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Comparison of ethylene, propylene and styrene 7,8-oxide in vitro adduct formation on N-terminal valine in human haemoglobin and on *N*-7-guanine in human DNA

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Abstract

Epoxides react at various nucleophilic sites in macromolecules such as haemoglobin and DNA. To study the reaction rate constants of ethylene oxide (EO), propylene oxide (PO) and styrene 7,8-oxide (SO) towards two of these positions, i.e., the N-terminal valine in haemoglobin and *N*-7-guanine in DNA was the central aim of this investigation. These two reactive sites are the most studied haemoglobin and DNA adducts, respectively. Further attention, therefore, was also paid to the applicability in vivo of the in vitro determined reaction constants. The determination of the second-order rate constants between EO and PO and N-terminal valine in Hb [2.7 l (mol Hb h)$^{-1}$ and 1.0 l (mol Hb h)$^{-1}$, respectively] were consistent with the literature values. The constants for the reaction with *N*-7-guanine [16×10^{-3} l (mol DNA nucleotide h)$^{-1}$ and 7.7×10^{-3} l (mol DNA nucleotide h)$^{-1}$, respectively] were lower than previously published values, probably due to differences in the methodology used. The use of the in vitro obtained values to model the in vivo situation lead to a consistent picture for EO and PO. In contrast, for SO the in vitro ratio between the adduct formation on N-terminal valine [1.5 l (mol Hb h)$^{-1}$] and *N*-7-guanine [0.71×10^{-3} l (mol DNA nucleotide h)$^{-1}$] was about two orders of magnitude greater than for the in vivo situation. This was probably due to a lower than expected reactivity of SO towards N-terminal valine in vivo. Further research is needed to elucidate whether the use of SO in vitro, contrasting with the in vivo experiments in which SO was metabolically formed from styrene, could entail an explanation for this discrepancy. Concerning the methodological part, the use of dipeptide standards to replace the alkylated globins as standard lead to an improvement of the method. Especially the commercial availability of the standards, their stability and accurately known adduct content will make them to the standards of choice in the future.

Keywords: Epoxides; Haemoglobin adducts; DNA adducts; Edman degradation

1. Introduction

The epoxides studied are the derivatives (oxides) of ethylene, propylene and styrene, respectively, which have a widespread use as monomers in the chemical industry. Moreover, ethylene oxide and to a certain extent propylene oxide, also have a number of specific applications. Especially, their use in the gaseous form as a disinfectant, sterilising agent, fumigant or insecticide leads to relatively high exposures. The highest exposures for styrene are observed

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in the production of fiberglass-reinforced plastics, where manual application techniques are used.

The overall evaluation of the carcinogenicity of these three chemicals and their epoxides is reconsidered by the IARC [1] and is summarized in Table 1.

The metabolism of the alkenes could lead to the formation of the corresponding reactive epoxides. These epoxides are able to bind covalently to nucleophilic sites in proteins and DNA (reviewed in Refs. [1,2]). The measurement of DNA and haemoglobin adducts is a useful tool for monitoring exposure to electrophilic agents. Binding with DNA gives some information about the potential risk since DNA adduct formation is in many cases the initial step in chemical carcinogenesis [3]. The determination of DNA adducts has focused mainly on the *N*-7-adducts on guanine, since these are by far the main DNA adducts formed with the three epoxides (reviewed in Refs. [1,2]). Regarding haemoglobin adducts, up to now, only the modified Edman degradation technique has found to be a practical method for the determination of N-terminal valine adducts formed by electrophilic agents. The method developed by Törnqvist et al. [4] provides a rapid, sensitive and specific technique for the determination of adducts on the N-terminal valine in haemoglobin.

For EO, a large number of studies are available. This compound, therefore, has been used as a model for dose monitoring and risk estimation [5–7]. Moreover, EO may be an important contributor to the collective genotoxic dose to human populations, mainly as metabolite of ethylene arising from endogenous production, from environmental sources (plant hormone and combustion product) or from cigarette smoking [6].

The reaction rate constants of the three epoxides towards N-terminal valine in haemoglobin has been determined in different in vitro experiments using human blood [8–10]. The reaction rate constants of EO and PO towards *N*-7-guanine in DNA, so far, has been determined in a solution of calf thymus DNA [11,9]. The comparison of the adduct formation by the three epoxides on N-terminal valine in haemoglobin and *N*-7-guanine in DNA in whole blood was the primary objective of this study. The second-order reaction rate constants between the three epoxides and either N-terminal valine in haemoglobin or *N*-7-guanine in DNA were calculated. Further attention was also paid to whether the in vitro determined reaction rate constants can be used in vivo by comparison of the ratio of the in vitro reaction rate constants towards N-terminal valine and *N*-7-guanine with the ratio between the adduct levels observed in a number of animal experiments.

Ring tests for the determination of N-terminal valine adducts of EO and SO indicated that the use of different standards for the Edman degradation method leads to differences in absolute adduct levels measured [12,13]. A secondary objective of this study, therefore, was to determine if the synthetic *N*-alkylated-dipeptide standards, which became recently available, could be used to replace the former

Table 1
Evaluations of the carcinogenicity of ethylene, propylene, styrene and their corresponding epoxides (IARC [1])

Agent	Degree of evidence of carcinogenicity		Overall evaluation of carcinogenicity for humans
	Human	Animal	
Ethylene	inadequate	inadequate	3
Ethylene oxide (EO)	limited	sufficient	1
Propylene	inadequate	inadequate	3
Propylene oxide (PO)	inadequate	sufficient	2B
Styrene	inadequate	limited	2B
Styrene 7,8-oxide (SO)	inadequate	sufficient	2A

Group 1: human carcinogen.
Group 2A: probable human carcinogen.
Group 2B: possible human carcinogen.
Group 3: not classifiable as to its carcinogenicity to humans.

standards, prepared through reaction between haemoglobin and the epoxides. In this way problems with absolute calibration of standards could be avoided in the future.

2. Materials and methods

2.1. Chemicals

Pentafluorophenyl isothiocyanate (PFPITC) was obtained from Fluka (Buchs, Switzerland) and was used without further purification. Formamide (analytical grade) was extracted with pentane before use. Ethylene oxide (50 mg/ml in methanol) was obtained from Supelco (Bellefonte, USA), 1,2-propylene oxide (> 98%) was delivered by Merck (Overijse, Belgium) and styrene 7,8-oxide (97%) by Aldrich (Bornem, Belgium). The dipeptide standards *N*-(2-hydroxyethyl)Val-Leu-anilide (> 98%) and *N*-DL-(2-hydroxypropyl)Val-Leu-anilide (> 95%) for the determination of the N-terminal valine adducts of EO and PO, respectively, were purchased from Bachem Biochemica (Heidelberg, Germany). Haemoglobin containing 192 pmol *N*-hydroxyethylvaline/mg Hb, EO-Hb, was a kind gift from N. Van Sittert, Shell, The Netherlands. The standard for the determination of SO adducts, SO-Hb, and the internal standard (I.S.) used for all analyses, [$^2H_8$]SO-Hb, was prepared as described in Severi et al. [14]. The amount of SO-Hb was found to be 2.03 nmol/mg Hb, whereas the [$^2H_8$]SO-Hb contained 1.73 nmol of *N*-(2-hydroxy-2-[$^2H_8$]phenylethyl)valine/mg Hb. The level of N-terminal valine SO-adduct in the reference globin and the I.S. was determined by acid hydrolysis of the globin, followed by the determination of the amount of alkylated valine as described in Severi et al. [14].

All other chemicals and solvents were of analytical grade and used without further purification.

2.1.1. Synthesis of N-7-(2-hydroxyethyl)guanine, N-7-(2-hydroxypropyl) guanine and N-7-(2-hydroxy-1-phenylethyl)guanine (N-7-α-SO-adduct)

The synthesis of these *N*-7-adducts was based on the method described by Latif et al. [15]. Guanosine (2.8 g) was suspended portion-wise in glacial acetic acid (75 ml) and was treated with either 5 ml EO, 10 ml PO or 12 ml SO. After 5 h stirring at 37°C the reaction mixture was diluted with 100 ml methanol. Adducts and unreacted guanosine were precipitated by the addition of 900 ml diethyl ether. After filtration the precipitate was resuspended in methanol. This allowed the removal of the unreacted guanosine by filtration. After evaporation of the solvent under N_2 the residue was dissolved in 0.1 N HCl and heated to 110°C for 30 min. After cooling to room temperature, neutralization of the solution with KOH resulted in precipitation of the adduct. After recrystallization only the *N*-7-(2-hydroxyethyl)adduct turned out to be pure by GC–MS. The propylene and styrene oxide adducts contained still some guanine and needed to undergo an additional purification step on a preparative Sep-Pak® Vac 12 cm^3 C_{18}-cartridge (Waters Chromatography Division). The cartridges were conditioned with MeOH, followed by a flushing with bidistilled water. The samples were dissolved in slightly acidified water and brought on the columns. After washing with water and 10% MeOH, the adduct was eluted from the cartridges with 40% MeOH. The procedure was repeated until the product turned out to be pure on GC–MS.

2.1.2. Synthesis of N-7-(2-hydroxy-2-phenyl)ethylguanine (N-7-β-SO-adduct) [15]

Guanosine (3 g) was suspended in ethanol: H_2O (1:1) (400 ml). The mixture was heated until the guanosine was dissolved. After addition of 4 ml styrene oxide, the solution was stirred during 3 days at 37°C. After evaporation of the solvent, the residue was redissolved in methanol. The unreacted guanosine was only partially soluble and the major part could be removed by filtration. The solvent was evaporated and the residue redissolved in 0.1 N HCl. The hydrolysis of the *N*-7-guanine adduct from the ribose was carried out at 110°C for 30 min. After cooling to room temperature the mixture was brought to pH 7 with KOH, resulting in the precipitation of the adduct together with some unreacted guanine.

The adduct was again separated from the unreacted guanine through the same C_{18}-purification technique as described above. In contrast with the reaction in acetic acid, which allowed to obtain pure *N*-7-α-SO-adduct, this reaction resulted in the formation of a mixture of *N*-7-α-SO en *N*-7-β-SO-adduct in a 3:2 ratio. The amount of *N*-7-β-SO-adduct

could be calculated by subtraction of the amount of *N*-7-α-SO-adduct from the total amount of adduct.

2.2. Elimination of the three epoxides from blood in vitro

The initial concentration of EO (328 μM) was different from the initial concentration of PO (30 μM) and SO (40 μM), due to reasons of sensitivity of detection. All initial concentrations, however, were well within the range of the linear dose–N-terminal valine adduct relationship (0–1000 μM). For two reasons, to determine the second-order rate reaction constant between SO and *N*-7-guanine an elimination constant at a higher initial concentration (50 mM) was calculated. Firstly, the determination of *N*-7-SO-guanine adducts required higher incubation concentrations of the epoxide compared to EO and PO. Secondly, the elimination constant of SO was relatively high compared to the other epoxides. Only for SO, therefore, and not for EO and PO, substantial effects on the second-order rate reaction constants were expected from a slower elimination at higher incubation concentrations (see Section 3).

The apparent first-order elimination constant (k_e) for EO was estimated as follows. All materials used for the handling of the solutions containing EO were kept at −20°C before use because of the low boiling point of EO (11°C). The incubation of blood with EO, was carried out in completely filled and tightly closed vials. The solution of 50 mg EO/ml methanol was diluted in order to obtain a stock solution of 6.5 mg/ml. To six different vials containing 1.5 ml blood EO solution was added to a final concentration of 328 μM EO. The blood was spiked with EO by inserting the needle of a cooled Hamilton syringe through the membrane, avoiding an increase of pressure in the vial. After mixing, the vials were placed in an oil bath at 37°C. At convenient intervals (0, 15, 30, 60, 90 and 120 min) the content of one of the vials was extracted in a standardized way with 750 μl ethyl acetate. The ethyl acetate layer was injected, without further purification on a HP 5890 series II gas chromatograph with a PORAPLOT Q fused silica capillary column (25 m × 0.32 mm, phase thickness 10 μm) from Chrompack (Bergen op Zoom, The Netherlands). The chromatograph was equipped with a flame ionization detector and an automatic injector (1 μl) with an inlet splitter (1:10). Helium was used as the carrier gas. The oven was programmed at 20°C from 130 to 200°C, which was held for an additional 7.5 min. The EO concentration was determined from established calibration curves. The relative concentration at 0 min was set to 100%.

Ten microliters of a stock solution of PO (105 μl in 100 ml saline) was added to 10 ml freshly drawn human blood resulting in a 30 μM initial concentration. The blood was incubated in an oil bath at 37°C and at 0, 5, 15, 30, 45, 60, 90 and 120 min intervals aliquots of 1 ml blood were removed and immediately extracted with 1 ml ethyl acetate. The amount of PO in the ethyl acetate layer was determined in the same way as described for EO.

The first-order elimination rate constant for SO human blood was estimated essentially as described in Rappaport et al. [16] and Yeowell-O'Connell et al. [10]. Briefly, freshly drawn human blood was incubated with SO at an initial concentration of 40 μM. One milliliter aliquots were taken at 0, 5, 10, 15, 20, 30, 60, 90 and 120 min intervals and immediately extracted with hexane containing [$^2H_8$]SO as the I.S. After drying of the hexane layer the SO concentration was determined by GC–MS. The elimination experiment was repeated with an initial concentration of 50 mM, since for the modification of DNA in vitro, higher incubation concentrations were required.

2.3. Modification of haemoglobin and DNA by in vitro incubation of whole blood

2.3.1. Modification of haemoglobin

Again, the necessary precautions were taken to minimalize the influence of the high volatility of EO on the results. The solution of 50 mg EO/ml methanol was diluted in order to obtain a stock solution of 6.5 mg/ml. Sufficient amounts of this solution were added to 1.5 ml blood, resulting in final concentrations of 9.8, 29.5, 98.4, 295 and 984 μM.

PO (639 μl/10 ml acetone) was added to 3 ml human blood to obtain the final concentrations of 0, 10, 30, 100, 300 and 1000 μM. The same final concentrations were obtained by addition of sufficient amounts of SO (144 μl in 10 ml isotonic saline) to 4 ml aliquots of human blood [10].

All blood samples were occasionally stirred during a 2 h incubation at 37°C.

2.3.2. Modification of DNA

Similar procedures were used for the incubation of blood to determine the formation of *N*-7-guanine adducts, except that the initial concentrations had to be higher, because of the lower sensitivity. For EO, sufficient amounts of a 1 M aqueous solution (stored at −80°C) were added to 10 ml blood to obtain initial concentrations of 1, 2, 5 and 10 mM. The blood samples were incubated for 3 h at 37°C.

Five milliliters freshly drawn blood was spiked with 2, 4, 18 and 35 μl PO, resulting in initial concentrations of 5, 11, 51 and 100 mM, respectively. Amounts of 5.5, 29, 57, 143 and 285 μl SO were added to 5 ml blood in order to obtain initial concentrations of 9.4, 49, 96, 240 and 460 mM, respectively. The samples were incubated for 2 h at 37°C, with occasional stirring.

2.4. Determination of N-terminal valine adducts

2.4.1. Isolation of Hb

Red blood cells were separated from plasma by centrifugation at 800 g for 10 min and were washed with 3 volume of saline prior to lysis with 1 volume of water. Haemoglobin was purified by the method of Mowrer et al. [17]. Briefly, acidic propanol was added to the red blood cells and, following centrifugation, the globin was precipitated by the addition of ethyl acetate. The globin was filtered and washed with ethyl acetate and pentane and then dried by passing a gentle stream of air over the globin.

2.4.2. Derivatization

N-terminal valine adducts were analysed using the method of Törnqvist et al. [4] as described in Severi et al. [14] and Pauwels et al. [13]. Fifty milligrams globin and 10 μl I.S. (containing 1.2 mg globin/ml and 1.73 nmol *N*-(2-hydroxy-2-[$^2H_8$]phenylethyl)-valine/mg Hb) were reacted with pentafluorophenyl isothiocyanate under basic conditions. The resulting thiohydantoin derivatives were extracted into diethyl ether, washed with $NaHCO_3$ and dissolved in toluene for analysis by GC–MS.

Table 2
Comparison of GC–MS conditions for the three epoxides studied

	EO	PO	SO
Standard(s)	globin alkylated in vitro with EO, containing 192 pmol adduct/g Hb *N*-(2-hydroxyethyl)Val-Leu-anilide (> 98%)	*N*-DL-(2-hydroxypropyl) Val-Leu-anilide (> 95%)	globin alkylated in vitro with SO, containing 2.03 nmol adduct/g Hb
Internal standard	globin alkylated in vitro with [$^2H_8$]styrene oxide, containing 1.73 nmol adduct/g Hb		
Oven program	2°/min from 170°C to 200°C 4°/min to 234°C 45°/min to 300°C	10°/min from 170°C to 210°C 2°/min to 245°C 45°/min to 300°C	5°/min from 150°C to 250°C 10°/min to 300°C
Retention times	adduct: 8.4 min internal standard (I.S.) diastereoisomer 1: 20.5 min diastereoisomer 2: 21.6 min	adduct diastereoisomer 1: 4.7 min diastereoisomer 2: 4.75 min I.S. diastereoisomer 1: 11.6 min diastereoisomer 2: 12.6 min	adduct diastereoisomer 1: 17.0 min diastereoisomer 2: 17.7 min I.S. diastereoisomer 1: 16.9 min diastereoisomer 2: 17.6 min
Monitored ions	EO-adduct: m/z 308 I.S.: m/z 326	PO-adduct: m/z 325 I.S.: m/z 326	SO-adduct: m/z 325 I.S.: m/z 326

2.4.3. Determination by means of GC–MS

The most important differences for the determination of the three adducts are given in Table 2. Adduct formation of SO on the N-terminal valine in haemoglobin occurs mainly through the β-carbon of SO resulting in the formation of two diastereoisomers of *N*-(2-hydroxy-2-phenyl)ethylvaline [18]. Also *N*-(2-hydroxypropyl)valine, but not the EO-adduct, contains two chiral carbons leading to the formation of two diastereoisomers. The analyses were carried out using a HP 5890 Series II gas chromatograph coupled to a HP 5970 quadrupole mass spectrometer (EI mode) and a standard chemstation G1701AA version 03.00. The chromatographic separation of the different components in the samples was made on a DB-5-ms fused silica capillary column (30 m × 0.32 mm, 0.12 μm phase thickness). Helium was used as the carrier gas.

The interface temperature was 270°C and the source pressure was 0.005 Pa. Analyses were carried out using selected ion monitoring. Due to the manual injection of the samples the observed retention times could deviate sometimes from the values given in Table 2. The identification of the peaks was assured by the determination of the retention time relative to the I.S.

2.4.4. Calibration

Calibration curves were prepared through the addition of different amounts of the calibration reference globin or alkylated dipeptide to a constant amount of I.S. The two calibration curves for EO were established between 0 and 449 pmol adduct/g Hb and 0 and 575 pmol adduct/g Hb for *N*-(2-hydroxyethyl)Val-Leu-anilide and EO-Hb, respectively. The calibration curve for PO was established from calibration with *N*-(2-hydroxypropyl)Val-Leu-anilide as standard between 0 and 523 pmol PO-adduct/g Hb and for SO with SO-Hb as standard between 0 and 406 pmol SO-adduct/g Hb. All established calibration curves were linear. Although it should be better to use different deuterated analogues as I.S. for each N-terminal valine adduct separately, the obtained correlations for the calibration curves were considered sufficient for the purposes of this study. The correlation coefficient for the calibration curve for SO was $r = 0.9995$, whereas the correlation coefficients for EO-Hb ($r = 0.993$), *N*-(2-hydroxyethyl)Val-Leu-anilide ($r = 0.997$) and *N*-(2-hydroxypropyl)Val-Leu-anilide ($r = 0.9986$) were only slightly lower.

2.5. Determination of N-7-guanine adducts

2.5.1. Isolation of DNA

The incubation experiments were carried out in Vacutainer® tubes, with EDTA or sodium citrate as anticoagulant. The isolation procedure for EO was slightly different, since the samples were initially prepared for HPLC/Electrospray Mass Spectrometry, requiring a higher purity. After the incubation with EO, blood lymphocytes were isolated using Leucoprep tubes (Becton Dickinson, Aalst, Belgium), following the manufacturer's protocol. For the other epoxides, after centrifugation at 1200 × *g* for 10 min, the lymphocytes were transferred to a clean tube. After washing with phosphate buffered saline (2×) and 0.9% NaCl, the cells were lysed by addition of a cold (4°C) hypotonic solution (20 mM Tris HCL, pH 8.0, 10 mM EDTA). After pelleting the cells at 4000 rpm the supernatant was discarded. To the pellet 5 ml DNAzol™ reagent (Gibco BRL, Life Technologies, Merelbeke, Belgium) was added. To reduce the RNA amount (< 3%) the solution was centrifuged for 10 min at 10,000 × *g*. DNA was precipitated from the supernatant, which was transferred to a clean tube, by the addition of 2.5 ml ethanol. The DNA pellet, obtained after centrifugation, was washed twice with 95% ethanol and twice with 70% ethanol. For the incubation with EO, the DNA was air dried and weighted with an analytical balance. The determination of the amount of DNA extracted from the blood for the PO and the SO-experiment, was done by solving the DNA in 1.5 ml water followed by spectrophotometrical determination, assuming that one A_{260} unit equals 50 μg double stranded DNA per ml. The A_{260}/A_{280} ratio, which was between 1.6 and 1.9 for all extractions, was routinely determined to see if the DNA was free of proteins. The DNA was dried by lyophilization.

2.5.2. Determination by means of HPLC-UV

The DNA samples were dissolved in 100 μl water and submitted to a neutral thermal hydrolysis

(100°C-30 min) in order to release the *N*-7-adducts. Samples (25 μl) were injected without any further derivatization on a HP 1090 Series II Liquid Chromatograph coupled with a HP 1040 Diode Array Detector. The products were separated on a 4 × 25 mm reverse phase C_{18}-column, filled with Nucleosil-100 with 5 μm particles (Machery–Nagel, Germany). The eluent used for HPLC was a 0.1 M ammonium acetate buffer (pH = 5.2). The best separation was obtained with a methanol gradient going from 6% to 10% in 12 min for EO, 6% to 10% in 14 min for PO and from 6% to 30% in 18 min for SO. The flow was 1 ml/min. For quantitative determinations the UV-signal was measured at two wavelengths: λ = 254 nm and 282 nm, with a band width of 4 nm.

2.5.3. Calibration

In contrast with the situation for EO, for PO theoretically the formation of two regioisomers of the *N*-7-guanine adduct is possible. The reaction with PO, however, resulted only in the formation of the β-adduct *N*-7-(2-hydroxypropyl)guanine in detectable quantities. In contrast, the reaction between *N*-7-guanine and SO results in the formation of two regioisomers, i.e., *N*-7-(2-hydroxy-1-phenyl)ethylguanine and *N*-7-(2-hydroxy-2-phenyl)ethylguanine [19]. The SO-adduct formation on *N*-7-guanine was determined as the sum of both isomers. Calibration curves for all adducts were prepared through the injection of different solutions containing 10^{-7}–10^{-6} M adduct. All absolute calibration curves were linear in the concentration range studied.

3. Results

3.1. Comparison of the calibration curves obtained with EO-Hb and N-(2-hydroxyethyl)Val-Leu-anilide as standard

In Fig. 1, calibration curves for the determination of *N*-(2-hydroxyethyl)valine are presented. The calibration curve obtained from haemoglobin alkylated with EO shows a less steep slope than the curve established from the alkylated dipeptide as standard. Although the difference between both curves is small, a possible explanation may be the difference in efficacy of the Edman degradation reaction between the dipeptide standard and the globin standard.

3.2. Elimination of the epoxides from whole blood

If the epoxides are uniformly mixed and the concentration is low relative to the pool of available

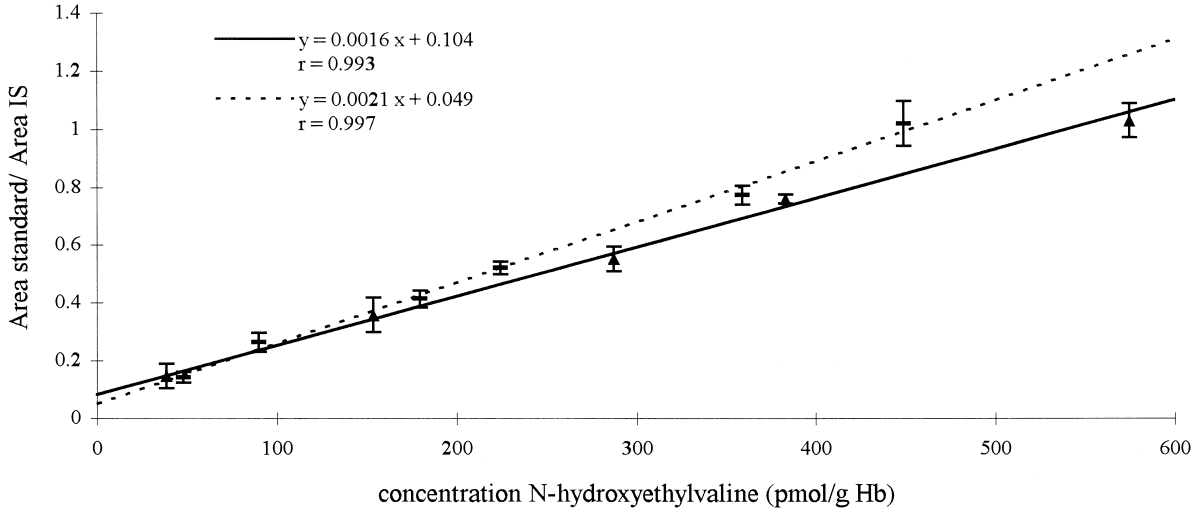

Fig. 1. Calibration curves (area standard/area I.S. in function of concentration standard) for the determination of *N*-(2-hydroxyethyl)valine with either EO-Hb (– – – –) or *N*-2-hydroxyethyl-Val-Leu-anilide (–▲–) as standard and [$^2H_8$]*SO* as I.S.

Table 3
Elimination constants and half-lives for the three epoxides in human blood

Agent	Initial concentration (μM)	k_e (h^{-1})	$t_{1/2}$ (h)
EO	328	0.20	3.5
PO	30	0.05	13.6
SO	40	0.78	0.90
	50,000	0.11	6.4

nucleophiles in DNA and haemoglobin, then the overall rate of reaction is pseudo-first-order, with the rate constant k_e representing the sum of all first-order rate constants of the individual reactions [20]. This fits with the observation that in all elimination experiments, even for the incubation with a high concentration of SO, first-order kinetics turned out to be the most appropriate model to describe the results. Accordingly, the concentration of the epoxide, [Epox], as a function of the incubation time can be described as $[\text{Epox}] = [\text{Epox}]_0 e^{-k_e t}$, with $[\text{Epox}]_0$ being the initial concentration. Values of the elimination constant, k_e and the corresponding half-life for the different epoxides are given in Table 3.

3.3. Reaction of N-terminal valine and N-7-guanine with the epoxides in vitro

The second-order rate constants for the reaction of the two site-specific adducts studied can be calculated using the following relationship (derived from Ref. [21]):

$$k_{\text{Epox}-Y} = \frac{[\text{Epox} - Y](k_e)}{[Y][\text{Epox}]_0(1 - e^{-k_e t})} = \frac{\beta_Y(k_e)}{[Y](1 - e^{-k_e t})}$$

with $k_{\text{Epox}-Y}$: second-order reaction constant (l mol^{-1} h^{-1}); $[\text{Epox} - Y]$: adduct concentration in μM; k_e: elimination constant of the epoxide (h^{-1}); $[Y]$: haemoglobin or DNA nucleotide concentration in human blood (M); $[\text{Epox}]_0$: initial epoxide concentration in μM; β_Y: slope of the linear regression of the adduct concentration on the initial epoxide concentration (μM/μM and forced through the origin); t: incubation time (h);

The value used for the blood concentration of haemoglobin (2.3 mM) was taken from the literature [22]. The DNA Isolation kit allowed to isolate an average yield of 350 μg DNA/10 ml blood collected from healthy human subjects (= 113 μM DNA nucleotide), ranging from 200–700 μg/10 ml. With these data, the equation was worked out and the results are presented in Table 4. The haemoglobin adduct formation was linear for the whole concentration range studied. For the determination of the DNA binding, the formation of *N*-7-EO and *N*-7-PO-guanine was linear up to epoxide concentrations of about 10 mM. For SO, the least reactive epoxide towards DNA, the dose–adduct relationship was linear up to 240 mM. At this concentration, however,

Table 4
Second-order rate constants [l (mol Hb or DNA nucleotide h)$^{-1}$] for the three epoxides in human blood

Agent	β^a_{Hb}	$k_{\text{Epox-Hb}}$	β^b_{DNA}	$k_{\text{Epox-DNA}}$	Ratio $k_{\text{Epox-Hb}}/k_{\text{Epox-DNA}}$
EO	12×10^{-3}	2.7	2.6×10^{-6}	16×10^{-3}	169
PO	4.5×10^{-3}	1.0	0.60×10^{-6}	5.1×10^{-3}	196
SO	3.5×10^{-3}	1.5[c]	0.13×10^{-6}	0.71×10^{-3}[d]	2113

[a] β_{Hb} is the slope (μM adduct/μM epoxide) of the relationship between dose and N-terminal valine adduct in haemoglobin (linear between 0 and 1000 μM epoxide and forced through the origin).
[b] β_{DNA} is the slope (μM adduct/μM epoxide) of the relationship between dose and *N*-7-guanine adduct in DNA (linear between 0–10 mM for EO, between 0–11 mM for PO and between 0–240 mM for SO; forced through the origin).
[c] As elimination constant $k_e = 0.78$ h^{-1}, obtained with an initial SO concentration of 40 μM, was used.
[d] As elimination constant $k_e = 0.11$ h^{-1}, obtained with an initial SO concentration of 50 mM, was used.

lysis of the blood was observed, which was even more pronounced at higher concentrations.

The reaction rate constant of N-terminal valine in haemoglobin as well as of *N*-7-guanine was highest for EO. N-terminal valine in haemoglobin showed the slowest reaction rate constant towards PO, whereas for *N*-7-guanine the slowest reaction rate constant was observed for SO. In Table 4 also the ratios between the second-order reaction rate constant for haemoglobin and DNA are presented. For EO and PO comparable ratios of 169 and 196, respectively, were obtained. In contrast, for SO the ratio between $k_{\text{SO-Hb}}$ and $k_{\text{SO-DNA}}$ was 2113, which reveals an increased preference for N-terminal valine relative to *N*-7-guanine of about one order of magnitude compared to the other epoxides studied.

4. Discussion

4.1. Use of dipeptides as standard

Peptide standards have already been used for the determination of N-terminal valine adducts of EO and SO [23,13]. This study clearly demonstrates that the use of an alkylated dipeptide standard is as suitable as an alkylated globin standard for the determination of N-terminal valine adducts by means of the modified Edman degradation technique. Moreover, advantages such as commercial availability, stability and accuracy allow a better comparability between labs.

4.2. Elimination of the epoxides from whole blood

A slower elimination at a higher initial concentration for SO can probably be attributed to the lowered contribution at this concentration of enzymatic processes and reactions with nucleophiles to the elimination of SO. When the elimination of the three epoxides at comparable concentrations is considered, the fastest elimination was observed for SO. Although ethylene oxide is clearly the most reactive of the three epoxides studied, this is not reflected in its half-life in human blood. This is in accordance with the observation of a relative long half-life of 4 days for ethylene oxide and 4.1 days for propylene oxide in salt water [1]. In contrast, a much faster non-enzymatic elimination of 3–4% SO per hour, resulting in a half-life between 12.5 and 16.7 h, was observed in a buffer solution [24]. Moreover, Dent and Schnell [25] concluded that EO and PO are poor substrates for epoxide hydrolase in contrast with SO. Also, Mendrala et al. [26] observed a high glutathione *S*-transferase activity towards SO in vitro. These arguments lead to the conclusion that the fast elimination for SO from whole blood at low concentration is according to expectations with literature data.

4.3. Second-order rate constants for the reaction with N-terminal valine and N-7-guanine

The second-order rate reaction constants for the reaction with N-terminal valine and *N*-7-guanine have previously been determined for both ethylene and propylene oxide. The constant for the reaction between human N-terminal valine and ethylene oxide was estimated to be 2.97 l (mol Hb h)$^{-1}$ [8], which is close to our estimate of 2.7 l (mol Hb h)$^{-1}$. For propylene oxide Segerbäck et al. [9] came to a $k_{\text{PO-Hb}}$ of 1.01 l (mol Hb h)$^{-1}$, virtually equal to our estimate.

The way of determination of the second-order rate reaction constant in DNA in this study is different from the method used in previous studies. The incubations in this study were done with whole blood, whereas the available literature values are based on in vitro expositions of calf thymus DNA to the epoxides. The lower accessibility of DNA in whole blood could explain our estimate of the second-order rate reaction constant for ethylene oxide being about half of the literature value of 30×10^{-3} l (mol DNA nucleotide h)$^{-1}$ [11]. For PO, a smaller difference between our 5.1×10^{-3} l (mol DNA nucleotide h)$^{-1}$ and the value of 7.7×10^{-3} l (mol DNA nucleotide h)$^{-1}$ obtained by Segerbäck et al. [9] was seen.

4.4. Comparison of literature values of the in vitro and in vivo ratio between N-terminal valine and N-7-guanine adduct formation

Osterman-Golkar et al. [27] suggest that adduct formation in short term experiments could be related

Table 5
Comparison of literature values of the ratio between adduct formation on N-terminal valine and *N*-7-guanine in vivo for the three epoxides studied

Compound	Target tissue for DNA adducts	Species (experiment duration)	Type of dosing, regimen and amounts	Ratio N-terminal valine/*N*-7-guanine	Reference
Ethylene	liver	rat (3 days)	inhalation 300 ppm, 12 h/day	243	[28]
EO	liver	rat (6 h)	i.p. injection (2.8–20.4 μmol/kg BW)	53–65	[29]
	liver	rat (6 h)	inhalation 3–33 ppm	267–342	[30]
	lung	mice (4 weeks)	inhalation 33–100 ppm (6 h/day, 5 days/week)	993–1254	[31]
	lung	rat (4 weeks)	inhalation 33–300 ppm (6 h/day, 5 days/week)	295–367	[31]
Propylene	liver	mice (7 h)	an estimated inhalation of 0.88 mmol/kg BW	71	[32]
	liver	rat (3 days)	inhalation 300 ppm, 12 h/day	174	[28]
PO	liver	mice (3 h)	i.p. injection 0.1 mmol/kg BW	66	[32]
	lung	mice (3 h)	i.p. injection 0.1 mmol/kg BW	285	[32]
	liver	mice (6–7 h)	inhalation or i.p. injection 0.05–0.32 mmol/kg BW	246–361	[9]
	lung	mice (7 h)	inhalation 0.11–0.32 mmol/kg BW	171–245	[9]
	liver	rat (6–7 h)	inhalation or i.p. injection 0.05–0.19 mmol/kg BW	318–731	[9]
	lung	rat (6–7 h)	inhalation or i.p. injection 0.05–0.19 mmol/kg BW	171–464	[9]
	liver	dog (4 h)	i.v. injection 0.07–0.35 mmol/kg BW	797–872	[9]
	lung	dog (4 h)	i.v. injection 0.07–0.35 mmol/kg BW	222–259	[9]
Styrene	liver	mice (3 h)	i.p. injection 0.28–4.35 mmol/kg BW	6–55	[33]
	lung	mice (3 h)	i.p. injection 0.28–4.35 mmol/kg BW	9–32	[33]

to the integrated dose of the epoxide according to the following relationship:

$$D = [\text{Epox} - Y]/k_{\text{Epox}-Y}[Y]$$

where D has units μM h. Assuming that the integrated dose of the epoxide in blood is the same for N-terminal valine in haemoglobin and *N*-7-guanine adduct formation in leucocytes, from this equation it can be deduced that:

$$k_{\text{Epox-Hb}}/k_{\text{Epox-DNA}} = ([\text{Epox-Hb}]/[\text{Hb}]) /([\text{Epox-DNA}]/[\text{DNA}])$$

In other words, the ratio of the second-order rate constants should agree with the ratio between the amount of N-terminal valine and *N*-7-guanine adduct in leucocytes expressed in mol adduct per mol macromolecule. Furthermore, this in vitro ratio is compared with a number of animal studies wherein the ratio of the adduct formation on haemoglobin and on DNA in different tissues has been studied (Table 5). At present it remains, however, unknown whether this ratio remains constant across species and tissues. Especially, the amount of *N*-7-guanine adducts could be different due to variations in the tertiary structure of the DNA.

Nevertheless, the data obtained by Walker et al. [31] for ethylene oxide show a good agreement between the levels of *N*-7-guanine adducts in leucocytes and in the lung. In the liver slightly lower *N*-7-guanine adduct levels were found. Literature values for the ratio between N-terminal valine and *N*-7-guanine adduct formation in liver and lung, the two most studied tissues, are presented in Table 5. The different ratio in the study of Walker et al. [31] with respect to our in vitro obtained value, can possibly be explained by RNA interferences, due to insufficient purification, with a different degree of alkylation than the DNA [28]. Another possible explanation could be the relatively long duration of the experiment. The life-time of haemoglobin adducts (120 days) compared to a half-life for *N*-7-guanine adducts between 1.0 and 5.8 days [31], results in an increased Hb/DNA adduct ratio for long term experiments. Our in vitro obtained value of 169 for EO-binding (Table 4), therefore, has preferentially to be compared to the values obtained by Osterman-Golkar et al. [29] and Potter et al. [30] resulting from short term experiments.

The ratio for propylene oxide adducts with Hb and DNA varies between 66 and 872, without any clear relationship across the species, the target tissue, the way of administration, the exposure regimen or the amount of epoxide administered. However, the average value for all ratios was 317, which is, given the high variation observed, not considered to be substantially different from our in vitro obtained 196.

In contrast with EO and PO-binding, for SO a deviation of about two orders of magnitude is seen between the in vitro and the in vivo observed ratio.

4.5. Discussion on the extrapolation from the in vitro obtained results to the in vivo situation

The applicability of the in vitro obtained reaction constant to the in vivo situation was already demonstrated in a study of Ehrenberg and Törnqvist [6] in which the adduct formation by EO on N-terminal valine in vivo is modelled. They observed that based on the $k_{\text{EO-Hb}}$ and other metabolic parameters for rodents at low dose a reasonably consistent picture was obtained concerning the estimates of the adduct level increments per unit of administered dose in vivo. Moreover, the authors came to the conclusion that for humans, although the value for the in vivo dose per unit of exposure was uncertain because of unreliable data, the most likely value for this relationship agrees with the rodent data. Due to the lower sensitivity of the DNA adduct determination technique, the experimental conditions for haemoglobin and DNA adduct determination in this study differed substantially. Nevertheless, our in vitro obtained ratio between N-terminal valine and *N*-7-guanine adduct formation by EO and PO, seems to agree very well with the in vivo obtained ratio in short term animal experiments.

Only for the N-terminal valine/*N*-7-guanine ratio for SO a major discrepancy was observed between the in vitro and in vivo results. This is probably due to lower than expected values for N-terminal valine adducts by SO in vivo, which was already concluded by Christakopoulos et al. [18], extrapolating from EO-data. Apparently, the ratio of the in vitro obtained second-order rate reaction constants for the

reaction of SO with N-terminal valine and *N*-7-guanine shows a 100-fold difference as to the in vivo adduct levels. Inaccuracy of the techniques and problems with the detection limit of the methods can only partly account for this discrepancy. Several studies indicate that our results showed no major deviations from the results of other laboratories using independent variants of the same technique or even completely different techniques [10,13,33,34]. Probably, one or more of the three assumptions that were made for the extrapolation does not hold in the specific case of SO. First of all, a linear extrapolation was assumed from the higher incubation concentrations of SO for DNA adduct formation to the concentration used for Hb adduct formation. Secondly, the species difference was not assumed to affect the ratio between both adducts. The in vitro results were determined in human blood, whereas the in vivo results for SO were obtained in mice. In this context, the potential protective role of the different (species specific) GST-types remains to be elucidated. Especially the erythrocytic GSTT1-1 enzyme could prevent SO and the other epoxides from reacting with N-terminal valine [35]. A last assumption was that the formation of *N*-7-guanine adduct levels in leucocytes is comparable to the levels in other tissues. Data on this issue for styrene 7,8-oxide are lacking, but could differ from the other epoxides due to its deviating chemical properties. In contrast with the other epoxides, styrene and its metabolites contain an aromatic ring, resulting in increased lipophilic properties. This certainly leads to higher concentrations in fat (reviewed in Ref. [1]), but could also work at the subcellular level with a higher affinity for, e.g., membranes. In this context, it should be emphasized that no data are available of both haemoglobin and DNA adduct formation in SO treated animals. Although treatment with the alkene compared to the epoxide did not lead to major differences in the adduct ratio for ethylene and propylene, due to its higher affinity for membranes the situation for styrene could be different.

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