in the petrochemical industry (IARC 1994). Its main source in the urban atmosphere is vehicle exhaust (Boström *et al.* 1994). Ethene is also one of the major volatile organic constituents of cigarette smoke (Törnqvist *et al.* 1986). Endogenous sources of ethene include lipid peroxidation of unsaturated fats and metabolism of intestinal bacteria (Törnqvist *et al.* 1989). *In vivo* ethene is metabolized by the cytochrome P-450 dependent mixed function oxidases to ethylene oxide (ETO) (Sipes and Gandolfi 1991), a direct-acting alkylating agent that is carcinogenic in rodents and genotoxic in humans. However, mutagenicity has not been demonstrated for ethene (IARC 1994).

Several experiments have demonstrated the *in vivo* formation and persistence of 7-HEG in DNA following single or multiple exposures of mice or rats to ETO. ETO reacts with DNA by an S_N2 mechanism, which favours efficient alkylation of the N-7 position of guanine (Walker *et al.* 1992). The proportions of 7-HEG, 3-(2-hydroxyethyl)-adenine and O^6-(2-hydroxyethyl)guanine in DNA are 200: 8.8 : 1 after *in vitro* incubation (Segerbäck 1985, 1990). The formation of 7-alkylguanines in DNA has also been demonstrated in rats exposed to ethene by inhalation (Segerbäck 1983, Eide *et al.* 1995, Swenberg *et al.* 1995). Eide *et al.* (1995) also demonstrated that among the C2–C8 1-alkenes, ethene caused the highest levels of 7-alkylguanines. The purpose of the present study was to quantify DNA adduct formation after ethene inhalation in rats and to compare the level and persistence of ethene-related DNA adducts in liver and in a surrogate tissue (lymphocytes). In addition, the background levels of 7-HEG and 7-MG in DNA were also studied as previous studies have shown various background levels of 7-alkylguanines (Föst *et al.* 1989, Walker *et al.* 1992, van Delft *et al.* 1994, Swenberg *et al.* 1995).

MATERIALS AND METHODS

Animals and exposures

Male Sprague–Dawley rats were provided by Møllegaard A/S, L1. Skensved, Denmark. The animals were acclimatized for at least 5 days before the start of exposure. Food and water were given *ad libitum* except during exposure. At the start of each experiment the weight of the animals ranged from 203 to 215 g.

The inhalation study was carried out as described previously (Eide *et al.* 1995). Animals were exposed to ethene in conically shaped 0.7 m^3 steel chambers with glass front doors and walls. There were five or six animals in each cage and a maximum of four cages in each inhalation chamber. Air exchange rate was 15 per hour. Temperature and humidity were kept within limits of 22 ± 0.3 °C and 65 ± 5% relative humidity, respectively. Ethene was delivered by HydroGas AS, Oslo, Norway with > 99.5% purity. The aimed concentration of ethene was generated by introducing a controlled stream of pure gas delivered by a two-stage precision pressure regulator. Exposures were performed during daytime for 12 h a day (09 : 00–21 : 00) for three consecutive days. Light/dark cycles of 14 h light (08 : 00–22 : 00) and 10 h dark (22 : 00–08 : 00) were kept during acclimatization and exposure. Control groups of animals were treated identically with the exposed groups except for the absence of ethene. The concentration of ethene in the inhalation chamber was monitored hourly by gas chromatography. The mean concentration (± SD) of ethene for 3 days' exposure was 296 ± 11 ppm.

Sampling

Immediately after the last exposure, four animals were removed from the chamber, one at a time as described previously (Eide *et al.* 1995), for immediate decapitation and sample preparation (day 0). In addition, groups of four animals were sacrificed at 18 h (day 1), 115 h (day 5) and at day 20 after the last exposure. Control animals were sacrificed at day 0 and day 20. Blood (5–7 ml) was collected directly into heparinized (1000 IU

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were isolated with NycoPrep 1.077 Animal TM before they were frozen in nitrogen and stored at – 80 °C. Liver tissue (1.5–2 g from lobus sinister) was transferred to glass vials and frozen at – 80 °C.

DNA isolation

DNA was isolated from cellular nuclei of livers and lymphocytes as described by Gupta (1982). Briefly, the nuclear pellets were treated with RNase A and T1 in 50 mM Tris Cl buffer, pH 8.0, followed by pancreatic ribonuclease and proteinase K treatment. Proteins were removed by phenol extraction followed by chloroform : isoamyl alcohol extraction. DNA was precipitated by ethanol and washed with 70% ethanol. The precipitated DNA was redissolved in water, concentration measured by absorption at 260 nm with a spectrophotometer and RNA contamination (< 2%) checked by HPLC after digestion to nucleosides, with nuclease P1 and alkaline phosphotase.

Preparation of standards

7-(2-Hydroxyethyl) and 7-methyl-deoxyguanosine-5′-monophosphate adducts were prepared by reaction of the unmodified nucleotide with ETO and dimethylsulphate (DMS) respectively followed by purification on HPLC (Kumar and Hemminki 1996). DNA standards for these two adducts were obtained by treating salmon testis DNA with ETO and DMS respectively and the level of 7-alkylguanine adducts was determined by depurination at neutral pH, as described earlier (Kumar *et al.* 1995).

^{32}P-postlabelling

Analysis of 7-alkylguanine in DNA by the postlabelling assay was performed as described previously (Kumar *et al.* 1995, 1996) with minor modifications. Briefly, 5 μg DNA from livers and lymphocytes, as well as DNA reacted *in vitro* with DMS and ETO, were incubated at 37 °C with micrococcal nuclease (80 mU per μg DNA, 2 μl) in 3 mM Bicine (0.5 μl), pH 9.0 and 0.2 mM $CaCl_2$ (0.5 μl) for 2 h followed by addition of spleen phosphodiesterase (1.6 mU per μg DNA, 2.0 μl) and 20 mM ammonium acetate (1.0 μl), pH 5.0. The incubation was continued for a further 2 h. The adduct enrichment was carried out by applying DNA digests to strong anion exchange cartridges. ^{32}P-postlabelling was carried out in a total volume of 2.0 μl containing 2 pmoles [γ-^{32}P]ATP, 40 mM bicine buffer, pH 9.6, 20 mM spermidine and 6U T4 polynucleotide kinase. The mixture was incubated at 37 °C followed by incubation with nuclease P1 (2.5 μg, 0.5 μl) for 15 min. ^{32}P-postlabelled mixtures were applied to pre-washed 10 × 20 cm PEI TLC plates and developed with 0.1 M ammonium formate, pH 5.2 in the first dimension (D1) and 0.6 M ammonium formate, pH 5.2, mixed with 40% *n*-propanol in the second dimension (D2). The plates were exposed to X-ray films (Kodak XAR-5) for 2 h at – 80 °C. The areas of the plates corresponding to adduct spots on autoradiographs were cut and counted in a Cerenkov counter. The corresponding areas of the plates containing samples depurinated prior to labelling were used as background.

Analysis of 7-HEG and 7-MG by HPLC was carried out as previously described (Kumar and Hemminki 1996). The areas of plates corresponding to 7-alkylguanine were extracted with 10 mM ammonium formate, pH 5.3, by sonication. For HPLC analysis, the filtered and freeze-dried samples were diluted to 20 μl with water and injected into the Beckman HPLC System Gold equipped with a Kromasil C18 (150 × 2.0 mm) column. The retention times of the adducts were confirmed by analysing the aliquots of TLC extracts spiked with synthesized 7-(2-hydroxyethyl) and 7-methyl-deoxyguanosine-5′-monophosphate adducts which were used as UV markers (Kumar and Hemminki 1996). The separation was performed using a gradient, started at 100 % 0.2 M ammonium formate buffer, pH 4.6 for 10 min, followed by a linear gradient over the next 10 min to 10% methanol, which was maintained for 10 min. The methanol concentration was increased to 100 % in the next 10 min. 7-HEG and 7-MG adducts eluted separately at retention times of 6.9 and 8.2 min, respectively.

Analysis of results

The *in vitro* modified DNA with DMS and ETO were labelled in parallel to each set of rat DNA samples. The absolute adduct level in DMS and ETO modified DNA was determined by depurination and measurement of released 7-alkylguanine adducts by HPLC coupled with a diode array detector (Kumar and Hemminki 1996). The recovery of adducts from these *in vitro* samples were used to correct the levels of adducts in the rat DNA samples.

Results

The level of 7-alkylguanine adducts in *in vitro* modified DNA with DMS and ETO was found to be 33.8 and 37.5 adducts per 10^6 nucleotides respectively. The recoveries of 7-alkylguanine adducts in ^{32}P-postlabelling experiments were determined by using DNA modified *in vitro* with DMS and ETO. The recoveries were determined in each individual experiment and the values obtained were used to correct the adduct levels determined in rat livers and lymphocytes. Figure 1(A) shows the ^{32}P-postlabelling analysis of a DMS–DNA standard. A similar pattern of adduct spots was found in the ETO–DNA standard. The total recovery of 7-HEG and 7-MG adduct was 9.5 ± 3.8 (n = 6) and 20.0 ± 4.2 (n = 6) percent, respectively.

The patterns of adduct spots were similar in liver and lymphocyte DNA samples from both rats exposed to 300 ppm ethene for 3 days (12 h per day) (Figure 1(B, D)) and control rats (Figure 1(C, E)). One of these spots (7-alkylguanine) co-chromatographed with the postlabelled and nuclease P1 treated standards; 7-(2-hydroxyethyl)-deoxyguanosine-5′-monophosphate and with 7-methyl-deoxyguanosine-5′-monophosphate. However, no 7-alkylguanine adducts other than 7-HEG and 7-MG were detected in the TLC spot of the DNA samples. Thus, the term 7-alkylguanine is used here to denote the combined fraction of 7-HEG and 7-MG. The adduct

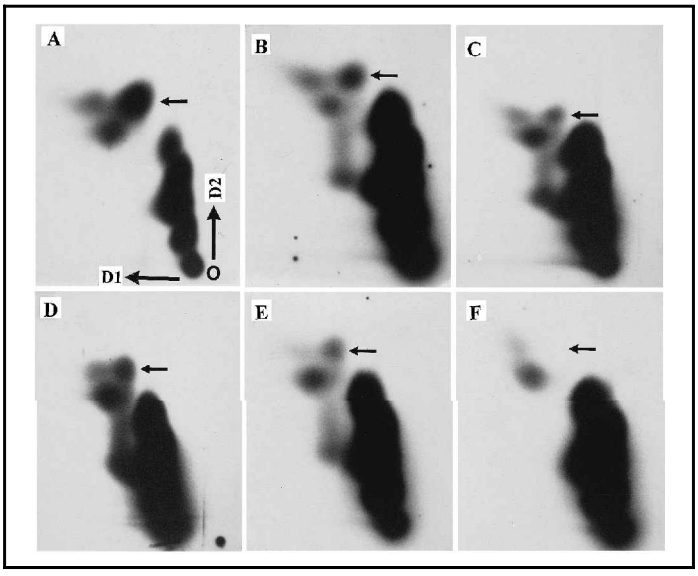

Figure 1. TLC analysis of (A) salmon testis DNA modified *in vitro* with DMS; (B) liver DNA from rats exposed to 300 ppm ethene for 3 days (12 h per day); (C) liver DNA from control rats; (D) lymphocyte DNA from rats exposed to 300 ppm ethene for 3 days (12 h per day); (E) lymphocyte DNA from control rats; (F) liver DNA as in (B) but depurinated prior to ^{32}P-postlabelling. 7-Alkylguanine adducts are indicated with arrows. D1 and D2 are the directions of chromatography and 'O' is the origin.

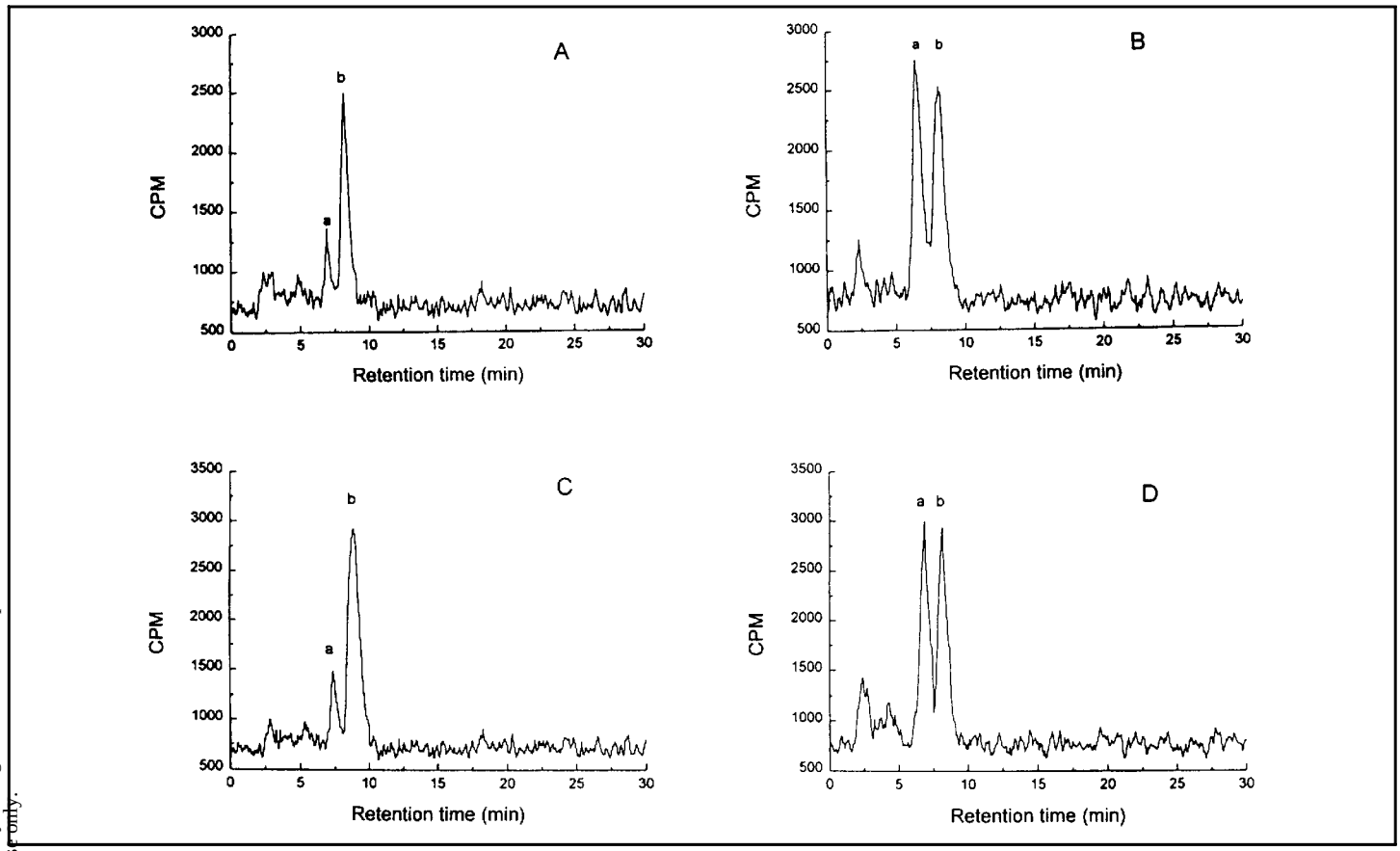

Figure 2. HPLC-radioactivity detector analysis of 7-HEG (peak 'a') and 7-MG adducts (peak 'b') in liver DNA from a control rat (A), exposed rat killed immediately after cessation of 3 days (12 h per day) of exposure to 300 ppm ethene (B); and in lymphocyte DNA from a control rat (C), exposed rat killed immediately after cessation of exposure (D). The radioactive peaks were obtained from excision of corresponding TLC spots.

spot was not present if the DNA samples were depurinated (boiled at 100 °C for 20 min) prior to ^{32}P-postlabelling (Figure 1(F)). The 7-alkylguanine adduct spots were analysed on HPLC after their extraction from TLC plates. This resulted in the separation of 7-HEG and 7-MG adducts whose identities were established by their co-migration with synthesized 7-alkyl-5′-dGMP standards used as UV markers. Figure 2 shows representative chromatograms using HPLC for separation of 7-HEG and 7-MG adducts in livers of control (A) and exposed rat killed immediately at the end of 3 days (12 h per day) of exposure to 300 ppm ethene (B), and also in lymphocytes of control (C) and exposed rat (D).

The levels of 7-alkylguanine, 7-HEG and 7-MG adducts in liver and lymphocyte DNA samples of the exposed and control rats killed up to 20 days after cessation of exposure are shown

Sample	Group	7-Alkyl guanine	7-HEG	7-MG
Liver				
	Exposed day 0	9.1 ± 0.7	7.0 ± 0.7	2.1 ± 0.1
	Exposed day 1	7.4 ± 0.4	5.4 ± 0.4	2.0 ± 0.1
	Exposed day 5	6.4 ± 0.3	4.0 ± 0.3	2.4 ± 0.2
	Exposed day 20	3.1 ± 0.7	0.9 ± 0.2	2.2 ± 0.6
	Controls day 0	3.0 ± 0.6	0.9 ± 0.2	2.1 ± 0.4
	Controls day 20	2.6 ± 0.3	0.8 ± 0.1	1.8 ± 0.2
Lymphocytes				
	Exposed day 0	9.6 ± 0.7	7.4 ± 0.7	2.2 ± 0.1
	Exposed day 1	7.7 ± 0.6	5.5 ± 0.6	2.2 ± 0.2
	Exposed day 5	5.2 ± 0.4	3.0 ± 0.4	2.2 ± 0.2
	Exposed day 20	3.0 ± 0.1	0.9 ± 0.1	2.1 ± 0.2
	Controls day 0	3.2 ± 0.1	0.9 ± 0.1	2.3 ± 0.1
	Controls day 20	3.0 ± 0.2	0.9 ± 0.1	2.1 ± 0.2

Table 1. Level of 7-alkylguanine, 7-HEG and 7-MG adducts (adducts per 10^7 nucleotides; mean ± SD) in livers and lymphocytes of the exposed and control rats killed up to 20 days after cessation of 3 days (12 h per day) of exposure to 300 ppm ethene. ^{32}P-postlabelling was used for DNA adduct analysis.

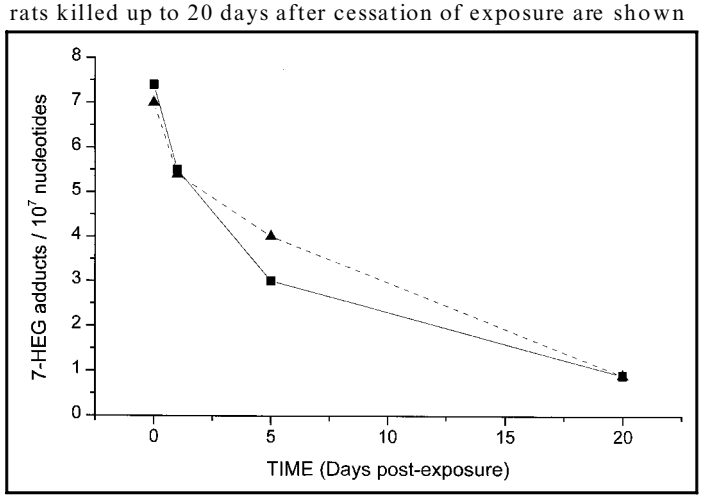

Figure 3. Persistence of 7-HEG in liver (▲) and lymphocyte (■) of rats killed up to 20 days after cessation of 3 days (12 h per day

in Table 1. A similar amount of 7-alkylguanine and 7-HEG adducts was found in surrogate tissue (lymphocytes) and internal tissue (liver) of rats killed immediately after 3 days of exposure to 300 ppm ethene. Thus, the mean levels of 7-alkylguanine and 7-HEG in liver were 9.1 and 7.0 adducts per 10^7 nucleotides and in lymphocytes they were 9.6 and 7.4 adducts per 10^7 nucleotides, respectively. The accumulated 7-HEG adduct level after 3 days of exposure was ~8-fold higher than that in the control rats. The level of 7-MG adduct was constant in liver and lymphocytes of both the exposed and the control rats during the 20-day time course of the study. The results also show that the background level of 7-MG was ~2.5-fold higher than that of 7-HEG in both liver and lymphocytes of control rats.

The persistence of 7-HEG in liver and lymphocytes was determined in DNA of rats killed up to 20 days following the 3-day exposure to 300 ppm ethene (Figure 3). The loss of 7-HEG was rapid from the liver and the lymphocytes, with a half-life of 5 and 3 days, respectively. Twenty days after exposure, the concentrations of 7-HEG in liver and lymphocytes of the treated rats were not statistically different from those found in the tissues of control rats. The half-life for 7-HEG in liver and lymphocyte was calculated from a semilog arithmetic plot (Figure 4).

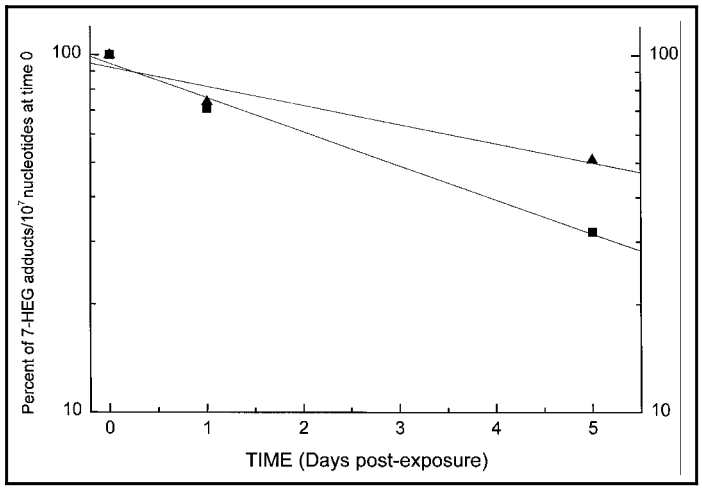

Figure 4. Removal of 7-HEG from DNA as a function of time after 3 days of exposure of rats (▲, liver; ■, lymphocyte) to 300 ppm ethene. The background levels are subtracted from the amounts of 7-HEG adducts, and the amount of 7-HEG in tissues from rats killed at 0 day after exposure was assumed 100%.

Discussion

Analysis of tissues of rats exposed to 300 ppm ethene (12 h per day; for 3 days) by inhalation demonstrated that 7-HEG accumulates in liver and lymphocytes. The concentration of 7-HEG in DNA of rats killed immediately after exposure was similar in liver (7.0 adducts per 10^7 nucleotides) and lymphocytes (7.4 adducts per 10^7 nucleotides). The determination of concentration of free ethene in rat tissues showed that they were similar in blood (0.3 μmol kg^{-1}) and liver (0.4 μmol kg^{-1}) of rats following ethene exposure identical to the present study (Eide *et al.* 1995). Previous studies also showed that the degree of 7-HEG formation was relatively similar in tissues of rats or mice given single or multiple exposures of ETO (Osterman-Golkar *et al.* 1983, Potter *et al.* 1989, Walker *et al.* 1990).

The levels of 7-alkylguanines are in very good agreement with those reported in a previous study (Eide *et al.* 1995). Furthermore, the levels of 7-HEG in liver are between those obtained in another study after exposure to 40 and 1000 ppm ethene for 4 weeks, and comparable to the levels obtained after inhalation of 10 ppm ETO for 4 weeks (Swenberg *et al.* 1995).

Determination of the persistence of 7-HEG *in vivo* demonstrated that 7-HEG concentrations declined rapidly in liver and lymphocytes. The estimated half-lives were 3 days in lymphocytes and 5 days in liver. This is in good agreement with a previous study, which showed the *in vivo* half-life of 2.9 and 3.9 days for white blood cells and liver, respectively, of rats exposed to 100 and 200 ppm of ETO by inhalation (Walker *et al.* 1992). The rapid removal of 7-HEG *in vivo* is probably due to spontaneous depurination, because the depurination half-lives from DNA at pH 7.4, 37 °C are 53–75 h *in vitro* (Koepke *et al.* 1988, Segerbäck 1990). It is reported that 7-HEG is a substrate for 3-methyladenine DNA glycosylase II (Habraken *et al.* 1991). The relative distribution and content of this enzyme in various tissues of rats, which is unknown, may account for the slight difference of the half-life between liver and lymphocyte. Another explanation is that the lymphocyte fraction may contain a portion of granulocytes, which have a short half-life.

The background levels of 7-HEG and 7-MG were detectable in DNA from control rats. The sources of background for these two adducts may be both exogenous and endogenous (Walker *et al.* 1990). 7-HEG adducts are formed by exogenous exposure to ETO, ethene, *N*-nitrosomorpholine, and *N*-nitrosodiethanolamine (NDELA) and its metabolites (Koepke *et al.* 1988, Ludeke and Kleihues 1988, Törnqvist 1988) or endogenously formed by lipid peroxidation products (Törnqvist *et al.* 1989, Kautiainen *et al.* 1993), whereas 7-MG adducts are formed by exogenous exposure to methylating agents such as nitrosamines (Shuker and Bartsch 1994) or by endogenous methylating agents such as betaine, choline and *S*-adenosylmethionine (Barrows and Magee 1982, Rydberg and Lindahl 1982, Näslund *et al.* 1983). We found that the background levels of 7-alkylguanine and 7-MG were 3.0 and 2.2 adducts per 10^7 nucleotides respectively. These values are comparable to recent results determined by ^{32}P-postlabelling (Eide *et al.* 1995, Widlak *et al.* 1995). In addition, the background level of 7-HEG (0.9 adducts per 10^7 nucleotides) is also comparable to that obtained recently using immunological assays (Van Delft *et al.* 1994) and gas chromatography–mass spectrometry (Swenberg *et al.* 1995). However, more than 10-fold higher levels of 7-HEG (1–2 adducts per 10^6 nucleotides) were reported in humans and rodents in two earlier studies involving a gas chromatography–mass spectrometry method (Föst *et al.* 1989, Walker *et al.* 1992). As the most recent data are consistently showing low background levels, it is suggested that this is due to generally improved methodologies.

In conclusion, the present study confirms that exposure to ethene causes DNA adducts (Segerbä

Swenberg *et al.* 1995). Our results showed that 7-HEG was removed rapidly by spontaneous depurination. Although the small 7-alkylguanine adducts are not considered to be promutagenic, apurinic sites have been shown to lead to miscoding during DNA replication (Takeshita and Eisenberg 1994), suggesting that 7-HEG can probably play a role in ethene-induced genotoxicity. The determined adduct levels can be used in risk estimation, by comparing DNA adduct levels induced by ETO, a known mutagen and carcinogen, to those induced by ethene, whose possible mutagenicity and carcinogenicity remains to be determined. The results show additionally that in the rat lymphocyte DNA adducts predict quite well the adduct levels in the liver, with implications to human biomonitoring.

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