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Toxicokinetics of ethylene and ethylene oxide in the male B6C3F1 mouse

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Toxicokinetics of inhaled ethylene and ethylene oxide in mice

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1 Introduction

1.1 Objective

Ethylene (ET) is quantitatively one of the most important high production volume chemicals worldwide. The gas is mainly used as feedstock in the production of polymers and industrial chemicals. It is ubiquitously present in the environment, arising predominantly from burning of organic material and from plants that form ET as ripening hormone. Mammals including humans exhale endogenously formed ET. A physiological function of ET is however unknown.

ET is epoxidized to ethylene oxide (EO), catalyzed by cytochrome P450-dependent monooxygenases (CYP) in the endoplasmic reticulum, as has been demonstrated in rats. EO is a directly DNA- and protein-alkylating agent in rodents and humans and is mutagenic and carcinogenic in rat and mouse. In spite of the formation of its carcinogenic metabolite EO, ET was negatively tested on carcinogenicity in a long-term study with rats. Therefore, this study could not be used to estimate the human tumor risk from ET. The negative outcome of the study had been predicted based on toxicokinetic investigations of inhaled ET and EO in rats: from a comparison of calculated internal EO burdens for various exposures to ET and for the exposures in the long-term studies of EO carcinogenicity in rats, it was concluded that, regardless of dose, the EO burdens upon ET exposures were too low to result in statistically significantly enhanced tumor incidences. In mice, no such comparison can be done because experimentally established toxicokinetic parameters of ET and of EO as a metabolite of ET are lacking.

The major aim of this work was to investigate the toxicokinetics of ET and EO in the mouse in order to evaluate whether positive results can be expected from a carcinogenicity study on ET in this species. Moreover, the endogenous ET production should be quantified and the thereby unavoidable body burden of EO calculated.

1.2 Properties, production, use, and occurrence of ethylene and ethylene oxide

ET (ethene or ethylene; CAS No.: 74-85-1; molecular formula: CH_2CH_2) is a colorless gas with a molecular mass of 28.05 g/mol. Its boiling point lies at -104°C and its melting point at -169°C at a pressure of 101.3 kPa (Lide, 1991). Mixtures of ET with air can be explosive. A mixture containing 2.75 vol% ET at 0.1 MPa and 20°C is reported to be the lower explosive limit (Zimmermann and Walzl, 2007). ET is lipophilic as evidenced by its logarithm of the octanol-water partition coefficient ($logP_{o/w}$) which is 1.13 (Hansch and Leo, 1979).

The worldwide production of ET was 113 million tons in 2005. Over 95% of ET is produced by steam cracking of petroleum hydrocarbon. About 60% of ET is used for the production of polymers, mostly polyethylene. ET serves also as feedstock for other industrial chemicals, like ethylene glycol, ethylene oxide, and 1,2-dichloroethane (Zimmermann and Walzl, 2007). Plants bio-synthesize ET as ripening hormone. Hence, ET is commercially used for the controlled ripening of fruits, vegetables and flowers that are harvested unripely (IARC, 1994a).

ET is ubiquitously present in the environmental atmosphere being both of natural (about three quarters) and of anthropogenic origin (about one quarter) (IARC, 1994a). Natural sources are emissions from plants and volcanoes and natural gas (Sawada and Totsuka, 1986). Anthropogenic ET results from incomplete burning of organic material – for example forest fires and combustion of fossil fuels by vehicle engines – and from the ET processing industry. In urban air, ET concentration can reach up to 700 ppb (Abeles and Heggestad, 1973).

Occupational exposure to ET occurs during fruit ripening (e.g., 0.3 ppm ET in fruit stores, Törnqvist et al., 1989a), during firefighting (up to 45 ppm ET, Jankovic et al., 1991), and in the ET processing industry. Exposure of the general population is due to environmental ET and to ET released from cigarette smoking (about 1 - 2 mg ET per cigarette, IARC, 1994a). In addition to external exposure, ET is also produced endogenously in mammals. The following potential sources of endogenous ET have been discussed: lipid

peroxidation (Frank et al., 1980; Lieberman and Hochstein, 1966; Sagai and Ichinose, 1980; Törnqvist et al., 1989b), oxidation of methionine (Kessler and Remmer, 1990; Lieberman and Kunishi, 1965; Lieberman and Mapson, 1964), oxidation of hemine in hemoglobin (Kessler, 1987), and metabolism in intestinal bacteria (Gelmont et al., 1981; Törnqvist et al., 1989b). However, no physiological function is known.

EO (ethylene oxide, 1,2-epoxyethane, or oxirane; CAS No.: 75-21-8; molecular formula: CH_2CH_2O) is a colorless gas with a molecular mass of 44.05 g/mol. Its melting and boiling points are at -112°C and 10.4°C, respectively, at a pressure of 101.3 kPa (Lide, 1991). The lower explosive limit of EO in air lies at 3.0 vol% (Dever et al., 2004). EO is amphiphilic as evidenced by its $\log P_{o/w}$ of -0.30 (Sangster, 1989).

The worldwide consumption of EO was 18 million tons in 2006 (Devanney, 2007). EO is predominantly synthesized by direct vapor phase oxidation of ET on a silver catalyst (Berglund et al., 1990; Rebsdat and Mayer, 2002). EO serves mainly as an intermediate in the production of ethylene glycol, glycolic ethers, and ethanolamine. It is used directly as a disinfectant, sterilizing agent for medical equipment, fumigant, and insecticide (summarized in IARC, 2008).

There is no information about the occurrence of EO in the general environment. Exposure of humans occurs mainly at the workplace during its production and use (OSHA, 2005). Concentrations of several hundreds ppm EO can be reached temporarily during opening of sterilization chambers (Hori et al., 2002). EO is also present in cigarette smoke (about 7 µg per cigarette, Hoffmann et al., 2001). Low dermal exposure of EO can be derived from unavoidable residues in cosmetics (Filser et al., 1994).

1.3 Metabolism of ethylene and ethylene oxide

A scheme of the metabolic pathways of ET and EO is presented Fig. 1.1. In ET exposed rats, the biotransformation of ET to EO has been proven by detection of EO in exhaled air (Erbach et al., 2005; Filser and Bolt, 1984) and in blood (Erbach et al., 2005; Fennell et al., 2004; Maples and Dahl, 1993).
Metabolism of ET was related to CYP because of the following findings. In incubations of rat liver microsomes with ET, EO was detected in the presence of NADPH (Schmiedel et al., 1983). In toxicokinetic studies on the ET metabolism *in vivo* (Bolt et al., 1984), the maximum rate of ET metabolism was enhanced after pretreatment of the animals with the CYP inducer Aroclor 1254, a mixture of polychlorinated biphenyls. ET metabolism was abolished when animals had been pretreated with diethyldithiocarbamate (dithiocarb). This compound acts an inhibitor of CYP-dependent monooxygenases (Sieger et al., 1978). At high ET concentrations, a suicidal inactivation of CYP was observed *in vitro* (Li et al., 2008; Ortiz de Montellano et al., 1980) and explained by *N*-2-hydroxyethylation of the prosthetic heme (Kunze et al., 1983).
As an electrophilic agent, EO reacts with nucleophilic groups, i.e. thiol, amino, and hydroxyl groups. As a result, 2-hydroxyethyl adducts in biological macromolecules have been found at amino acid residues of proteins, e.g. cysteine, histidine and *N*-terminal valine in hemoglobin, and at DNA bases such as guanine and adenine (IARC, 1994b, 2008). Exposure to ET results in the formation of the same adducts to proteins and to DNA of different organs as exposure to EO. This was demonstrated at first in hemoglobin of mice (Ehrenberg et al., 1977) and later in DNA of different organs of mice (Segerbäck, 1983). The findings on adduct formation upon ET and EO exposure were confirmed and extended in more recent studies with mice (Walker et al., 2000; Wu et al., 1999a, 1999b) and rats (Eide et al., 1995; Rusyn et al., 2005; Walker et al., 2000; Wu et al., 1999a, 1999b). Background adduct levels in untreated animals were related to the endogenous formation of ET and its metabolism to EO (Bolt, 1996). Also in humans, exposure to EO (IARC 1994b) or ET (Filser et al., 1992; Törnqvist et al., 1989a) led to a significant increase in hemoglobin adducts over a background level.

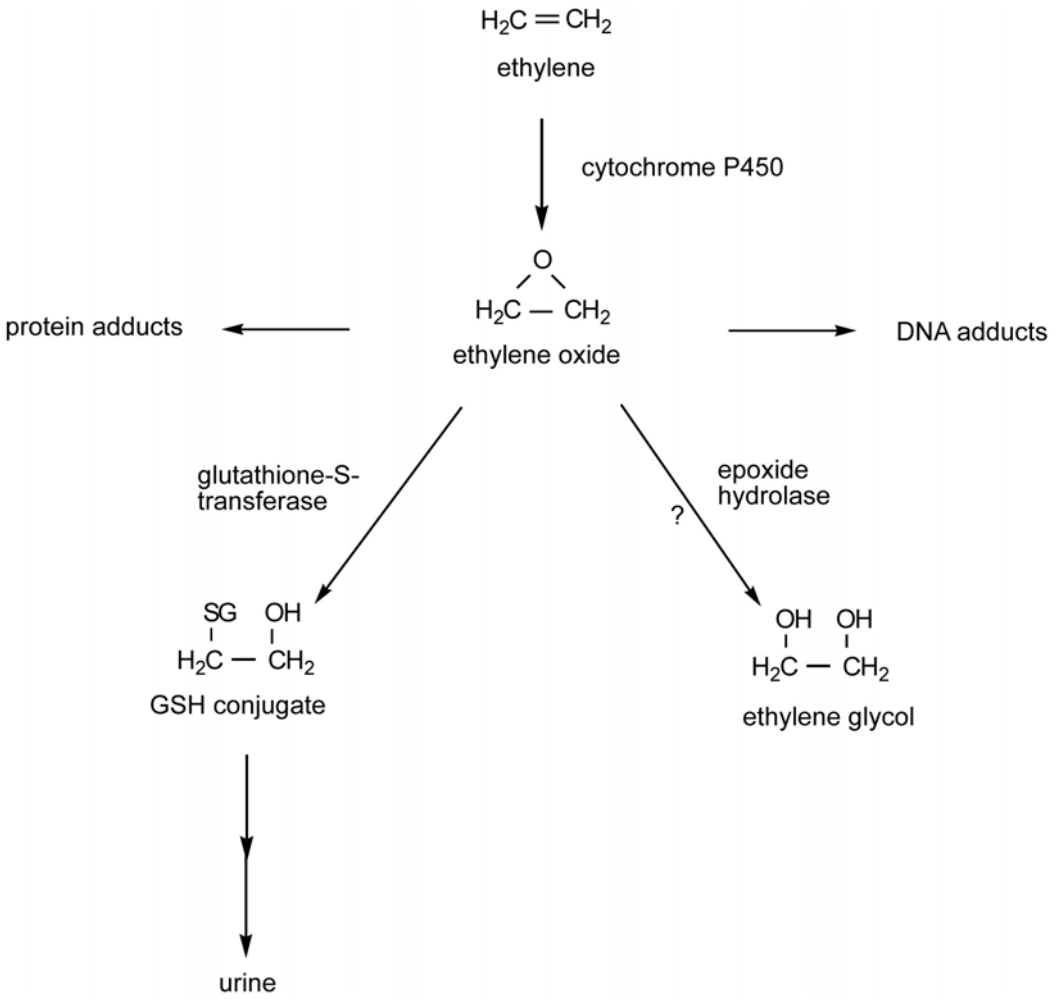


Figure 1.1 Metabolic pathway of ET according to Greim and Filser (1994). The "?" indicates that the existence of this pathway has not yet been proven.

However, no significant increase over a highly varying background level was seen in DNA adducts of granulocytes of EO exposed workers (Yong et al., 2007). 2-Hydroxyethyl adducts in hemoglobin and DNA have frequently been used as a measure of the internal EO dose upon exposure of animals and humans to EO (IARC, 1994b, 2008; Kolman et al., 2002) or ET (IARC, 1994a).

EO is metabolically eliminated by conjugation with reduced glutathione (GSH), catalyzed by cytosolic glutathione S-transferase (GST) in liver, kidney, and testes of rat, mouse, and human (Brown et al., 1996) and in human blood (Föst et al., 1991). Accordingly, thioether excretion in urine was increased following EO exposure of mice and rats (Tardif et al., 1987) and of humans (Burgaz et al., 1992). Exposure of mice (Brown et al., 1998; McKelvey and

Zemaitis, 1986) and rats (McKelvey and Zemaitis, 1986) to EO concentrations ≥100 ppm led to a GSH depletion in various tissues, which was more pronounced in mice than in rats. In the former species, this effect was considered to cause the reduced metabolic elimination of EO observed at an exposure to 300 ppm EO (Brown et al., 1998; Sega et al., 1991). Hydrolysis to ethylene glycol, catalyzed by epoxide hydrolase, was generally assumed as a second elimination pathway of EO. But unlike other epoxide homologues, e.g. propylene oxide, the rate of EO hydrolysis observed in rat liver microsomes was extremely low (Brown et al., 1996) or not measurable (Li et al., 2009). Ethylene glycol was only tentatively identified in urine samples of EO exposed mice and rats (Tardif et al., 1987).

1.4 Mutagenicity and carcinogenicity of ethylene and ethylene oxide

Although ET exposure of rodents led to alkylation of DNA bases, ET showed no positive effects in mutagenicity studies. Up to a concentration of 20% in air, the gas was not mutagenic in Salmonella typhimurium TA 100 with and without a metabolizing system (Victorin and Ståhlberg, 1988). No ET-dependent micronuclei in bone marrow cells (Vergnes und Pritts, 1994) and HPRT mutations in splenic T cells (Walker et al., 2000) were seen in rats and mice exposed to ET concentrations up to 3000 ppm (6 h/day, 5 days/weeks, 4 weeks). Also, no tumorigenic effects were seen in long-term studies in rats exposed up to 3000 ppm ET (Hamm et al., 1984). A carcinogenicity study on ET in mice has not been performed.
The International Agency for Research on Cancer (IARC, 1994a) evaluated ET as ‘not classifiable as to its carcinogenicity to humans’ (Group 3). The German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK commission) concluded ET to have a cancer inducing potential for the human because of its metabolism to EO and classified ET into Category 3B. Consequently, no exposure limit (MAK value) was established (DFG, 1993).

EO alkylates DNA bases (see above) and produces chromosomal aberrations and micronuclei in rodents and humans (summarized in IARC, 2008; Kolman

et al., 2002; U.S. EPA, 2006). It is mutagenic in microorganisms and Drosophila melanogaster (summarized in IARC, 1994b, 2008; Kolman et al., 2002; U.S. EPA, 2006), mice (Houle et al., 2006; Sisk et al., 1997; Walker and Skopek, 1993), rats (Tates et al., 1999; van Sittert et al., 2000), and humans (Tates et al., 1991). Tumorigenic effects of EO were observed in several long-term inhalation studies in rodents. Exposure of female A/J mice to 0, 70 or 200 ppm EO (Adkins et al., 1986) and of B6C3F1 mice of both genders to 0, 50 or 100 ppm EO (NTP, 1987) led to dose-dependent significant increases in pulmonary adenomas and carcinomas. In the B6C3F1 mice, increases in benign and malignant tumors of other organs and tissues were also observed. In male Fischer 344 rats exposed to 0, 50 or 100 ppm, Lynch et al. (1984) detected dose-dependent significant incidences of brain tumors, peritoneal mesotheliomas in the region of the testes, and mononuclear-cell leukemia. In another long-term study, male and female F344 rats were exposed to 0, 10, 33 or 100 ppm. The same types of tumors as in the former study were significantly increased in a dose-dependent manner in both genders with the exception of mesotheliomas in females but, additionally, with an increase in subcutaneous fibromas in males at the high dose (Snellings et al., 1984). A local, dose-dependent tumor formation was seen in long-term studies upon weekly subcutaneous injection of EO to mice (0, 0.1, 0.3, and 1 mg per mouse; Dunkelberg, 1981) and upon twice weekly intragastric intubation of EO (0, 7.5 and 30 mg/kg) to rats (forestomach, Dunkelberg, 1982).

The results of epidemiological studies on the tumor formation in workers exposed to EO are equivocal (summarized and interpreted in: DFG, 1993; IARC, 2008; U.S. EPA, 2006). Negative findings were obtained by Greenberg et al. (1990), Kiesselbach et al. (1990), Morgan et al. (1981), and Thiess et al. (1981). Results of Gardner et al. (1989) were considered to be doubtful by the authors. Evidences of carcinogenic effect of EO in humans were found by Högstedt et al. (1979a, 1979b, 1986) and by Steenland et al. (1991). From the largest study (around 18.000 workers in sterilizing plants, Steenland et al., 2004) the authors concluded: „We found no overall evidence of excess cancer mortality in this cohort, with the exception of bone cancer based on small numbers. However, in exposure-response analyses we found evidence

of an association between increased exposure and some types of haematopoietic cancer, particularly for males. There is also some evidence for a positive exposure-response for breast cancer mortality“.
As to the carcinogenicity of EO, the International Agency for Research on Cancer (IARC, 2008) stated 'limited evidence in humans' and 'sufficient evidence in experimental animals' and classified EO as carcinogenic to humans (Group 1). The German MAK commission classified EO as carcinogenic to human (Category 2) based on animal studies (DFG, 1984).

1.5 Toxikokinetics of ethylene and ethylene oxide

In rat and human, toxicokinetics of ET (rat: Andersen et al., 1980; Bolt et al., 1984; Denk, 1990; Shen et al., 1989; human: Filser et al., 1992; Shen et al., 1989) and EO (rat: Denk, 1990; Filser and Bolt, 1984; Krishnan et al., 1992; human: Filser et al., 1992), were investigated in gas uptake and exhalation experiments using the closed chamber technique. Analysis of concentration-time courses measured in the atmosphere of the exposure chambers using a two-compartment model (Filser, 1992) yielded parameters of inhalation uptake, exhalation and metabolism as well as of the endogenous production of ET and its metabolite EO (Bolt et al., 1984; Bolt and Filser, 1987; Denk, 1990; Filser and Bolt, 1984; Filser et al., 1992; Shen et al., 1989). In mice, corresponding studies have not been performed yet.
Blood burdens of EO were determined in rats during inhalation exposures to ET (Fennell et al., 2004; Maples and Dahl, 1993) and in mice (Brown et al., 1996, 1998) as well as in rats (Brown et al., 1996) during and after inhalation exposure to EO. All available toxicokinetic data were reanalyzed by means of a sophisticated physiological toxicokinetic model developed for ET and EO (Csanády et al., 2000). Fennell and Brown (2001) presented a physiological toxicokinetic model for EO solely.
The toxicokinetic analyses of ET in the rat revealed the following: only a small percentage of inhaled ET is systemically available (17%). The overwhelming part is immediately exhaled before entering the blood stream. As was shown by Csanády and Filser (2001), this effect results from the small partition coefficient blood/air ($P_{blood/air}$) of ET (between 0.11 und 0.48, determined at

37°C in blood of various species (Csanády et al., 2000; Steward et al., 1973). Because of its hydrophobic characteristics (solubility in olive oil 40 times higher than in serum or plasma, Steward et al., 1973), ET enriches predominantly in the fat tissue. The thermodynamic equilibrium constant whole body/air (K_{eq}) of 0.7 in rats (Bolt et al., 1984) indicates, however, a low enrichment in the organism. The rate of ET metabolism in the rat follows first-order kinetics below exposure concentrations of about 125 ppm (Csanády et al., 2000). In this range, 24% of bioavailable ET is metabolized and 76% is exhaled, which means that only 3.7% of inhaled ET is biotransformed (Csanády et al., 2000). At higher concentrations, ET metabolism becomes increasingly saturated, reaching in rats a maximum rate of 8.5 µmol/(h*kg) above 1000 ppm (Andersen et al., 1980; Bolt et al., 1984; Csanády et al., 2000), the half-maximum rate being at about 200 ppm (Andersen et al., 1980; Bolt and Filser, 1987). After analyzing the data of Bolt et al. (1984) by means of a physiological toxicokinetic model, Csanády et al. (2000) estimated the concentration at half of the maximum rate of metabolism to be 125 ppm. Saturation of ET metabolism was also concluded from measurements of DNA and hemoglobin adducts as biomarkers of EO following exposures of mice and rats to low (≤40 ppm) and high (≥1000 ppm) ET concentrations (Ehrenberg et al., 1977; Segerbäck, 1983; Walker et al., 2000).
The endogenous production of ET was quantified by exhalation studies in rats (Denk, 1990; Filser, 1992; Shen et al., 1989) using the closed chamber technique presented by Filser (1992). Taking into account the toxicokinetic behavior of ET, a mean endogenous ET production of 10.3 nmol/(h*kg) was calculated.

In contrast to ET, most of inhaled EO is systemically available due to its large $P_{blood/air}$ of 61 (Csanády et al., 2000). Because of its amphiphilicity, EO distributes uniformly in the organism (Csanády et al., 2000). Metabolism of EO is rapid and follows linear kinetics up to 200 ppm in mice (concluded from EO measurements in blood, Brown et al., 1998) and up to at least 300 ppm in rats (Csanády et al., 2000). In this concentration range, more than 90% of EO taken up is eliminated by metabolism (Csanády et al., 2000). Deviation from linearity at concentrations higher than 200 ppm in mice was ascribed to the

inhibition of the GST pathway by depletion of the cofactor GSH (Brown et al., 1998).

Surprisingly, the formation of EO in rats exposed to high, constant ET concentrations (330 – 3000 ppm) was time dependent. Concentration-time courses of exhaled EO (Erbach et al., 2005; Filser and Bolt, 1984) or EO in blood (Erbach et al., 2005; Fennell et al., 2004; Maples and Dahl, 1993) were complex showing a rapid initial increase to a maximum followed a subsequent decline to a plateau. Fennell et al. (2004) assumed this picture to result from an inactivation of CYP2E1 during its catalysis of the ET metabolism.

1.6 Aim

The major aim of this investigation was to evaluate on the basis of the internal EO burden whether a carcinogenicity study of ET in mice can be expected to give positive results. In order to calculate the dose-dependent internal burden of EO in mice for various kinds of exposure to ET or EO, the following work should be done:

Determination of the

- inhalation toxicokinetics of ET,
- inhalation toxicokinetics of EO,
- formation of endogenous ET,
- internal EO burden resulting from exogenous and endogenous ET.

Adult male B6C3F1 mice should be chosen for the experiments since this strain had been used in the carcinogenicity study of EO (NTP, 1987). Large ranges of ET (1 – 10000 ppm) or EO (10 – 3000 ppm) concentrations should be prepared in the atmosphere of closed exposure chambers and the gas uptake from the atmosphere should be monitored subsequently up to 7 h by gas chromatography. The ranges should cover high concentrations as used in the carcinogenicity studies as well as low ones close to the human exposure situation. Some animals should be pretreated with dithiocarb in order to investigate the role of CYP activity in the ET metabolism. The endogenous production of ET should be quantified from exhalation experiments in naïve animals and animals pretreated with dithiocarb in order to circumvent the

metabolic elimination of ET. In a further set of experiments, animals should be exposed to a large range of constant ET concentrations (1 – 10000 ppm) and the time course of the metabolite EO exhaled into the atmosphere of the closed exposure chamber should be monitored until steady state is reached.
The experimental data should be analyzed by means of a toxicokinetic two-compartment model in order to obtain the toxicokinetic parameters of uptake by inhalation, elimination by exhalation and by metabolism of ET and EO as well as of the endogenous formation of ET. Hereof, the parameters describing the body burden by both compounds and the body burden of EO as a metabolite of ET should be calculated. The results should be compared with corresponding findings obtained in the rat which had been negatively tested in a long-term carcinogenicity study with ET.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Air (synthetic) 4.5	Linde, Unterschleißheim, Germany
Ethylene 3.5	Linde, Unterschleißheim, Germany
Ethylene oxide 3.0	Linde, Unterschleißheim, Germany
$^{13}C_2$-Ethylene oxide, 99 Atom% ^{13}C	Sigma-Aldrich, Taufkirchen, Germany
Grease Glisseal	Borer Chemie, Zuchwil, Switzerland
Hydrogen 5.0	Linde, Unterschleißheim, Germany
Sodium diethyldithiocarbamate trihydrate	Sigma-Aldrich, Taufkirchen, Germany
Nitrogen 5.0	Linde, Unterschleißheim, Germany
Oxygen 4.5	Linde, Unterschleißheim, Germany
Soda lime Drägersorb 800Plus	Dräger Medical AG, Lübeck, Germany
Water, deionised	GSF, Neuherberg, Germany

2.1.2 Instruments

Analytical systems

Gas chromatograph GC-8A, equipped with:	Shimadzu, Duisburg, Germany
- Flame ionization detector	Shimadzu, Duisburg, Germany
- Septum injector	Shimadzu, Duisburg, Germany
- Gas sample inlet MGS 4 with sample loops of 1.5 or 5 ml	Shimadzu, Duisburg, Germany
- Stainless steel column Tenax TA 60-	Chrompack, Frankfurt,Germany

80 mesh, 3.5 m x 2 mm ID	
- Stainless steel column Tenax TA 60-80 mesh, 2.5 m x 2 mm ID	Chrompack, Frankfurt, Germany
Integrator C-R5A Chromatopac	Shimadzu, Duisburg, Germany
Gas chromatograph HP 6890Plus equipped with:	Agilent Technologies, Waldbronn, Germany
- Mass selective detector HP 5973 Network	Agilent Technologies, Waldbronn, Germany
- Capillary column Poraplot U, 25 m x 0.32 mm ID, 10 µm film thickness	Chrompack, Frankfurt, Germany
- Chemical ionization gas purifier	Supelco, Bellefonte, PA, USA
- Thermal desorption cold trap injector CP4010	Chrompack, Frankfurt, Germany
- Cold trap CP Sil8CB, 30 cm x 5 µm x 0.53 mm	Chrompack, Frankfurt, Germany
- Infusion pump Precidor	Infors, Bottmingen, Switzerland

Laboratory instruments

Balance PT 6, U 3600	Sartorius, Göttingen, Germany
Desiccator, glass, 6.5 l, 2.8 l, 0.8 l, Duran	Schott, Mainz, Germany
Disposable hypodermic needle, 100 Sterican	Braun, Melsungen, Germany
Disposable syringes 1 ml, 2 ml, 5 ml, 10 ml	Braun, Melsungen, Germany
Glass adaptor for oxygen supply with water trap and grinding valve	Glasbläserei Höhn, Garching, Germany
Glass syringe, gas tight, 0,5 ml (no. 1750)	Hamilton, Darmstadt, Germany
Glass syringes, gas tight, 5 ml (no. 1005) and 10 ml (no. 1010)	Hamilton, Darmstadt, Germany
Magnetic stirrer, Ikamag® RCT	IKA Laboratorik, Staufen, Germany
Polypropylene disposable syringes 1 ml,	Novico, Ascoli Piceno, Italy

2.5 ml, 5 ml, 10 ml, 20 ml, 30 ml, 60 ml, and needles, Icogamma plus	
Punched stainless steel disk	GSF, Neuherberg, Germany
Room temperature and pressure reader	Wilh. Lambrecht KG, Göttingen, Germany
Thermostat EC for water bath	Julabo, Seelbach, Germany

2.1.3 Experimental animals

Male B6C3F1 mice (25 – 30 g) were purchased from Charles River (Sulzfeld, Germany). In total, 161 animals were used for this study. Five animals each were housed in macrolon cages type III and provided with HEPA-filtered air in a TOP FLOW-IVC-system (Tecniplast, Buggugiate, Italy). The animal room was air-conditioned (25°C, 60% air humidity). A 12-h light/dark cycle was maintained with light from 7:00 am to 7:00 pm. Animals were fed with standard chow (No. 1324, Altromin, Lage, Germany) and received tap water *ad libitum*. Animal husbandry and experimental protocols were in accordance with the German National Animal Protection Law.

2.2 Methods

2.2.1 Exposure systems and exposure conditions

Mice were exposed in gas-tight all-glass chambers (Filser et al., 2004; Fig. 2.1). Each closed exposure system consisted of a desiccator (0.9 – 6.5 l) in which the animals were placed on a punched stainless steel disk. On a stainless steel wire lattice, 25 g (gas uptake studies and exposures to constant concentrations of ET) or 50 g (exposures to quantify the endogenous production of ET) soda lime was placed to absorb exhaled CO_2. Loss of CO_2 was immediately compensated by purified O_2 (oxygen 4.5) delivered to the exposure system by means of an O_2 line which was connected to two washing flasks arranged in parallel (see Fig. 2.1). This arrangement together with a Teflon coated iron bar driven by a magnetic stirrer enabled a constant O_2 atmosphere in the chamber.

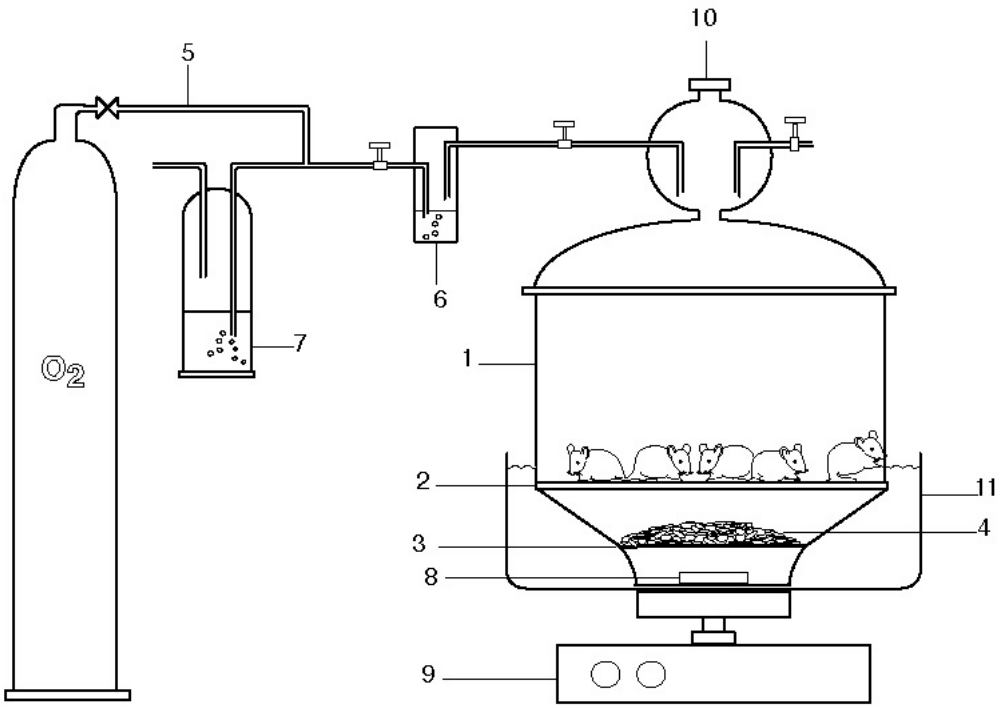


Figure 2.1 Closed gas-tight desiccator system for exposure of laboratory animals.
Numbers: (1) desiccator; (2) punched stainless steel wire lattice; (3) stainless steel wire lattice to place soda lime; (4) soda lime; (5) O_2 line; (6), (7) washing flasks; (8) iron bar; (9) magnetic stirrer; (10) teflon coated rubber septum; (11) thermostat controlled water bath.

ET or EO was injected via a Teflon coated rubber septum into the atmosphere of the chamber. Samples of chamber air were taken through the septum and injected into a gas chromatograph (GC) in order to monitor the concentration-time course of ET or EO in the chamber atmosphere (see 2.2.3). Each exposure chamber was immersed in a thermostat-controlled water bath which maintained a constant temperature of 23 – 25°C in the chamber. The temperature was surveyed by a thermometer kept inside the chamber.

Gas uptake studies with ethylene or ethylene oxide

For studying inhalation kinetics of ET or EO, five mice were exposed together for 7 h in closed desiccators of 2.8 l (ET) or 6.5 l (EO). For ET, the smaller

chamber was chosen because of an expected slow metabolic ET elimination, as had been found in ET exposed rats (Bolt et al., 1984). The larger chamber was chosen for the EO exposures taking into account the fast EO elimination observed in EO exposed rats (Filser and Bolt, 1984). Initial concentrations of about 1, 3, 10, 30, 100, 300, 1000, 3000, or 10000 ppm (ET) or of about 10, 30, 100, 300, 1000, or 3000 ppm (EO) were established by injecting the required amount of ET gas or gaseous EO (see 2.2.2.3, Eq. 1). Immediately thereafter, the chamber atmosphere was mixed by 5 times pumping chamber air using a 50 ml disposable syringe equipped with a stainless steel needle of 20 cm. During the first 30 min of exposure, air samples were collected every 5 min and, thereafter, every 30 or 60 min. All air samples (4 ml for ET or 10 ml for EO) were taken by means of disposable syringes to which a 20 cm stainless steel needle was connected in order to reach the center of the exposure chambers. Immediately after collection, air samples were analyzed by GC (2.2.2.1).

In order to test whether ET elimination from exposure chambers in the presence of animals resulted solely from oxidative metabolism, 5 animals were pretreated with dithiocarb. A solution of 250 mg dithiocarb was prepared in 5 ml physiological saline. Of this solution, 0.2 ml per mouse of 25 g was injected intraperitoneally 30 min prior to exposure, resulting in a dithiocarb dose of 400 mg/kg body weight. The pretreated mice were exposed together for 2 h in a desiccator of 0.8 l starting with an initial ET concentration of 10.6 ppm. The small chamber size established a high sensitivity concerning any concentration loss. The subsequent concentration-time course of ET was monitored as described above.

Exhalation of endogenously produced ethylene

In order to investigate the exhalation of ET in unexposed mice, 6 animals were placed together in each experiment for 7 h in a desiccator of 0.9 l that contained initially only synthetic air and soda lime. Air samples of 10 ml were collected every 60 min by means of disposable syringes and analyzed for ET by GC (2.2.2.1). In order to inhibit the metabolism of endogenously produced ET, some animals were pretreated with dithiocarb as described above.

Exposures to constant concentrations of ethylene

In order to investigate the formation of EO from inhaled ET, EO was determined as exhaled metabolite in the atmosphere of chambers (2.8 l) containing groups of five mice each exposed to constant ET concentrations of 1, 3, 10, 30, 100, 300, 1000, 3000, or 10000 ppm. Atmospheric ET concentrations were maintained quasi-constant for 7 h by injecting ET repeatedly in order to compensate the loss of ET by metabolism. Immediately after each injection of ET, the chamber atmosphere was mixed and atmospheric ET was measured in air samples of 4 ml by means of a GC as described in 2.2.2.1. The concentration of EO exhaled into the atmosphere of the closed chamber was determined by two GC methods depending on the ET exposure concentration. At ET concentrations ≥100 ppm, air samples of 0.5 ml were collected every 5 min for the first 60 min and every 15 min thereafter until the end of exposure. The air samples were immediately analyzed for EO using a GC equipped with a flame ionization detector (FID) (see 2.2.2.1). At ET exposure concentrations ≤100 ppm, a GC equipped with a mass selective detector (MSD) was used for the analysis of EO. At start of exposure and at time periods of 30 min thereafter, air samples of 2 ml each were taken at ET concentrations of 10, 30, and 100 ppm and of 8 ml each at ET concentrations of 1 and 3 ppm, respectively. All air samples were immediately subjected to GC/MSD analysis (see 2.2.2.2).

2.2.2 Analytical methods

2.2.2.1 Determination of atmospheric ethylene and ethylene oxide using gas chromatographs with flame ionization detectors

Concentrations of atmospheric ET and EO (at all EO exposures and at ET exposures ≥100 ppm) were determined by GC/FID using GC-8As equipped with stainless steel columns packed with Tenax TA 60-80 mesh. For the determination of ET, air samples of 4 ml (at ET exposure studies) or 10 ml (at ET exhalation studies) were injected via the gas sample inlet MGS 4 equipped with loops of 1.5 ml (at ET exposure) or 5 ml (at ET exhalation). For the determination of EO from 10 ml air samples, a gas sample inlet equipped

with a 5 ml loop was used. Direct injection onto column was carried out for EO analysis from air samples of 0.5 ml. Separations were done isothermally. The detector signals were recorded and integrated using C-R5A integrators. Gas chromatographic parameters are listed in Tables 2.1 and 2.2.

2.2.2.2 Determination of atmospheric ethylene oxide using a gas chromatograph with mass selective detector

Gaseous EO at ET exposures ≤30 ppm and $^{13}C_2$-EO added as a reference compound were analyzed using a GC HP 6890 equipped with a Thermal Desorption Cold Trap (TCT) injector CP-4010 and a MSD HP 5973. Gas samples of 2 or 8 ml were collected by means of a 5 or a 10 ml gastight glass syringe, respectively. The samples were injected within 2 or 7 min, respectively, into the injector system using helium with a flow of 20 ml/min. The cold trap CPSil 5 CB 30 cm x 0.53 mm was precooled to -150°C. Immediately after the injection, the cold trap was heated within a few seconds to 200°C and maintained at this temperature for 5 min. Separation was done on a capillary column PoraPlot U, 25 m length, 0.32 mm i.d., 10 µm film thickness, using helium as carrier gas with a flow of 1.9 ml/min. The temperature program of the column oven was started in parallel with the heating of the trap. The initial temperature of 70°C was held for 1 min. Then it increased with a rate of 8°C/min to 140°C and remained constant for 1 min. Thereafter, it increased further with a rate of 20°C/min to 170°C and remained constant for 10 min. Finally, it decreased again to 70°C. The temperature of the transfer line to the MSD was kept at 280°C. For ionization, the MSD was used in the positive chemical ionization mode with methane as reactant gas.

Table 2.1 Parameters for the analysis of ET from air samples using GC/FIDs.

Injection volume	1.5 ml	5 ml
Injector temperature	room temperature	room temperature
Separation column	3.5 m x 2 mm ID	2.5 m x 2 mm ID
Column temperature	60°C	30°C
Retention time	2.5 min	2.1 min
Detector temperature	200°C	200°C
Carrier gas nitrogen, pressure	2.0 kg/cm^2	2.0 kg/cm^2
FID gas hydrogen, pressure	0.6 kg/cm^2	0.6 kg/cm^2
FID gas synthetic air, pressure	0.6 kg/cm^2	0.6 kg/cm^2

Table 2.2 Parameters for the analysis of EO from air samples using GC/FIDs.

Injection volume	5 ml	0.5 ml
Injector temperature	room temperature	200°C
Separation column	2.5 m x 2 mm ID	2.5 m x 2 mm ID
Column temperature	70°C	110°C
Retention time	9.5 min	2.8 min
Detector temperature	200°C	200°C
Carrier gas nitrogen, pressure	2.0 kg/cm^2	2.0 kg/cm^2
FID gas hydrogen, pressure	0.6 kg/cm^2	0.6 kg/cm^2
FID gas synthetic air, pressure	0.6 kg/cm^2	0.6 kg/cm^2

The temperatures of the ion source and the quadrupole were 250°C and 150°C, respectively. EO and $^{13}C_2$-EO were quantified in the single ion mode using the M-H$^+$ ions m/z 45 and 47, respectively. Chromatograms were recorded and integrated manually with the HP Chemstation software. The retention time of EO and $^{13}C_2$-EO varied around 6 min, depending on the humidity of the gas sample.

2.2.2.3 Validation of the Methods

Defined atmospheric concentrations of ET or EO were used to construct calibration curves. For establishing a defined atmospheric concentration C_a (ppm) in a desiccator of the volume V_{desc} (ml), the required volume V_{gas} (ml) of pure gaseous ET or EO (10^6 ppm) to be injected was calculated according to the equation:

$$V_{gas} = \frac{C_a \cdot V_{desc}}{10^6} \qquad (1)$$

Accordingly, the concentrations obtained with pure gases were further diluted in order to achieve concentrations in the low range.

The detector response was tested for linearity in the required concentration ranges of ET and EO. For each experiment, a one-point calibration was carried out within the range of the expected ET or EO concentrations. The precision of each method was determined from repeated measurements and expressed as the coefficient of variation.

2.2.3 Toxicokinetic analysis

Toxicokinetic model

For the toxicokinetic analysis of the concentration-time courses of ET or EO in the atmospheres of closed chambers containing mice, a two-compartment model was used (Fig. 2.2, Filser, 1985, 1992; Filser et al., 1995). It contains processes of uptake by inhalation, of elimination by exhalation and metabolism, and of endogenous production.

Compartment 1 represents the chamber atmosphere with the volume V_1 and the actual substance concentration y_1. Compartment 2 represents the sum of the five concurrently exposed mice with their total body volume V_2 and the actual average substance concentration y_2 in their bodies. The transfer rates between both compartments describe the rates of inhalation uptake (dN_{up}/dt) and exhalation (dN_{ex}/dt) of the substance. Both rates with the rate constants k_{12} and k_{21} are directly proportional to the actual concentrations y_1 and y_2, respectively:

$$\frac{dN_{up}}{dt} = k_{12} \cdot V_1 \cdot y_1 \qquad (2)$$

$$\frac{dN_{ex}}{dt} = -k_{21} \cdot V_2 \cdot y_2 \qquad (3)$$

The rate of metabolism (dN_{met}/dt) with the rate factor k_{met} is related to y_2:

$$\frac{dN_{met}}{dt} = -k_{met} \cdot V_2 \cdot y_2 \qquad (4)$$

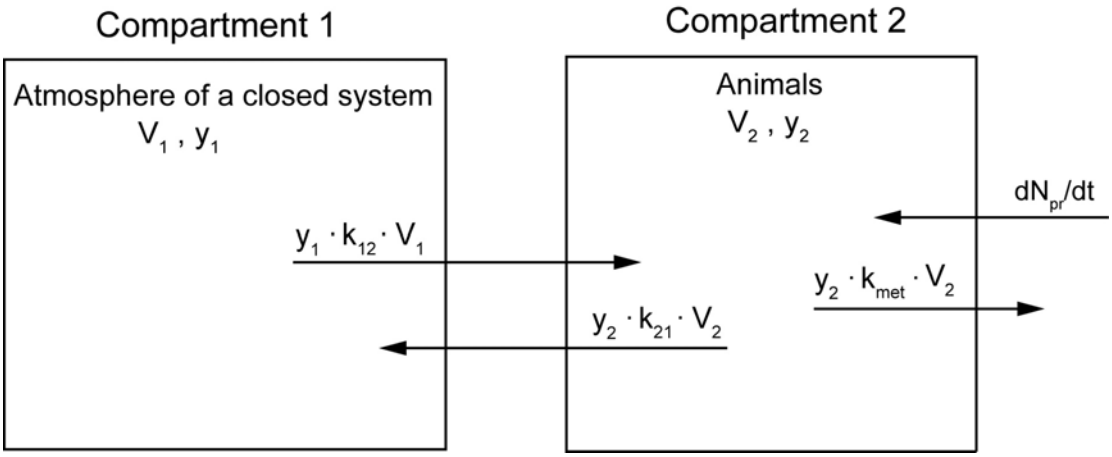


Figure 2.2 Two-compartment model for ET or EO determined in the atmosphere of closed exposure chambers (gas uptake studies). Abbreviations are specified in text.

In contrast to k_{12} and k_{21}, the value of k_{met} is constant only when metabolism follows first-order kinetics. If metabolism follows saturation kinetics according to Michaelis and Menten, k_{met} becomes smaller with increasing y_2 and is expressed by:

$$k_{met} = \frac{V_{max}}{V_2 \cdot (K_m + y_2)} \qquad (5)$$

V_{max} is the maximum rate of metabolism and K_m the apparent Michaelis constant, representing the concentration in compartment 2 at which $V_{max}/2$ is reached. In contrast to V_{max}, K_m is independent of V_2 (see below).

A production of the substance in the body (dN_{pr}/dt) is modeled to be constant in the case of endogenously formed ET and to depend on the rate of the ET metabolism in the case of EO.

Upon a single initial administration of ET or EO into the chamber atmosphere, the concentration y_1 is given in dependence of the time (t) by the following function:

$$y_1 = C_1 \cdot e^{\lambda_1 \cdot t} + C_2 \cdot e^{\lambda_2 \cdot t} + C_3 \qquad (6)$$

Accordingly, the concentration y_2 is given by:

$$y_2 = B_1 \cdot e^{\lambda_1 \cdot t} + B_2 \cdot e^{\lambda_2 \cdot t} + B_3 \qquad (7)$$

The rate constants λ_1 and λ_2 are complex expressions of k_{12}, k_{21} and k_{met}:

$$\lambda_1 = -\frac{1}{2} \cdot \left[k_{12} + k_{21} + k_{met} + \sqrt{(k_{12} + k_{21} + k_{met})^2 - 4 \cdot k_{12} \cdot k_{met}} \right] \qquad (8)$$

$$\lambda_2 = -\frac{1}{2} \cdot \left[k_{12} + k_{21} + k_{met} - \sqrt{(k_{12} + k_{21} + k_{met})^2 - 4 \cdot k_{12} \cdot k_{met}} \right] \qquad (9)$$

C_1, C_2, B_1, and B_2 are expressions of the rate constants and of the initial concentrations in the atmosphere $y_{1(0)}$ and in the animals $y_{2(0)}$. In addition, B_1, B_2, and B_3 depend also on V_2:

$$C_1 = \frac{-y_{1(0)} \cdot (k_{12} + \lambda_2)}{\lambda_1 - \lambda_2} + \frac{k_{21}}{V_1 \cdot (\lambda_1 - \lambda_2)} \cdot \left[\frac{\frac{dN_{pr}}{dt}}{\lambda_1} + V_2 \cdot y_{2(0)} \right] \qquad (10)$$

$$C_2 = \frac{y_{1(0)} \cdot (k_{12} + \lambda_2)}{\lambda_1 - \lambda_2} - \frac{k_{21}}{V_1 \cdot (\lambda_1 - \lambda_2)} \cdot \left[\frac{\frac{dN_{pr}}{dt}}{\lambda_2} + V_2 \cdot y_{2(0)} \right] \quad (11)$$

$$B_1 = \frac{y_{2(0)} \cdot V_2 \cdot (k_{21} + k_{met} - k_{12}) - 2 \cdot y_{1(0)} \cdot V_1 \cdot k_{12}}{2 \cdot V_2 \cdot (\lambda_2 - \lambda_1)} + \\ + \frac{\frac{dN_{pr}}{dt} \cdot (k_{21} + k_{12} - k_{met}) + \left[V_2 \cdot y_{2(0)} \cdot k_{met} - \frac{dN_{pr}}{dt} \right] \cdot (\lambda_2 - \lambda_1)}{2 \cdot V_2 \cdot k_{met} \cdot (\lambda_2 - \lambda_1)} \quad (12)$$

$$B_2 = \frac{-y_{2(0)} \cdot V_2 \cdot (k_{21} + k_{met} - k_{12}) + 2 \cdot y_{1(0)} \cdot V_1 \cdot k_{12}}{2 \cdot V_2 \cdot (\lambda_2 - \lambda_1)} + \\ + \frac{-\frac{dN_{pr}}{dt} \cdot (k_{21} + k_{12} - k_{met}) + \left[V_2 \cdot y_{2(0)} \cdot k_{met} - \frac{dN_{pr}}{dt} \right] \cdot (\lambda_2 - \lambda_1)}{2 \cdot V_2 \cdot k_{met} \cdot (\lambda_2 - \lambda_1)} \quad (13)$$

In the presence of endogenous production of ET or EO at t = 0 and in the absence of exogenous exposure, $y_{2(0)}$ is expressed by:

$$y_{2(0)} = \frac{\frac{dN_{pr}}{dt}}{V_2 \cdot (k_{21} + k_{met})} \quad (14)$$

C_3 and B_3 depend on dN_{pr}/dt:

$$C_3 = \frac{k_{21} \cdot \frac{dN_{pr}}{dt}}{k_{12} \cdot k_{met} \cdot V_1} \quad (15)$$

$$B_3 = \frac{\frac{dN_{pr}}{dt}}{k_{met} \cdot V_2} \quad (16)$$

The sum of C_1, C_2 and C_3 equals $y_{1(0)}$ and the sum of B_1, B_2, and B_3 equals $y_{2(0)}$. In the presence of endogenous production of ET or EO, $y_{2(0)}$ is ≥0. When there is no endogenous production, B_3 and C_3 equal zero and the sum of B_1 and B_2 gives $y_{2(0)}$= 0, and therefore B_1 equals -B_2. In inhalation experiments with high concentrations of ET or EO, any initial concentration in the animals was disregarded and C_3 and B_3 were set to zero treating $y_{2(0)}$ as zero.

According to the model, the clearances of inhalation uptake Cl_{up}, related to the atmospheric concentration, and of exhalation Cl_{ex}, related to the concentration in the organism, are expressed as:

$$Cl_{up} = k_{12} \cdot V_1 \tag{17}$$

$$Cl_{ex} = k_{21} \cdot V_2 \tag{18}$$

These clearances are flows (volume/time). If multiplied with the actual concentration in air ($Cl_{up} \cdot y_1$) or in animals ($Cl_{ex} \cdot y_2$), they give the actual amounts inhaled or exhaled per time. V_1 is inverse proportional to k_{12}. Therefore, Cl_{up} is independent of V_1.

K_{eq} represents the thermodynamic equilibrium constant whole body/air and can be expressed as:

$$K_{eq} = \frac{Cl_{up}}{Cl_{ex}} \tag{19}$$

According to Csanády et al. (1994), K_{eq} can also be calculated from the sum of the products of all partition coefficients “tissue i/ air” ($P^i_{tissue/air}$) with their corresponding tissue volumes (V_i) and considering the total body volume of one animal V_a.

$$K_{eq} = \frac{\sum_{i=1}^{n} (P^i_{tissue/air} \cdot V_i)}{V_a} \tag{20}$$

In the present thesis, K_{eq} was calculated using Eq. 20. The values of $P^{i}_{tissue/air}$ were taken from Table 2.3. The corresponding V_i-values were obtained by multiplying the volume fraction of the matching tissue i (Table 2.3) with 0.0229 l. This value represents 91.4% of the total body volume of a mouse of 25 g. It reflects the body volume without the volume of the skeleton: bones were considered not to be targets for ET or EO. Accordingly, for the calculation of K_{eq} by means of the values in Table 2.3 and Eq. 20, V_a was allocated a value of 0.0229 l.

Table 2.3 Volume fractions of tissue i[a] and partition coefficients tissue i/air ($P^{i}_{tissue/air}$)[b] of ET and EO in mice.

Tissue i	Volume of tissue i as fraction of body weight	$P^{i}_{tissue/air}$	
		ET	EO
Blood	0.049	0.48	61
Fat	0.10	2.06	43
Muscles	0.66	0.63	66
Liver	0.055	0.57	54
RPT*	0.050	0.50	66

[a] Arms and Travis (1988)
[b] Csanády et al. (2000)
* Richly perfused tissue group (sum of lung, brain, kidney, heart, and intestines)

Johanson and Filser (1992) demonstrated that Cl_{up} can be expressed by means of the alveolar ventilation Q_{alv}, the cardiac output Q_{card}, a factor f respecting a "wash-in-wash-out" effect in the upper airways, and the substance specific $P_{blood/air}$:

$$Cl_{up} = \frac{Q_{alv} \cdot f \cdot Q_{card} \cdot p_{blood/air}}{Q_{alv} \cdot f + Q_{card} \cdot p_{blood/air}} \qquad (21)$$

According to Arms and Travis (1988), the values of the alveolar ventilation Q'_{alv} and the cardiac output Q'_{card} of one standard mouse at rest (body weight 25 g; V'_2 = 0.025 l) are 1.5 l/h and 1.02 l/h, respectively. Johanson and Filser

(1992) derived an f value of about 0.6 in rodents. Consequently, the clearance of inhalation uptake of a standard mouse $Cl_{up}^{Õ}$ was obtained as:

$$Cl'_{up} = \frac{Q'_{alv} \cdot f \cdot Q'_{card} \cdot p_{blood/air}}{(Q'_{alv} \cdot f) + Q'_{card} \cdot p_{blood/air}} \qquad (22)$$

According to Csanády et al. (2000), the value of f was set to 0.60 for the calculation of $Cl_{up}^{Õ}$ of the lipophilic ET. For calculating $Cl_{up}^{Õ}$ of the amphiphilic EO, f was reduced in the present work by 22% to f = 0.47 because the measured concentration-time courses of inhaled EO could not be fitted reasonably with the higher f value (see results). The clearance of exhalation of one standard mouse $Cl_{ex}^{Õ}$ was obtained according to Eq. 19:

$$Cl'_{ex} = \frac{Cl'_{up}}{K_{eq}} \qquad (23)$$

The experimental clearance Cl representing Cl_{up} or Cl_{ex} depends on the number n of the exposed animals and their summed volume V_2. The Cl values were calculated allometrically from the corresponding standard clearance $Cl^{ć}$ by means of the body surface factor "body volume$^{2/3}$" using the following equation:

$$Cl = Cl' \cdot n \cdot \left(\frac{V_2}{V'_2 \cdot n} \right)^{2/3} \qquad (24)$$

The experimental rate constants k_{12} and k_{21} were calculated from the obtained Cl values by using Eqs. 17 and 18.

Gas uptake studies with ET or EO

Considering that the metabolism of ET obeys saturation kinetics, the metabolic parameters V_{max} and K_m of ET were obtained from gas uptake experiments by the following procedure:

At the three highest $y_{1(0)}$ of ET, metabolism of ET was considered to be saturated and V_{max} was assumed to be reached. At such a condition, the dependence of k_{met} from y_2 (see Eq. 5) can be circumvented when using the following function for y_1 (instead of Eq. 6):

$$y_1 = C_1 + (C_2/\lambda)\cdot e^{\lambda\cdot t} + C_3\cdot t \tag{25}$$

with

$$\lambda = -(k_{12} + k_{21}) \tag{26}$$

$$C_1 = y_{1(0)} + \frac{k_{21}\cdot V_{max}}{V_1\cdot (k_{12}+k_{21})^2} - \frac{y_{1(0)}\cdot k_{12}}{k_{12}+k_{21}} \tag{27}$$

$$C_2 = \frac{k_{21}\cdot V_{max}}{V_1\cdot (k_{12}+k_{21})} - k_{12}\cdot y_{1(0)} \tag{28}$$

$$C_3 = -\frac{k_{21}\cdot V_{max}}{V_1\cdot (k_{12}+k_{21})} \tag{29}$$

In Eqs. 25 – 29, dN_{pr}/dt was not taken into account because the influence of the very small dN_{pr}/dt on y_1 in the ppm range was negligible. After inserting the values of k_{12} and k_{21} (obtained as described above) into Eqs. 26 – 29 and the experimental values of V_1 into Eqs. 27 – 29, Eq. 25 was fitted to the three measured concentration-time courses to quantify V_{max} as fit parameter.
The standard value V'_{max} (related to a body weight of 1 kg representing 40 standard mice) was then calculated allometrically:

$$V'_{max} = \frac{V_{max}}{n}\cdot\left(\frac{V'_2\cdot n}{V_2}\right)^{2/3}\cdot 40 \tag{30}$$

At low concentrations, when metabolism follows first-order kinetics, Eq. 5 simplifies to:

$$k_{met} = \frac{V_{max}}{V_2 \cdot K_m} \quad (31)$$

with k_{met} being constant.

At the three lowest $y_{1(0)}$ of ET, the declines of ET in the chamber atmosphere were modeled to follow first-order kinetics. The values of k_{12} and k_{21} were inserted in Eqs. 8 – 11. Considering $y_{2(0)}$ to be zero and neglecting dN_{prET}/dt, Eq. 6 was fitted to the measured concentration-time courses in order to obtain the value of k_{met} as the only fit parameter.

The clearance of metabolism Cl_{met} is defined as:

$$Cl_{met} = k_{met} \cdot V_2 \quad (32)$$

Its standardized form Cl'_{met} was obtained by means of Eq. 24 to:

$$Cl'_{met} = k'_{met} \cdot V'_2 \quad (33)$$

The standardized rate of metabolism dN'_{met}/dt was calculated per kg body weight of 40 standard mice as the product of Cl'_{met} and y_2, multiplied with 40.

K_m was calculated as:

$$K_m = \frac{V'_{max}}{Cl'_{met} \cdot 40} \quad (34)$$

For the ET gas uptake experiments starting with the remaining $y_{1(0)}$ (between the three highest and the three lowest $y_{1(0)}$ values), concentration-time curves were constructed by the following procedure. The concentrations expressed by Eqs. 6 and 7 were iteratively calculated for short time periods. At each iteration step, the appropriate k_{met} value was calculated using Eq. 5 and inserted into the Eqs. 6 and 7.

In the case of EO, only k_{met} had to be obtained by curve fitting because the elimination of EO from the chamber atmosphere followed first-order kinetics. After obtaining k_{met} as described for ET, the corresponding Cl'_{met} was calculated as described above.

Endogenous ET production

The endogenous production of ET (dN_{prET}/dt) was determined from measurements of the ET concentration-time courses observed in closed chambers with initially zero concentrations of ET ($y_{1(0)} = 0$). The chambers contained 6 mice either naïve or pretreated with dithiocarb which inhibited the metabolism of ET completely (see results).

In the first case, y_1 rose to a plateau at which y_2 was proportional to y_1 with K_{eq} as proportionality factor:

$$y_2 = K_{eq} \cdot y_1 \tag{35}$$

At plateau, the amount of ET metabolized equaled the amount of endogenously produced ET:

$$\frac{dN_{prET}}{dt} = k_{met} \cdot V_2 \cdot y_2 \tag{36}$$

Since dN_{prET}/dt was considered to be proportional to the volume of the animals, the standardized value dN'_{prET}/dt (related to a body weight of 1 kg representing 40 standard mice) was calculated from the experimental value as follows:

$$\frac{dN'_{prET}}{dt} = \frac{dN_{prET}}{dt} \cdot \frac{V'_2}{V_2} \cdot 40 \tag{37}$$

In the dithiocarb treated mice, the exhaled endogenously produced ET concentration in the atmosphere of the closed chamber (y_{1end}) was modeled by the following function:

$$y_{1end} = \frac{k_{12} \cdot \frac{dN_{prET}}{dt}}{V_1 \cdot (k_{12} + k_{21})^2} \cdot \left[1 - e^{-(k_{12}+k_{21})t}\right] + \frac{k_{21} \cdot \frac{dN_{prET}}{dt}}{V_1 \cdot (k_{12} + k_{21})} \cdot t \qquad (38)$$

Using the known rate constants k_{12} and k_{21}, dN_{prET}/dt was obtained by fitting Eq. 38 to the experimental data. All fittings were done using the program Prism 4 for Macintosh (GraphPad Software, San Diego, USA).

Parameters valid only in open exposure systems

The atmospheric exposure concentration y_1 is constant only in an open exposure system. At steady-state conditions, the amount inhaled per time by a standard mouse equals the sum of the amounts exhaled and metabolized per time:

$$Cl'_{up} \cdot y_1 = Cl'_{ex} \cdot y_2 + Cl'_{met} \cdot y_2 \qquad (39)$$

Also at steady state, the ratio of the concentration of a gas in a standard animal to its constant concentration in the air is given by the bioaccumulation factor K'_{st}:

$$K'_{st} = \frac{y_2}{y_1} = \frac{Cl'_{up}}{Cl'_{ex} + Cl'_{met}} = \frac{V'_1 \cdot k'_{12}}{V'_2 \cdot (k'_{12} + k'_{met})} \qquad (40)$$

It has to be stressed that the value of K'_{st} depends on the exposure concentration because k'_{met} describes saturation kinetics. K'_{st} increases with increasing concentration and approaches K_{eq} at high concentrations ($y_2 >> K_m$):

$$K'_{st} = \frac{y_2}{y_1} \leq K_{eq} \qquad (41)$$

At low concentrations where the rate of metabolism follows first-order kinetics, K'_{st} becomes concentration independent and therefore constant. For such

conditions, Cl'_{met} (which is related to y_2) can also be related be related to y_1 when multiplying it with K'_{st}. The product was named Cl'_{metA}:

$$Cl'_{metA} = Cl'_{met} \cdot K'_{st} \tag{42}$$

The standardized alveolar retention (R'_{alv}), expressed as percent of Q'_{alv}, is a well known parameter which is often used to calculate the rate of metabolism of inhaled gaseous compounds at steady state. It is obtained using Cl'_{metA}:

$$R^{Õ}_{alv} = \frac{100 \cdot Cl^{Õ}_{met}}{Q^{Õ}_{alv}} \tag{43}$$

During exposure in an open system, y_2 reaches a steady state described by the product of y_1 with K'_{st} (see Eq. 40). After stopping the exposure, the substance is eliminated from the organism by exhalation and metabolism. If the metabolism follows first-order kinetics, the standardized biological half-life ($t'_{1/2}$) of the substance in an open system is obtained by the toxicokinetic model to:

$$t'_{1/2} = \frac{\ln 2}{k'_{21} + k'_{met}} \tag{44}$$

Formation of EO from ET

The EO formation from exogenous ET was studied by repeatedly administering ET into the atmosphere of the closed chamber (maintaining the ET concentration constant) and by monitoring the concentration-time course of the produced EO accumulating in the atmosphere of the closed chamber. These experiments imply, toxicokinetically spoken, an open system for ET and a closed one for EO. The concentration-time courses of exhaled EO were predicted by means of Eq. 6 inserting into λ_1 (Eq. 8), λ_2 (Eq.9), C_1 (Eq.10), C_2 (Eq. 11), and C_3 (Eq. 15), the toxicokinetic parameters of EO obtained from the gas uptake studies described above. The also in Eqs. 10, 11, and 15 required rate of metabolically produced EO (dN_{prEO}/dt) was modeled to be a

part of metabolized ET. Accordingly, the rate of ET metabolism (see Eq. 4) was split into two parts:

$$\frac{dN_{met}}{dt} = -k_{met} \cdot V_2 \cdot y_2 \cdot F - k_{met} \cdot V_2 \cdot y_2 \cdot (1-F) \quad (45)$$

F represents the fraction of metabolized ET which becomes bioavailable as EO. The production rate of EO (in the EO compartment 2) was modeled to be represented by the first term of Eq. 46. Considering Eq. 40, dN_{prEO}/dt was described by:

$$\frac{dN_{prEO}}{dt} = +k_{met} \cdot F \cdot V_2 \cdot K_{st} \cdot y_1 \quad (46)$$

Here, y_1 means the concentration of ET in the chamber atmosphere and K_{st} the experimental bioaccumulation factor of ET at steady state which was calculated from K'_{st} (Eq. 40).

Species scaling

Clearance values obtained in one species can be compared to those in another one by means of an allometric formula:

$$Cl'_{species1} = Cl'_{species2} \cdot \left(\frac{b.w._{species1}}{b.w._{species2}} \right)^{2/3} \quad (47)$$

Accordingly, the ratio of a clearance in a standard rat (b.w. 250 g) to that in a standard mouse (b.w. 25 g) is expected to be 4.

2.2.4 Calculations

GC detector responses of ET and EO were analyzed by linear regression using the program Prism 4 for Macintosh. The coefficient of determination (r^2) was used as quality criterion of linearity.

Exposure concentrations of ET in the chambers were kept constant for the determination of exhaled EO. For each exposure, the mean of the ET concentration was calculated from the area under the concentration-time course (AUC) divided by the exposure time. The AUC was obtained by the trapezoidal method.

AUCs of exhaled EO were obtained by the trapezoidal method. When first-order kinetics apply, the AUC until infinity of an EO concentration-time course increasing during ET exposure from zero to a plateau and decreasing after the end of exposure to zero was calculated as the product of the plateau value with the exposure duration because it is equal with a rectangle limited by the plateau value and the exposure time (Fig. 2.3, Filser, 1985). The corresponding AUC in the body was calculated by multiplying the AUC in air with the K_{eq} of EO.

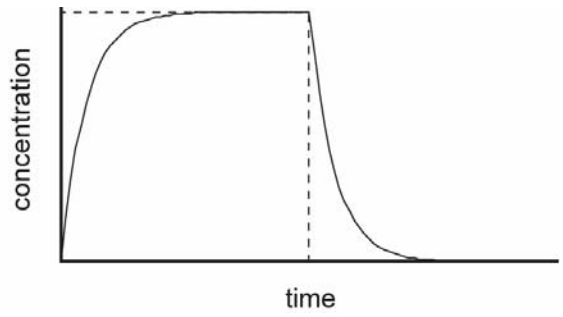


Figure 2.3 Calculation of an AUC of a concentration-time curve rising to a plateau value. The area under the curve (solid line) equals the area of the rectangle (broken lines).

A molar volume of 25.13 l/mol (25°C, 987 hPa) was used as factor to convert ppm into µmol/l air:

$$\left[\mu mol/l\right] = \frac{\left[ppm\right]}{25.13\ \left[l/mol\right]} \qquad (48)$$

2.2.5 Statistics

The arithmetic mean and the standard deviation (SD) were calculated using equations 49 and 50, respectively (Geigy Documenta, 1985):

$$\bar{x} = \frac{\sum_{i=1}^{i=n} x_i}{n} \tag{49}$$

$$SD = \sqrt{\frac{\sum \left(x_i - \bar{x}\right)^2}{n-1}} \tag{50}$$

with $\bar{x}$ representing the mean value of n analyses, x_i the value of the ith sample, and n the number of measurements.
The coefficient of variation (CV) in percent was obtained as follows:

$$CV = \frac{SD}{\bar{x}} \cdot 100 \tag{51}$$

The value of K_m of ET is defined as the ratio of $V_{max}^{\tilde{O}}$ to Cl'_{met} (Eq. 34). Therefore, K_m and its SD was calculated as given for the quotient of two means and their SDs (Sachs, 1997):

$$\frac{\bar{x}_1}{\bar{x}_2} \pm \frac{1}{\bar{x}_2^2} \sqrt{\bar{x}_1^2 \cdot SD_2^2 + \bar{x}_2^2 \cdot SD_1^2} \tag{52}$$

In order to examine whether dN'_{prET}/dt in naïve animals and dN'_{prET}/dt in dithiocarb pretreated animals were significantly different ($p<0.05$) from each other, Student's two-sided t-test was used.

3 Results

3.1 Analytical methods

3.1.1 Determination of atmospheric ethylene and ethylene oxide using gas chromatographs with flame ionization detectors

Typical gas chromatograms of ET and EO obtained from gas samples taken from chamber atmospheres are given in Figs. 3.1 and 3.2. No disturbing signals were observed at the retention times of the analytes. In gas uptake studies, all measurements were done using gas sample loops for injection. Since this injection mode resulted in relatively broad signals (see Fig. 3.2 A and B) it was not suitable for the measurement of the low concentrations of exhaled EO during ET exposure because of a chromatographic disturbance by an adjacent signal. Instead, those gas samples were injected directly on-column giving narrower signals, which were baseline-separated from adjacent signals (see Fig. 3.2 C, D and E). Due to the low sample volume, this method could be used only to determine exhaled EO at ET exposures ≥100 ppm because of the small injection volume.

The linearity of the detector response was tested for each method by injecting pure ET or EO or defined air mixtures of ET or EO into desiccators of known volumes. Curves were linear in the range of 10 – 80 ppb and of 1 – 10000 ppm for ET, of 0.1 – 5000 ppm for EO when injected via sample loops, and of 0.1 – 1 ppm for EO when injected on-column (Figs. 3.3, 3.4 and 3.5). For daily determinations, one-point calibrations were performed in the range of the expected ET and EO concentrations. The precision determined as CV was for ET ≤11% (10 – 80 ppb, n = 3) and ≤1.3% (0.25 – 10000 ppm, n = 3), for EO (sample loop injection) ≤5.7% (0.1 ppm, n = 3) and ≤1.4% (11 – 5000 ppm, n = 3) and for EO (on-column injection) 17% (0.1 ppm, n = 5) and ≤6% (0.2 – 1 ppm, n = 5). Concentrations of ET and of EO injected via the sample loops during inhalation experiments were far above the corresponding detection limits defined as three times the signal-to-noise ratios.

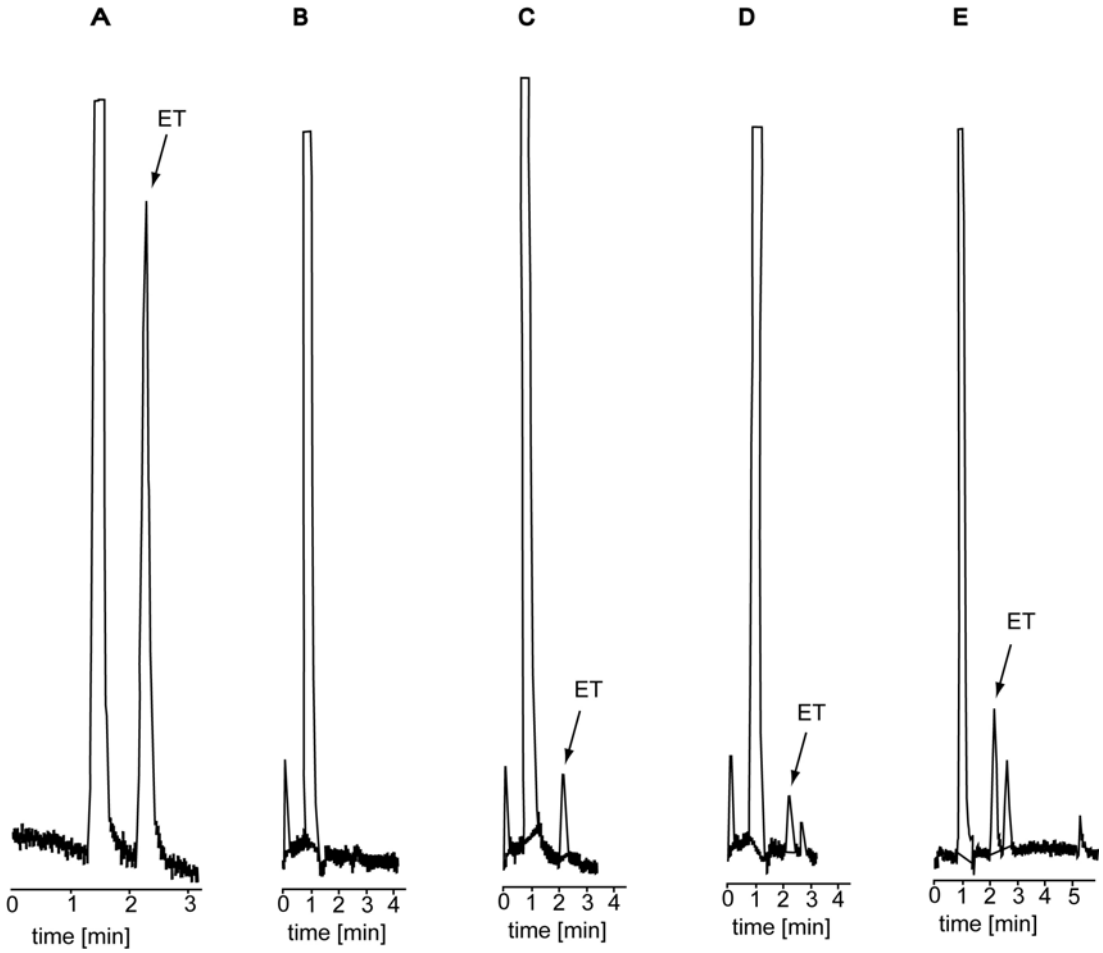


Figure 3.1 Typical gas chromatograms of atmospheric ET obtained by means of GC/FID (conditions see Tables 2.1). (**A**) ET in the closed system (2.8 l) during exposure of 5 mice to ET (0.9 ppm), (**B**) closed system (0.9 l) without ET, (**C**) desiccator containing ET (21 ppb), (**D**) endogenously produced ET (11 ppb) in the closed system (0.9 l) containing 6 mice, (**E**) endogenously produced ET (34 ppb) in the closed system (0.9 l) containing 6 mice pretreated with 400 mg dithiocarb/kg body weight.

The limits of detection of exhaled ET injected via the sample loop and of exhaled EO injected on-column were at 9 ppb and 0.1 ppm, respectively.

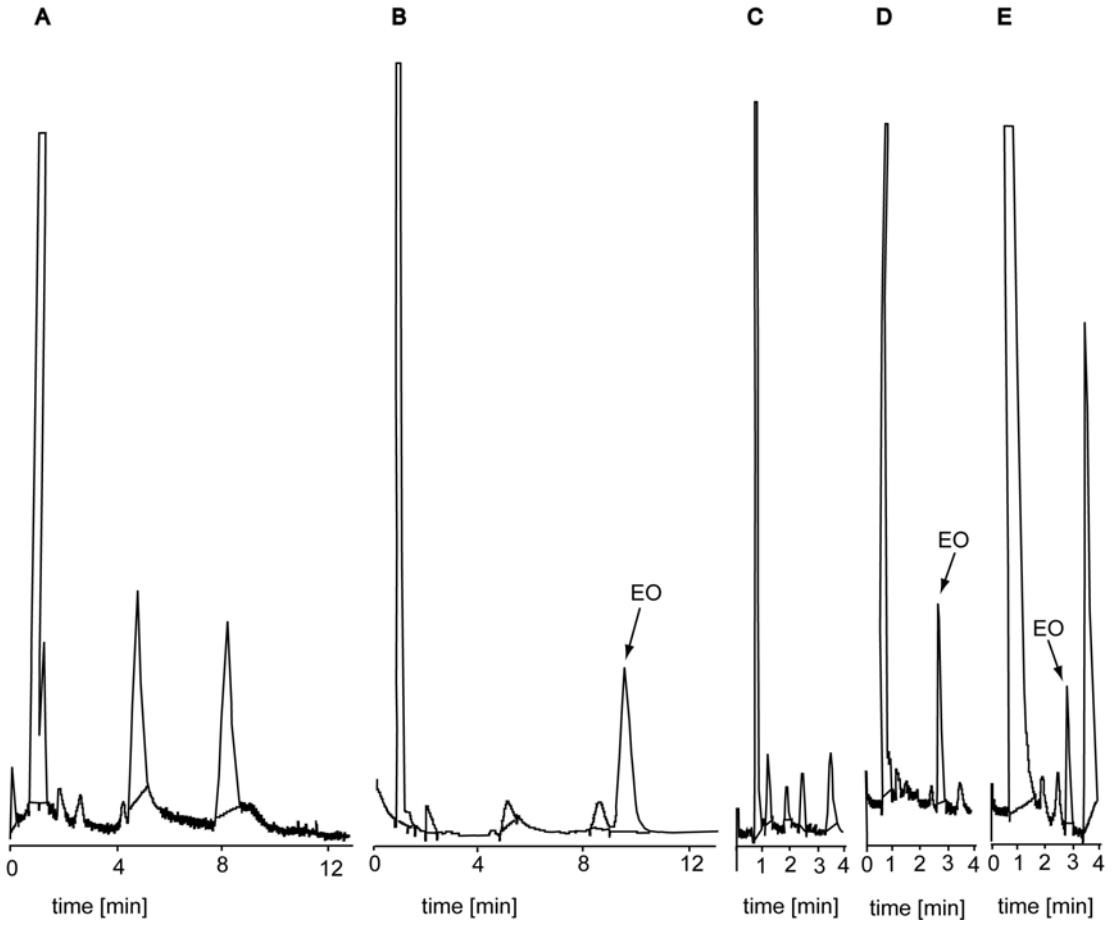
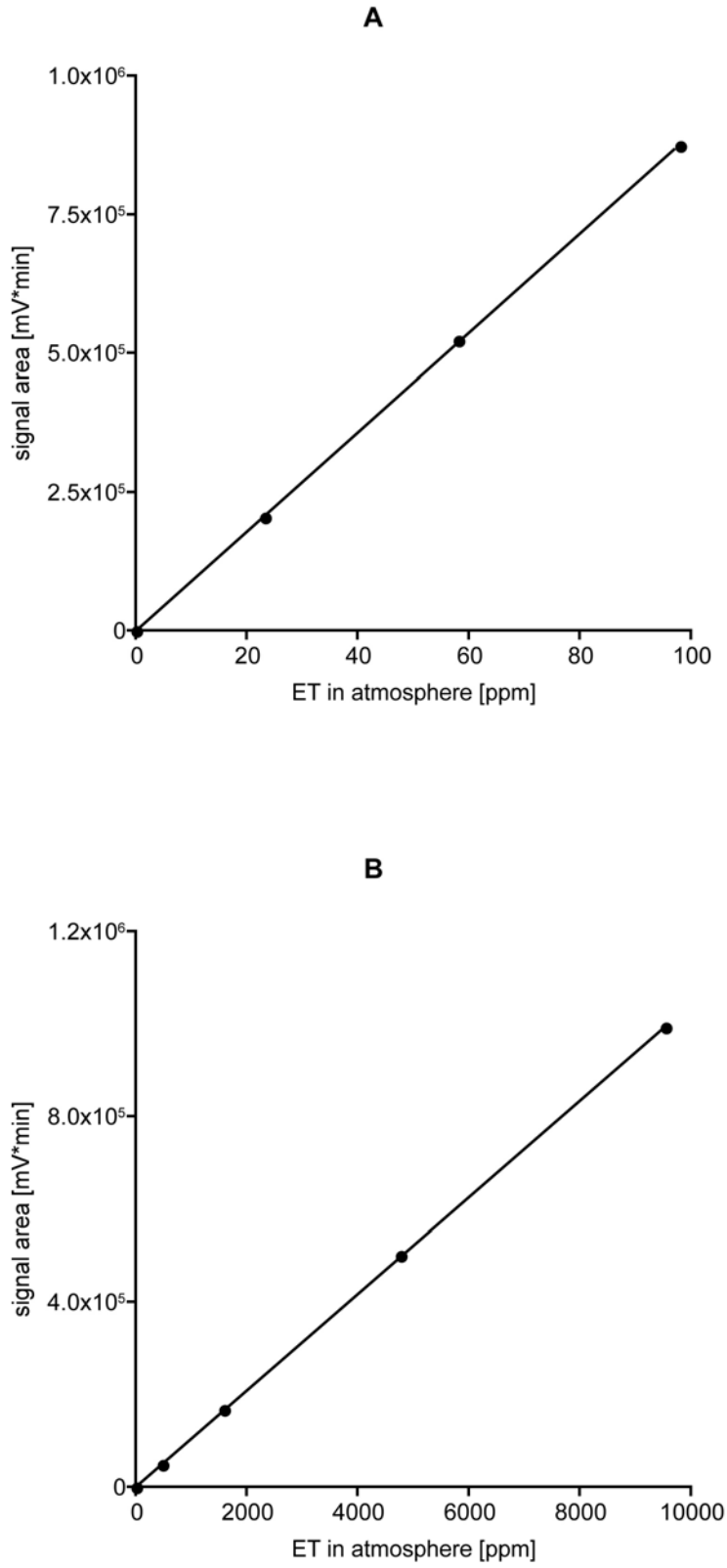


Figure 3.2 Typical gas chromatograms of atmospheric EO obtained by means of GC/FID (conditions see Tables 2.1). (**A**) closed system (6.5 l) without EO (sample loop injection, attenuation 0), (**B**) EO in the closed system (6.5 l) during exposure of 5 mice to EO (10 ppm, sample loop injection, attenuation 4), (**C**) closed system (6.5 l) without EO (on-column injection), (**D**) desiccator containing EO (1 ppm, on-column injection), (**E**) exhaled EO (0.7 ppm, on-column injection) in the closed system (2.8 l) containing 5 mice exposed for 7 h to 1000 ppm ET.


A
1.0x10⁶
7.5x10⁵
5.0x10⁵
2.5x10⁵
0
signal area [mV*min]
0
20
40
60
80
100
ET in atmosphere [ppm]
B
1.2x10⁶
8.0x10⁵
4.0x10⁵
0
signal area [mV*min]
0
2000
4000
6000
8000
10000
ET in atmosphere [ppm]

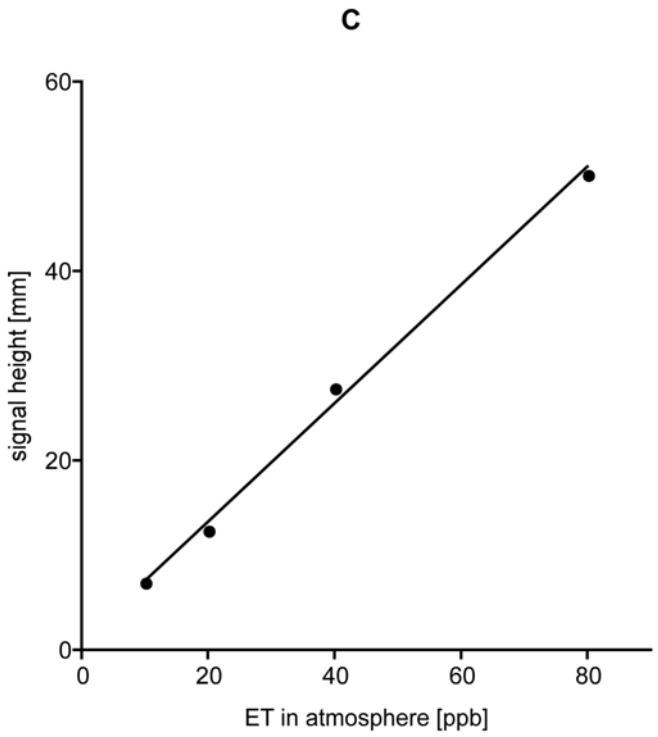


Figure 3.3 Calibration curves of atmospheric ET determined by means of a GC/FID. (**A**) Concentration range of 1 – 100 ppm (FID range 1). Symbols: means ± SD of three measurements; line: linear regression, y = 8950 x, r^2 = 0.9999. (**B**) Concentration range of 1 – 10000 ppm (FID range 100). Symbols: means ± SD of three measurements; line: linear regression, y = 104 x, r^2 = 0.999. If not visible, SD is smaller than the symbol. (**C**) Concentration range of 10 – 80 ppb. Symbols: means ± SD of three measurements; line: linear regression, y = 0.6194 x, r^2 = 0.9952. SD is always smaller than the symbol.

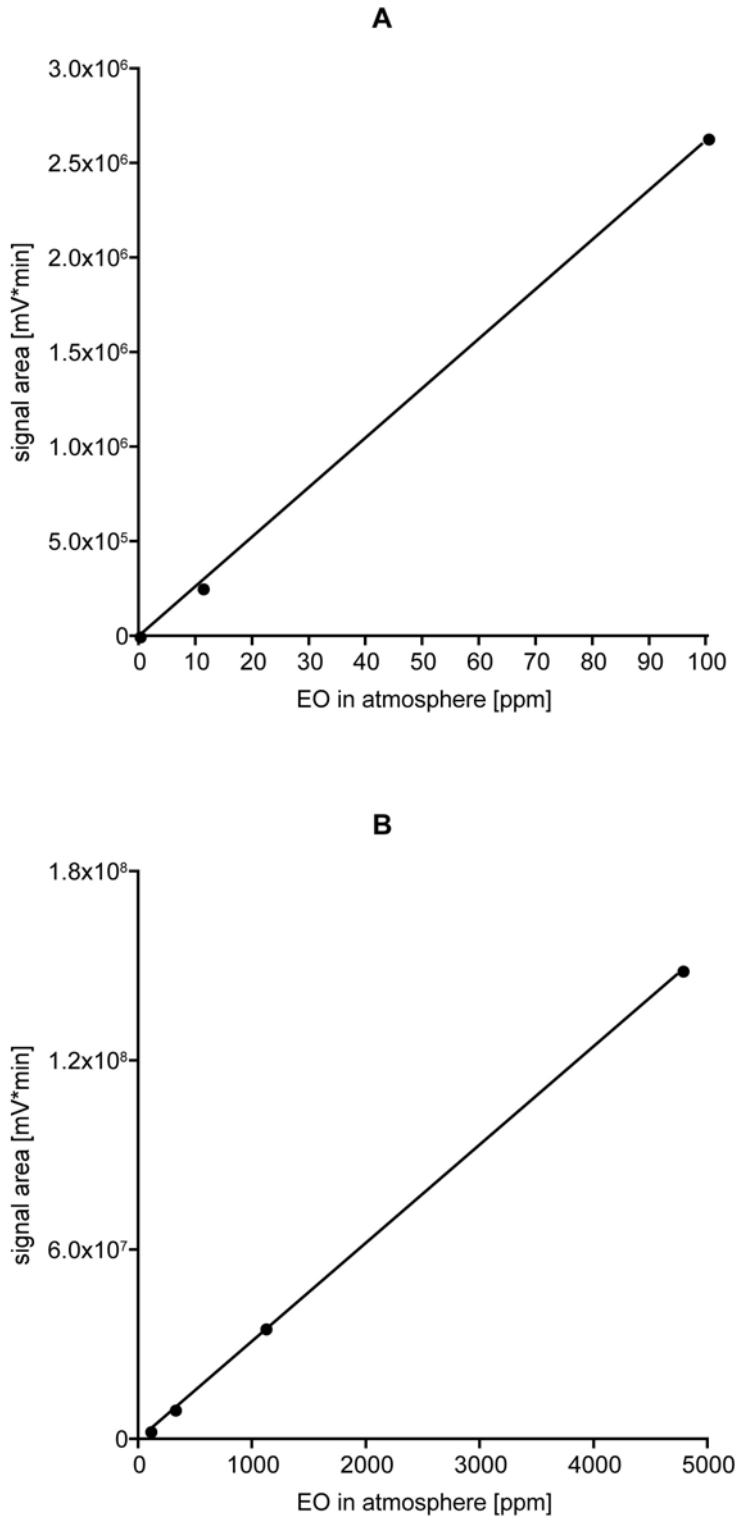


Figure 3.4 Calibration curves of atmospheric EO determined by means of a GC/FID (sample loop injection). (**A**) Low concentration range, Symbols: means ± SD of three measurements; line: linear regression, $y = 26220\ x$, $r^2 = 0.9997$. (**B**) High concentration range. Symbols: means ± SD of three measurements; line: linear regression, $y = 31160\ x$, $r^2 = 0.9999$. SD is always smaller than the symbol.

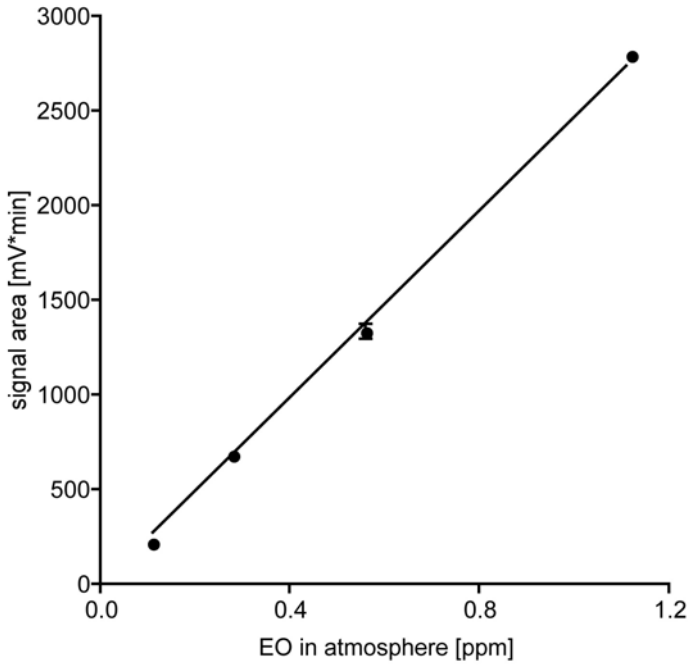


Figure 3.5 Calibration curve of atmospheric EO determined by means of a GC/FID (on-column injection). Symbols: means ± SD of three measurements; line: linear regression, $y = 2470\ x$, $r^2 = 0.996$. If not visible, SD is smaller than the symbol.

3.1.2 Determination of atmospheric ethylene oxide using a gas chromatograph with mass selective detector

Due to the low sensitivity of the GC/FID method with on-column injection, a GC/MSD method was developed to enable the measurement of exhaled EO at ET exposure concentrations ≤30 ppm. The MSD was run in the positive chemical ionization mode with methane as reactant gas in order to avoid fragmentation of EO into less specific ions. The mass spectrum of EO obtained in the scan mode is shown in Fig. 3.6. The single mass of m/z 45 representing the protonated EO molecule was used for quantification. Due to the influence of humidity on the separation phase of the capillary column, the retention time of EO decreased when samples were injected which were taken from the atmosphere of chambers containing animals.

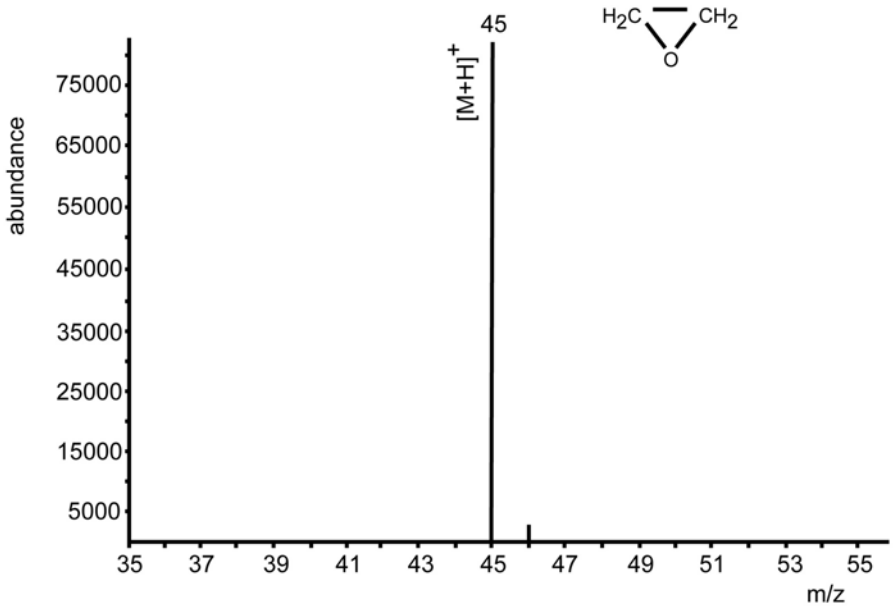


Figure 3.6 Mass spectrum of EO obtained in the scan mode of the MSD run in the positive chemical ionization mode with methane as reactant gas.

In order to verify the actual retention time, ^{13}C-EO was added into the atmosphere of the closed system before the final EO measurement. ^{13}C-EO was quantified using the ion m/z 47. Typical gas chromatograms in the single ion mode, given in Fig. 3.7, demonstrate that there was no EO detectable in an empty desiccator. However, EO was found when mice were exposed to ET.

Detector responses were linearly correlated to the EO concentration in the range of 0.7 – 40 ppb. A typical curve in the lowest range is given in Fig. 3.8 for EO gas samples of 8 ml. For each experiment, a one-point calibration was performed in the range of the expected EO concentration. The precision, expressed as CV, was around 0.9, 0.8, and 1.6% (n = 3), at 3, 7, and 21 ppb, respectively. The limit of detection, defined as three times the signal-to-noise ratio, was 0.45 ppb.

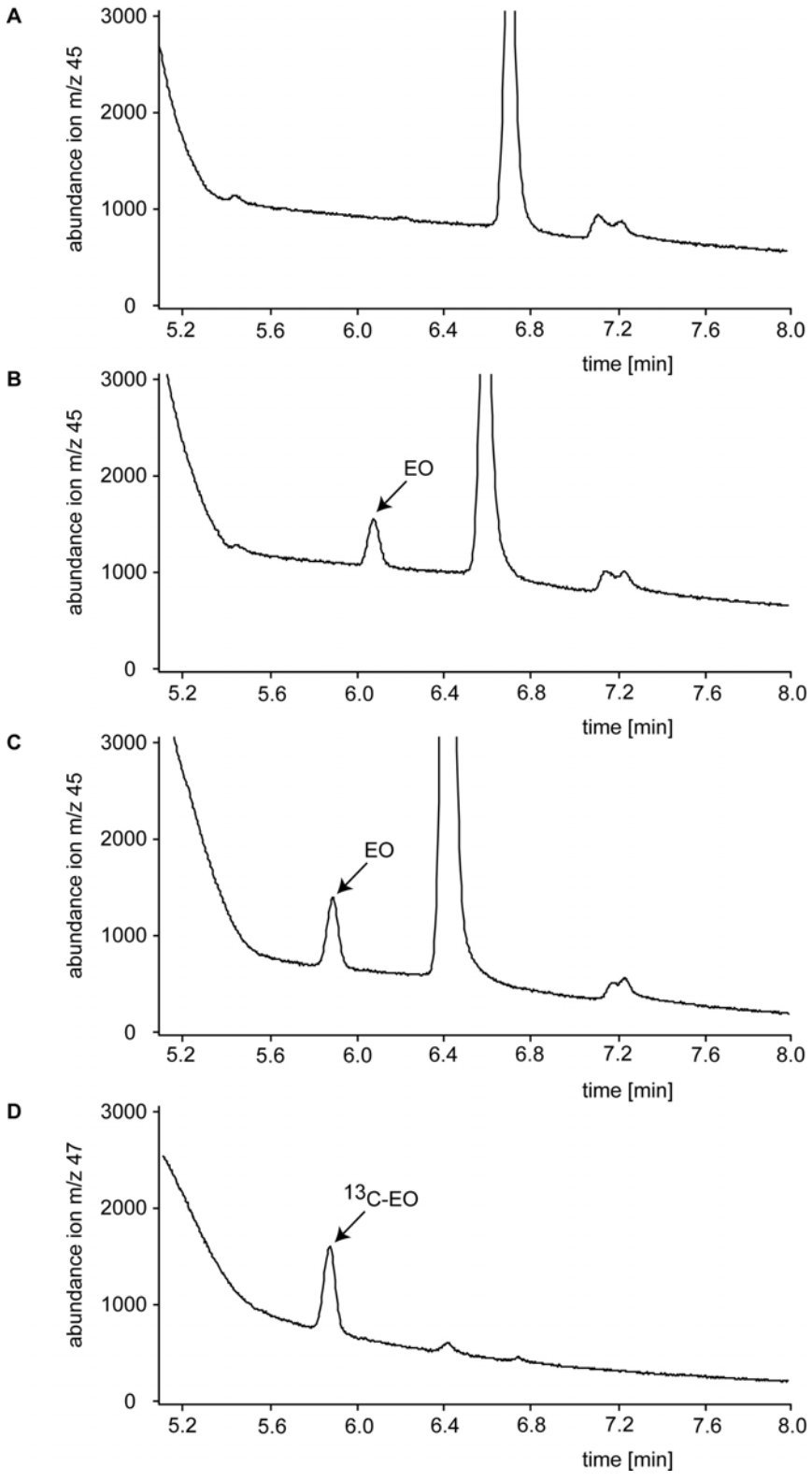


Figure 3.7 Typical gas chromatograms of EO and ^{13}C-ethylene oxide (^{13}C-EO) in the atmosphere obtained by means of a GC/MSD in the single ion mode. (**A**) Empty closed system (2.8 l). (**B**) EO concentration of 5 ppb prepared in a dry desiccator. (**C**) Closed system containing 5 mice exposed for 7 h to 3 ppm ET. (**D**) ^{13}C-EO (12 ppb) added to the closed system containing 5 mice exposed for 7 h to 3 ppm ET.

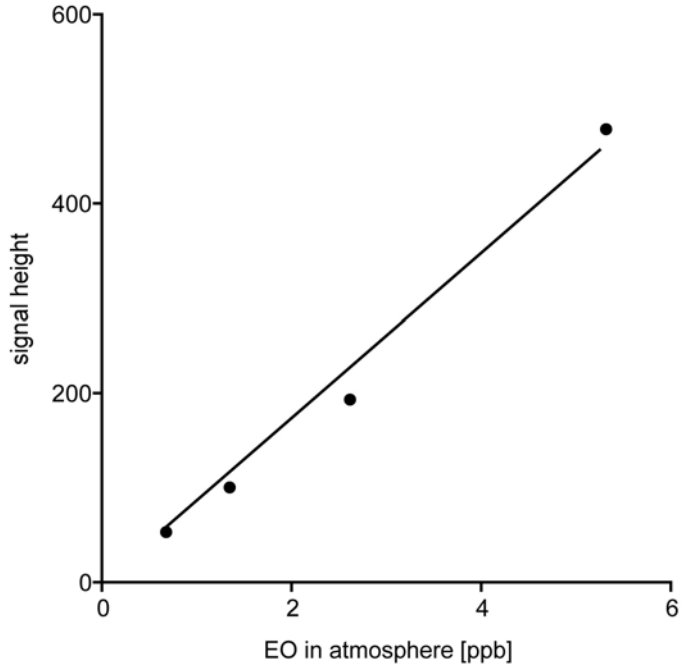


Figure 3.8 Calibration curve of EO determined by means of a GC/MSD. Symbols: single measurement; line: linear regression, y = 87 x, r^2 = 0.992.

3.2 Toxicokinetics of ethylene

3.2.1 Inhalation uptake, exhalation and maximum enrichment of ethylene

The thermodynamic equilibrium constant K_{eq} and the clearances of uptake by inhalation Cl'_{up} and of exhalation Cl'_{ex} in a standard mouse are listed in Table 3.1. The parameters were calculated from literature values as described in 2.2.3.

3.2.2 Metabolism of ethylene

In gas uptake experiments, ET was administered at various initial concentrations in the atmosphere of closed chambers (2.8 l), each containing 5 naïve mice. Fig. 3.9 shows in the semilogarithmic scale the resulting concentration-time courses of ET (filled symbols). At ET concentrations below 100 ppm, ET metabolism follows first-order kinetics as indicated from the parallel, linear concentration-time curves. At higher concentrations, the slopes of the curves flatten as a result from the saturation kinetics of ET metabolism. A control experiment, in the absence of mice, is also included in Fig. 3.9 (open symbols; dashed line). Here, the closed system was pre-humidified by exposing 5 mice to pure air for 5 h. The very small loss of ET over time (half-life about 280 h) results from the amount of ET taken away from the chamber atmosphere by the sampling for GC analysis. When comparing the two concentration-time courses obtained with and without mice, both starting at almost the same initial ET concentration, the steeper slope of the time course monitored in the presence of mice becomes evident. This difference can be regarded as experimental proof that ET is metabolized in the mouse. The solid lines in Fig. 3.9 represent modeled curves, the parameters being adjusted allometrically to each experiment. The three most upper curves served for the determination of V'_{max}. In the first-order range, Cl'_{met} was obtained from fits to the three lowest curves. The value of K_m was calculated as the quotient of V'_{max} and Cl'_{met} (see 2.2.3). The parameters are listed in Table 3.1.

In order to find out whether ET is metabolized by other enzymes than CYP, an ET gas uptake experiment was performed with 5 mice pretreated with the CYP inhibitor dithiocarb. The monitored concentration-time course shows a fast initial decrease of ET in the chamber atmosphere to a plateau concentration which remained constant until the end of exposure indicating complete inhibition of metabolism (Fig. 3.10).

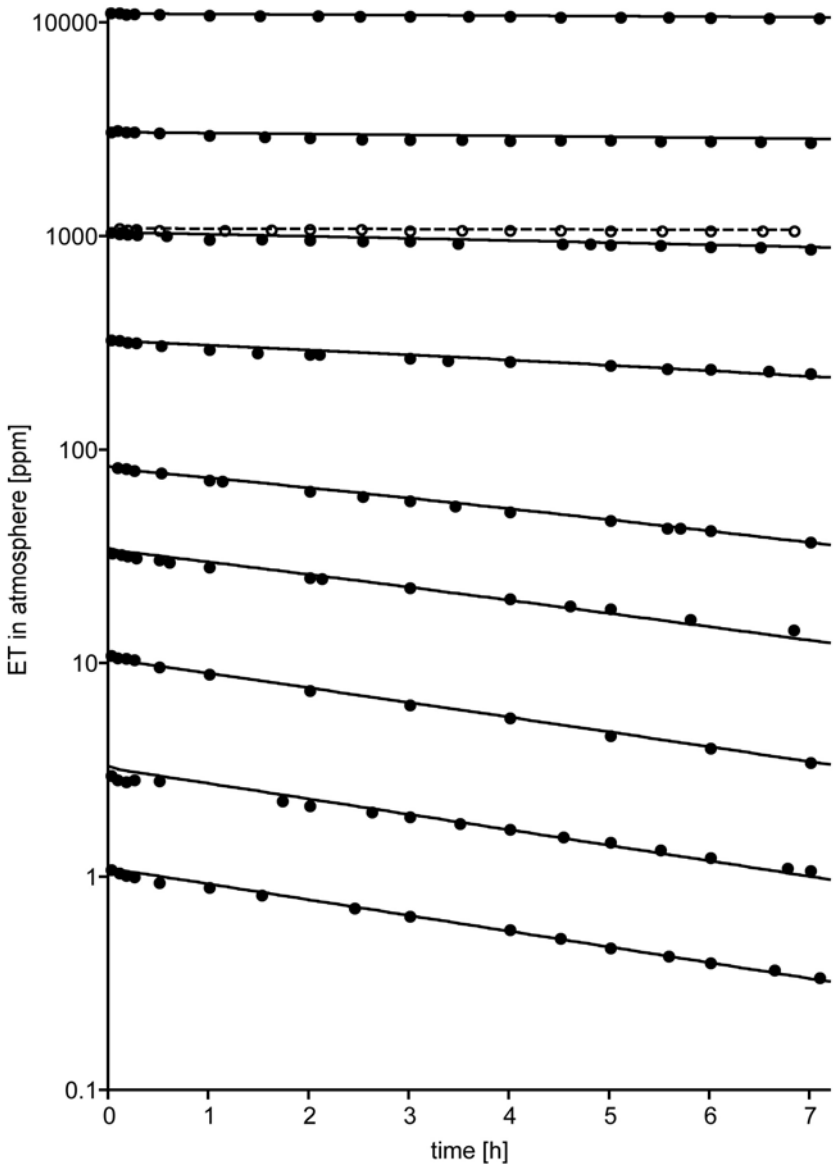


Figure 3.9 Concentration-time courses of ET at various initial concentrations in the atmosphere of closed exposure chambers (2.8 l). Symbols: measured data, filled circles from chambers occupied by 5 mice each, open circles from a chamber without mice; solid lines: constructed using the two-compartment model; dashed line: fit of an exponential function.

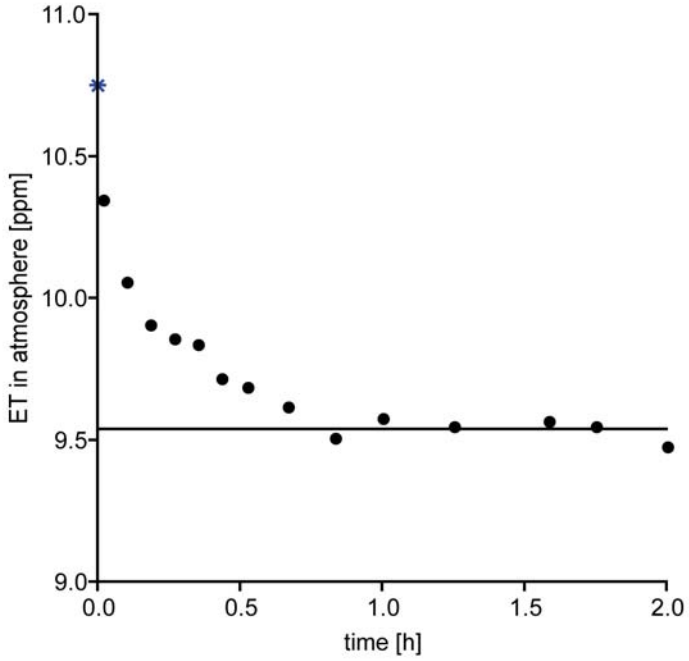
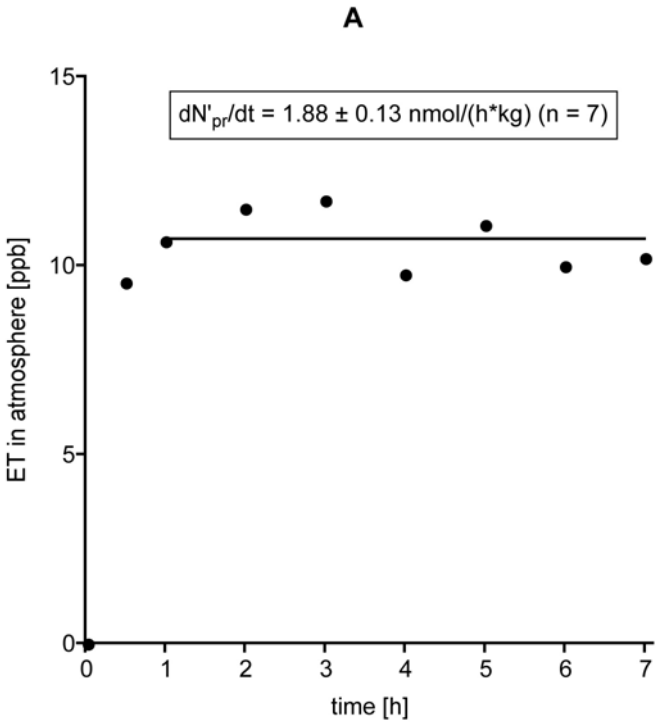
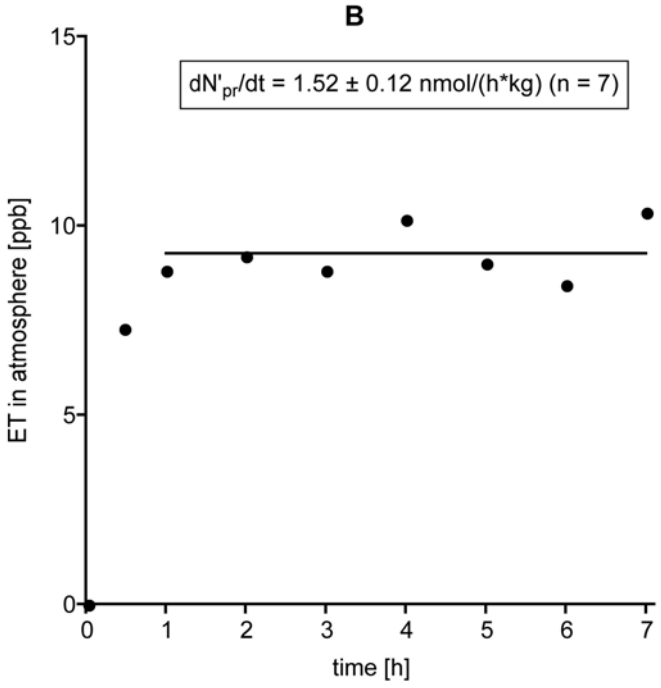


Figure 3.10 Time course of the ET concentration in the atmosphere of a closed chamber (0.8 l) containing 5 mice pretreated with the CYP inhibitor dithiocarb 30 min before start of exposure to ET. Star symbol: calculated initial concentration; filled symbols: measured data; line: plateau concentration at steady state.

3.2.3 Endogenous production of ethylene

Mice exhaled ET when kept in a closed system containing only air proving the existence of the endogenous production of ET. Atmospheric concentration-time courses of ET by six naïve mice kept together in a chamber (3 experiments) are depicted in Fig. 3.11. Steady-state concentrations of about 10 ppb ET were reached within 1 – 2 h. Because at steady state, the rate of uptake equals that of elimination, the experimental endogenous production rates of ET (dN_{prET}/dt) were calculated using Eqs. 35 and 36. The mean standard value $dN'_{prET}/dt \pm SD$ (n=20) obtained from the three experiments was calculated to be 1.62 ± 0.23 nmol/(h*kg).


A
dN'pr/dt = 1.88 ± 0.13 nmol/(h*kg) (n = 7)
ET in atmosphere [ppb]
time [h]
0
5
10
15
0
1
2
3
4
5
6
7


B
dN'pr/dt = 1.52 ± 0.12 nmol/(h*kg) (n = 7)
ET in atmosphere [ppb]
time [h]
0
5
10
15
0
1
2
3
4
5
6
7

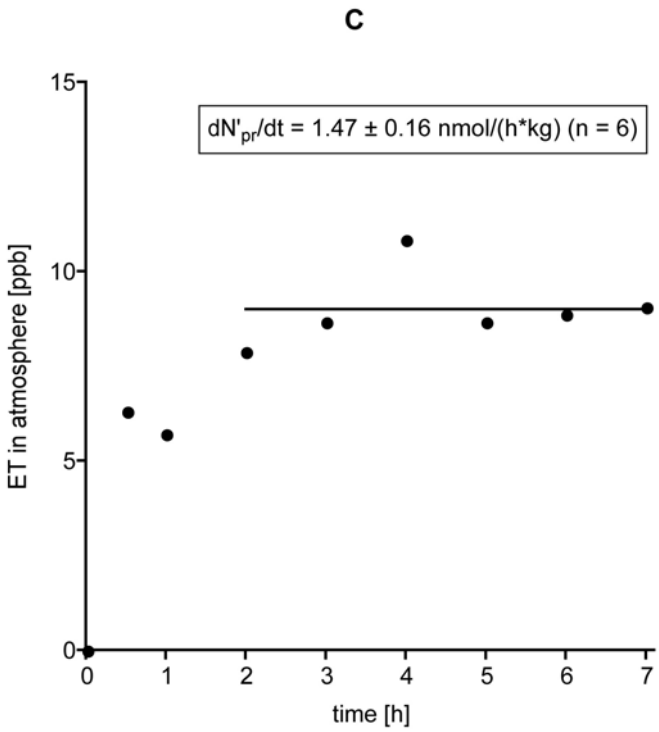
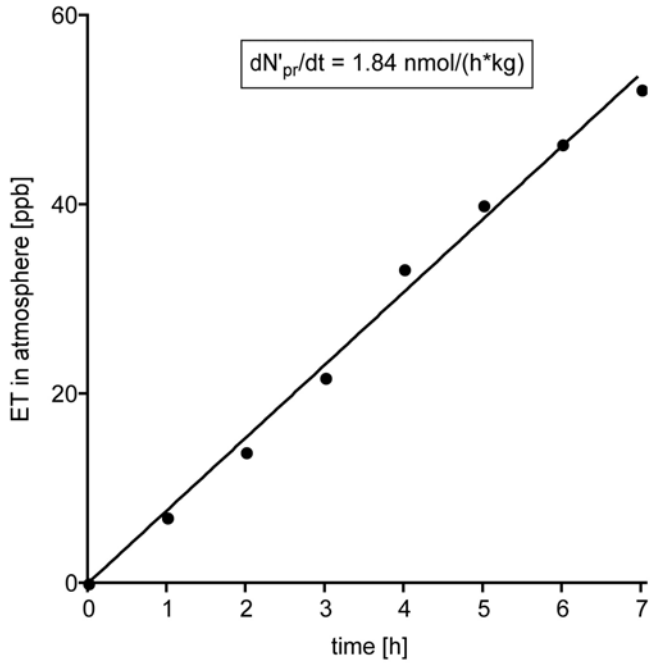


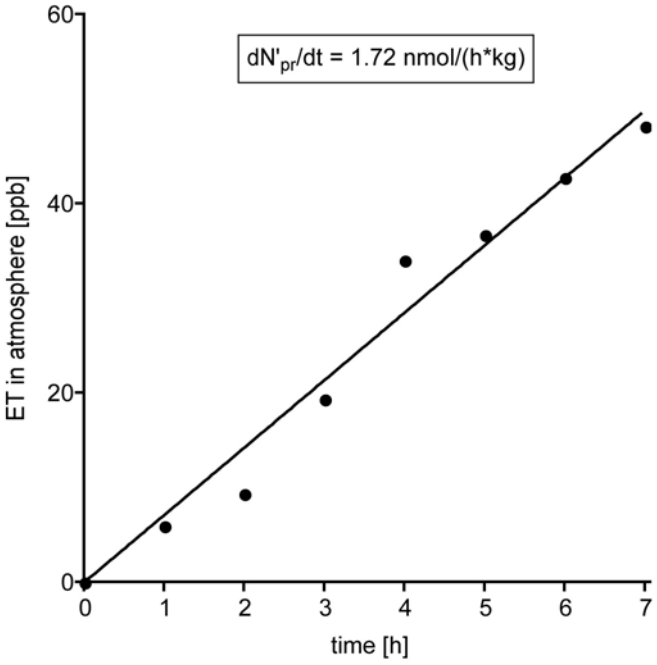
Figure 3.11 Concentration-time courses of ET in the atmosphere of closed chamber systems each containing 6 naïve mice. (**A**) Exposure system with V_1= 0.771 l and V_2= 0.139 l. (**B**) Exposure system with V_1= 0.738 l and V_2= 0.172 l. (**C**) Exposure system with V_1= 0.733 l and V_2= 0.177 l. Symbols: measured data; lines: average atmospheric ET concentrations at steady state; boxes: standardized endogenous production rates calculated from the corresponding steady state concentrations; n: number of data points used for calculation of endogenous production rates.

When six mice were treated with dithiocarb (400 mg/kg) and then kept together in a chamber that contained initially only synthetic air, the concentration of exhaled ET increased continuously until the end of exposure as shown in Fig. 3.12 for three experiments. Because of the dithiocarb-mediated complete inhibition of the ET metabolism, the linear concentration-time courses proved that dN_{prET}/dt was constant. Experimental values of dN_{prET}/dt were obtained by fitting Eq. 38 to the measured data. The obtained mean $dN'_{prET}/dt \pm SD$ of 1.88 ± 0.18 nmol/(h*kg) did not differ statistically significantly ($p<0.05$) from that found in naïve animals. Consequently, a mean value ± SD was calculated from all of the 6 experiments (Table 3.1).

A


dN'pr/dt = 1.84 nmol/(h*kg)
ET in atmosphere [ppb]
time [h]
0
20
40
60
0
1
2
3
4
5
6
7

B


dN'pr/dt = 1.72 nmol/(h*kg)
ET in atmosphere [ppb]
time [h]
0
20
40
60
0
1
2
3
4
5
6
7

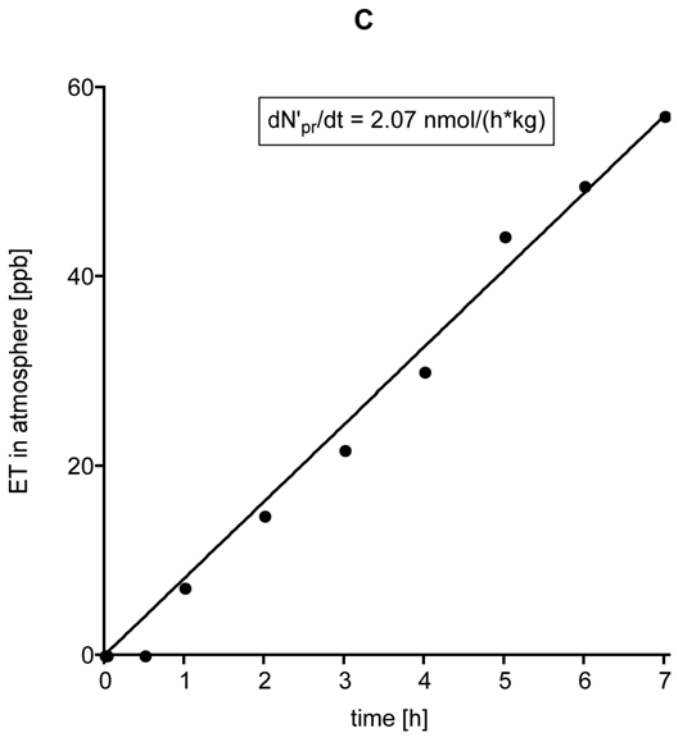


Figure 3.12 Concentration-time courses of ET in the atmosphere of closed chamber systems each containing 6 mice treated with 400 mg dithiocarb/kg. (**A**) Exposure system with V_1 = 0.760 l and V_2 = 0.151 l. (**B**) Exposure system with V_1 = 0.761 l and V_2 = 0.149 l. (**C**) Exposure system with V_1 = 0.768 l and V_2 = 0.142 l. Symbols: measured data; lines: model fits to the data; boxes: standardized endogenous production rates calculated from the corresponding model fits.

From the values of dN'_{prET}/dt obtained in naïve and dithiocarb-pretreated animals a mean ET concentration in the body of 0.072 nmol/l was calculated for a standard mouse (Table 3.1).

3.2.4 Standardized toxicokinetic parameters for steady-state conditions

The toxicokinetic parameters of ET obtained in 3.2.1, 3.2.2, and 3.2.3 were standardized as described in 2.2.3 and are listed in Table 3.1.

Table 3.1 Standardized toxicokinetic parameters of ET in the mouse.

Parameter	Formula	Value[a]	Dimension
Clearance of inhalation uptake (Cl'_{up})	$k'_{12} \cdot V'_1$	0.317	l/h
Clearance of exhalation (Cl'_{ex})	$k'_{21} \cdot V'_2$	0.453	l/h
Thermodynamic equilibrium constant body/air (K_{eq})	$\frac{k'_{12} \cdot V'_1}{k'_{21} \cdot V'_2}$	0.701	–
Bioaccumulation factor body/air at steady state (K'_{st})[b]	$\frac{k'_{12} \cdot V'_1}{(k'_{21} + k'_{met}) \cdot V'_2}$	0.550	
Maximum rate of metabolism (V'_{max})	–	15.5 ± 0.4 (n=3)	µmol/(h*kg)
Apparent Michaelis constant (K_m, related to average concentration in animal)	–	2.52 ± 0.07 (n=6)	µmol/l
Atmospheric conc. at $V'_{max}/2$	–	105	ppm
Clearance of metabolism (Cl'_{met})	$k'_{met} \cdot V'_2$	0.154 ± 0.0023 (n=3)	l/h
Clearance of metabolism related to the concentration in air (Cl'_{metA})[b]	$K'_{st} \cdot k'_{met} \cdot V'_2$	0.085	l/h
Alveolar retention (R'_{alv})[b]	$Cl'_{metA} \cdot 100 / Q'_{alv}$	5.66	%
Endogenous production rate (dN'_{pr}/dt)	–	1.75 ± 0.26 (n=6)	nmol/(h*kg)
Endogenous ET burden (y'_{2end})	–	0.072	nmol/l

[a]: Means ± SD obtained by model fitting. When no SD is given, in vitro data or mean values were used. [b]: valid for atmospheric concentrations up to 10 ppm (linear range).

The ratio of Cl'_{up} to Q'_{alv} shows that 21% of inhaled ET is bioavailable at ET exposure concentrations of up to 10 ppm. The value of Cl'_{met} is 25% and that of Cl'_{ex} is 75% of the sum of both elimination clearances. This signifies that the largest fraction of the bioavailable ET is exhaled unchanged. The clearance of metabolism can be related either to the average concentration in the organism expressed as Cl'_{met} or, when expressed as Cl'_{metA}, to the ET concentration in air. The product of Cl'_{metA} with the actual exposure concentration in air gives the actual rate of ET metabolism. The value of R'_{alv} is constant at low exposure concentrations where metabolism of ET can be described by first-order kinetics. It signifies that a maximum of only about 6% of inhaled ET is metabolized. With increasing ET exposure concentrations, the percentage of inhaled ET that is metabolized becomes even smaller due to the saturation kinetics of ET. Because of the saturation kinetics, all parameters influenced by metabolic elimination are concentration-dependent. Plots of the standardized rate of metabolism (dN'_{met}/dt) versus y_2 and y_1, calculated using V'_{max} and K_m, are given in Figs. 3.13 and 3.14. The curves given in Figs. 3.13 and 3.14A are almost parallel because the concentration dependent change of the ET bioaccumulation factor – given by K'_{st} (Eq. 40) – varies only by 27% from its smallest value at concentrations below 10 ppm ET to its maximum value (K_{eq}) at very high ET concentrations (see Table 3.1 and Fig. 3.15). dN'_{met}/dt increases linearly until an atmospheric concentration of about 10 ppm. At 40 ppm, the linear curve overpredicts the actual dN'_{met}/dt by 30%. At higher concentrations, the curve flattens increasingly since kinetics of ET metabolism change continuously from first-order to zero order due to saturation kinetics. The atmospheric ET concentration at $V'_{max}/2$ is 105 ppm; 90% of V'_{max} is reached at about 840 ppm.

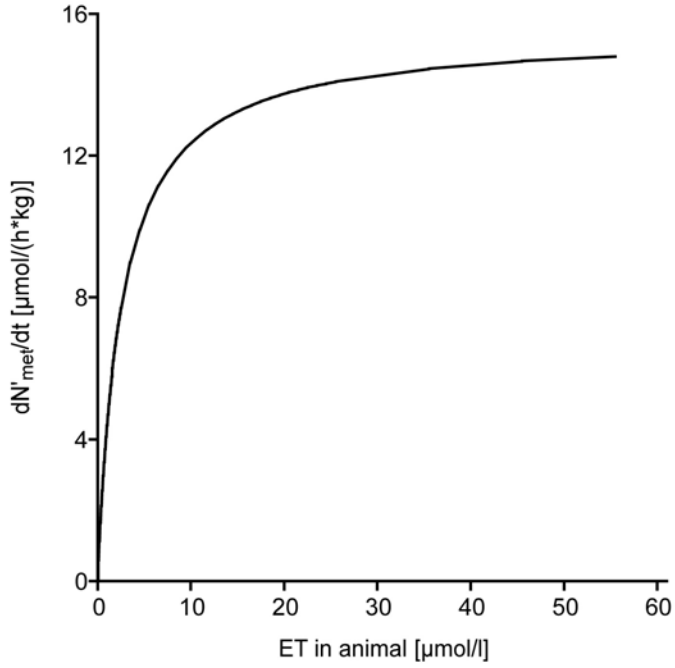


Figure 3.13 Dependence of the standardized metabolic elimination rate of ET (dN'_{met}/dt) on the average ET concentration in the body of mice exposed to ET in an open system. Line: model calculation using V'_{max} and K_m.

The relationship between y_2 and y_1 is shown in Fig. 3.16 for average ET concentrations in the animal body below 2.50 µmol/l body volume and in the atmosphere below 105 ppm. The deviation of the curve from linearity is only marginal reflecting the small difference between K'_{st} (below 10 ppm ET) and K_{eq}.

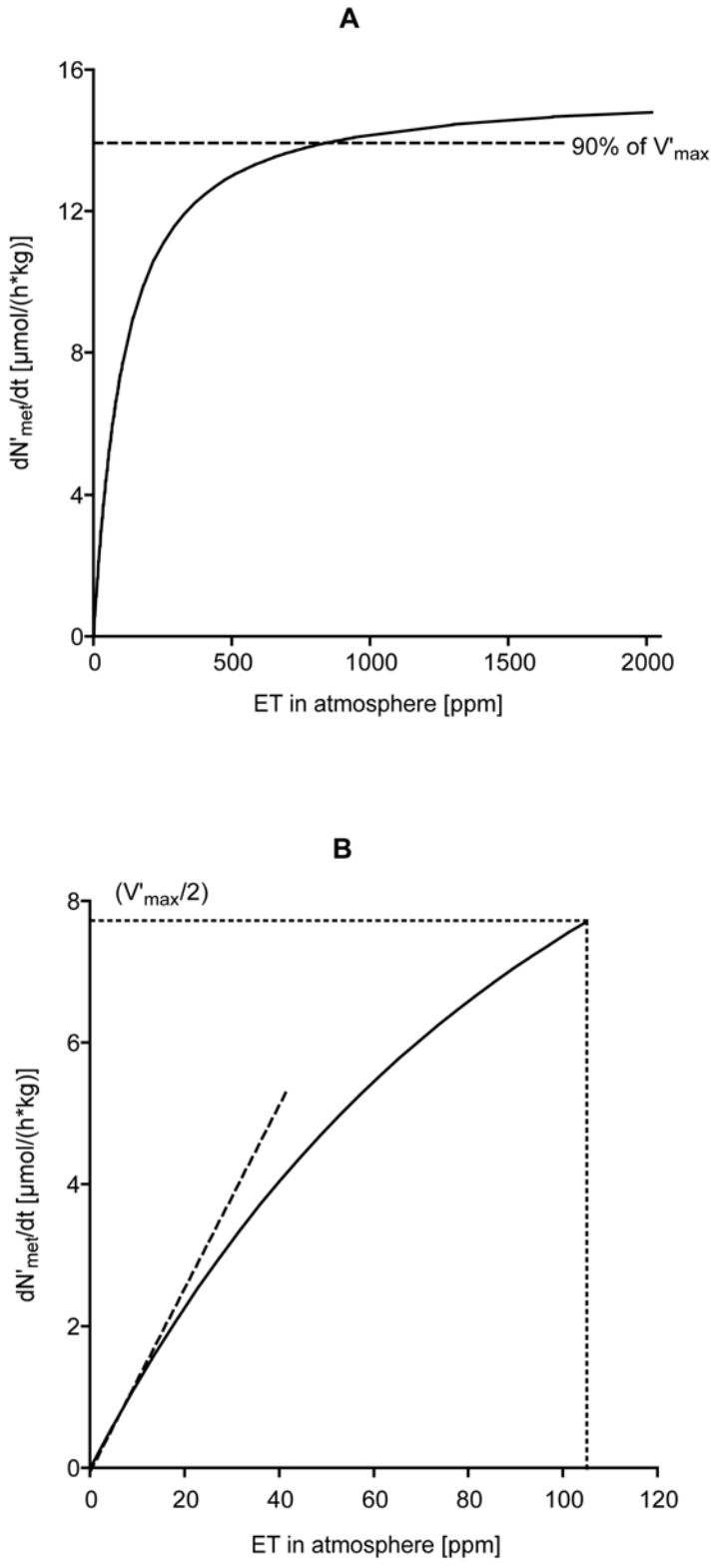


Figure 3.14 Dependence of the standardized metabolic elimination rate of ET (dN'_{met}/dt) on the atmospheric ET concentration in mice exposed to ET in an open system. (**A**) High concentration range. Line: calculated from Fig. 3.13 using K'_{st} and the micromolar-to-ppm conversion factor 25.13. (**B**) Low concentration range. Line: calculated as in **A**; dashed line: slope of linear kinetics.

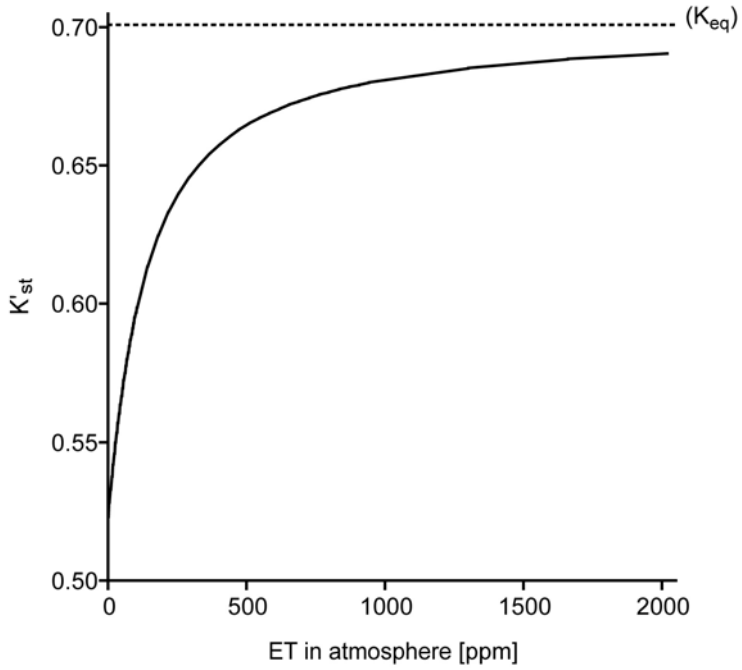


Figure 3.15 Concentration dependence of the bioaccumulation factor at steady state (K'_{st}) of ET in a standard mouse of 25 g exposed to constant ET concentrations in the atmosphere. Solid line: model calculation; dashed line: K_{eq}.

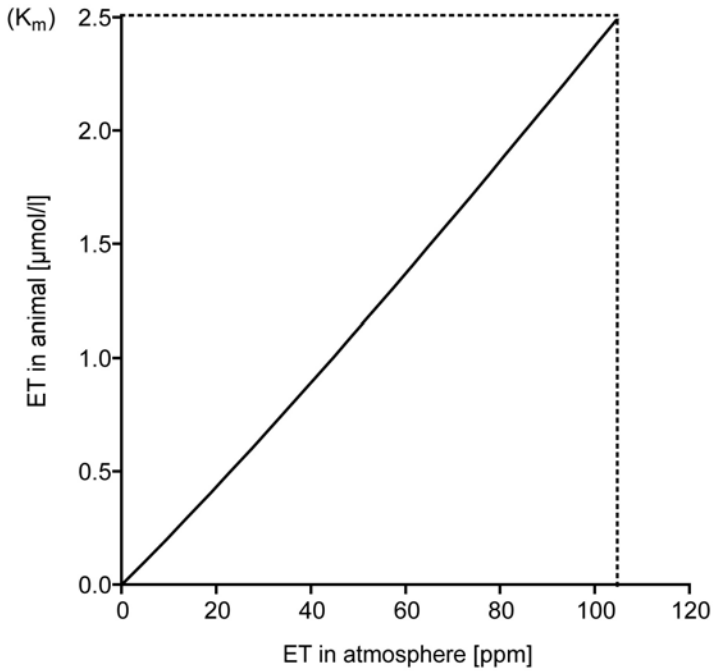


Figure 3.16 ET concentrations at steady state in a standard mouse of 25 g versus constant ET exposure concentrations in the atmosphere. Line: model calculation.

3.3 Toxicokinetics of ethylene oxide

3.3.1 Inhalation uptake, exhalation and maximum enrichment of ethylene oxide

As for ET, the thermodynamic equilibrium constant K_{eq} and the clearances of uptake by inhalation Cl'_{up} and of exhalation Cl'_{ex} in a standard mouse were calculated from literature values as described in 2.2.3. These parameters are listed in Table 3.2. The factor f respecting a "wash-in-wash-out" effect of the amphiphilic EO in the upper airways (see Eq. 22) was set to 0.47. This value gave the best fits of modeled curves to the concentration-time courses measured in the gas uptake experiments summarized as semilogarithmic plots in Fig. 3.17. In these experiments, 5 mice each were exposed in closed exposure chambers of 6.4 l to EO with initial concentrations ranging from 10 to 3000 ppm.

3.3.2 Metabolism of ethylene oxide

The semilogarithmic plots of the gas uptake experiments with 5 mice shown in Fig. 3.17 reveal linearly decreasing parallel concentration-time courses of up to an initial EO concentration of 300 ppm. This figure indicates that there was no saturation of the metabolic EO elimination within this concentration range. At initial EO concentrations of ≥1000 ppm, the decreases deviate from linearity and the slopes flatten with time. This effect resulted most probably from depletion of GSH leading to a reduced metabolic elimination via the GST pathway (see Discussion). At the initial concentration of 3000 ppm, the high internal EO concentration led to acute effects after 4 h (reduction of physical activity), enforcing the termination of the experiment. Cl'_{met}, characterizing the metabolic elimination in the first-order range, was obtained from model fits to the four lowest curves as described in 2.2.3. Cl'_{met} is given in Table 3.2. A control experiment was performed in an empty chamber the air of which was humidified by keeping 5 mice in the chamber for 5 h before starting the experiment.

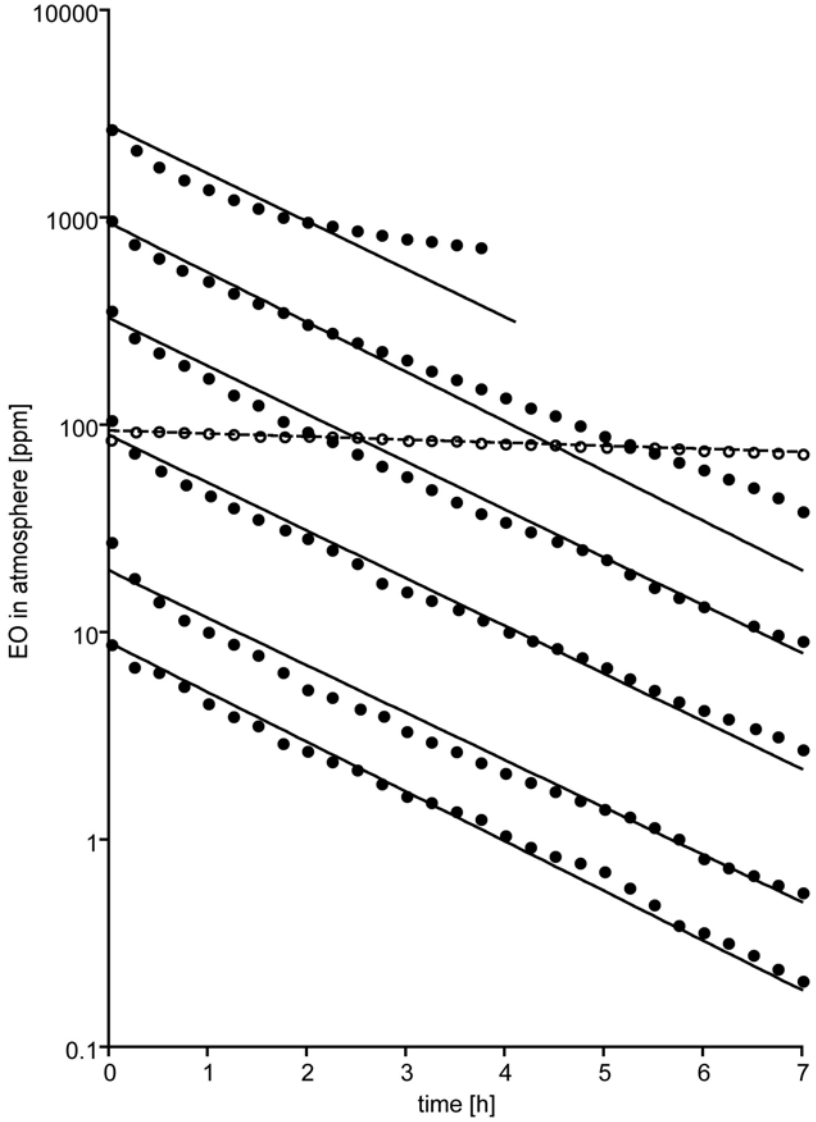


Figure 3.17 Concentration-time courses of EO at various initial concentrations in the atmosphere of closed exposure chambers (6.4 l). Symbols: measured data, filled circles from chambers occupied by 5 male B6C3F1 mice each, open circles from a chamber without mice; solid lines: constructed using the two-compartment model; dashed line: fit of an exponential function.

A small loss of EO (half-life 20.5 h) was found in the humidified chamber. It was larger than that calculated from the gas volume taken for GC analysis (Fig. 17). The small concentration loss resulted mainly from hydrolysis of EO in the humidified soda lime. It was neglected when yielding Cl'_{met} from the concentration-time courses obtained in the experiments with mice.

3.3.3 Standardized toxicokinetic parameters for steady-state conditions

The toxicokinetic parameters of EO obtained in 3.3.1 and 3.3.2 were standardized as described in 2.2.3 and are presented in Table 3.2. The ratio of Cl'_{up} to Q'_{alv} shows that 46% of inhaled EO is bioavailable at EO exposure concentrations of up to 200 ppm. The value of Cl'_{met} is 94% and that of Cl'_{ex} is 6% of the sum of both elimination clearances. This signifies that by far most of the bioavailable EO is metabolized. The similarity of the Cl'_{met} value with the hepatic blood flow (0.255 l/h, Arms and Travis, 1988) points to a limitation of the rate of EO metabolism by the transport to the metabolizing enzymes in the liver. The alveolar retention R'_{alv} signifies that at steady state 43% of inhaled EO is metabolized. For EO concentrations higher than 200 ppm, no Cl'_{met} value was calculated since the metabolic elimination changed with the time of exposure.

Fig. 3.18 shows the dependence of dN'_{met}/dt of EO from the average EO concentration in the mouse (A) and from the EO concentration in the atmosphere (B) in the concentration ranges in which dN'_{met}/dt followed first-order kinetics. The slope in Fig. 3.18A represents $Cl^{Õ}_{met} \cdot 40$ because dN'_{met}/dt is the rate of EO metabolism in 40 mice of 25 g each. The slope in Fig. 3.18B is $Cl^{Õ}_{metA} \cdot 40/25.13$.

K'_{st} is more than an order of magnitude lower than K_{eq} due to the effective metabolism of EO. Fig. 3.19 demonstrates the linear dependence of y_2 from y_1 up to 200 ppm with the slope $K'_{st}/25.13$.

Table 3.2 Standardized toxicokinetic parameters of EO in the mouse.

Parameter	Formula	Value[a]	Dimension
Clearance of inhalation uptake (Cl'_{up})	$k'_{12} \cdot V'_1$	0.694	l/h
Clearance of exhalation (Cl'_{ex})	$k'_{21} \cdot V'_2$	0.0122	l/h
Thermodynamic equilibrium constant body/air (K_{eq})	$\frac{k'_{12} \cdot V'_1}{k'_{21} \cdot V'_2}$	56.9	
Bioaccumulation factor body/air (K'_{st})[b]	$\frac{k'_{12} \cdot V'_1}{\left(k'_{21} + k'_{met}\right) \cdot V'_2}$	3.43	
Clearance of metabolism (Cl'_{met})[b]	$k'_{met} \cdot V'_2$	0.19 ± 0.022 (n=4)	l/h
Clearance of metabolism related to concentration in air (Cl'_{metA})[b]	$K'_{st} \cdot k'_{met} \cdot V'_2$	0.65	l/h
Alveolar retention (R'_{alv})[b]	$Cl'_{metA} \cdot 100 / Q'_{alv}$	43	%
Half-life ($t'_{1/2}$)[b]	$\frac{\ln 2}{k'_{21} + k'_{met}}$	5.1	min

[a]: Means ± SD obtained by model fitting. When no SD is given, in vitro data or mean values were used.
[b]: valid for atmospheric EO concentrations up to 200 ppm (linear range).

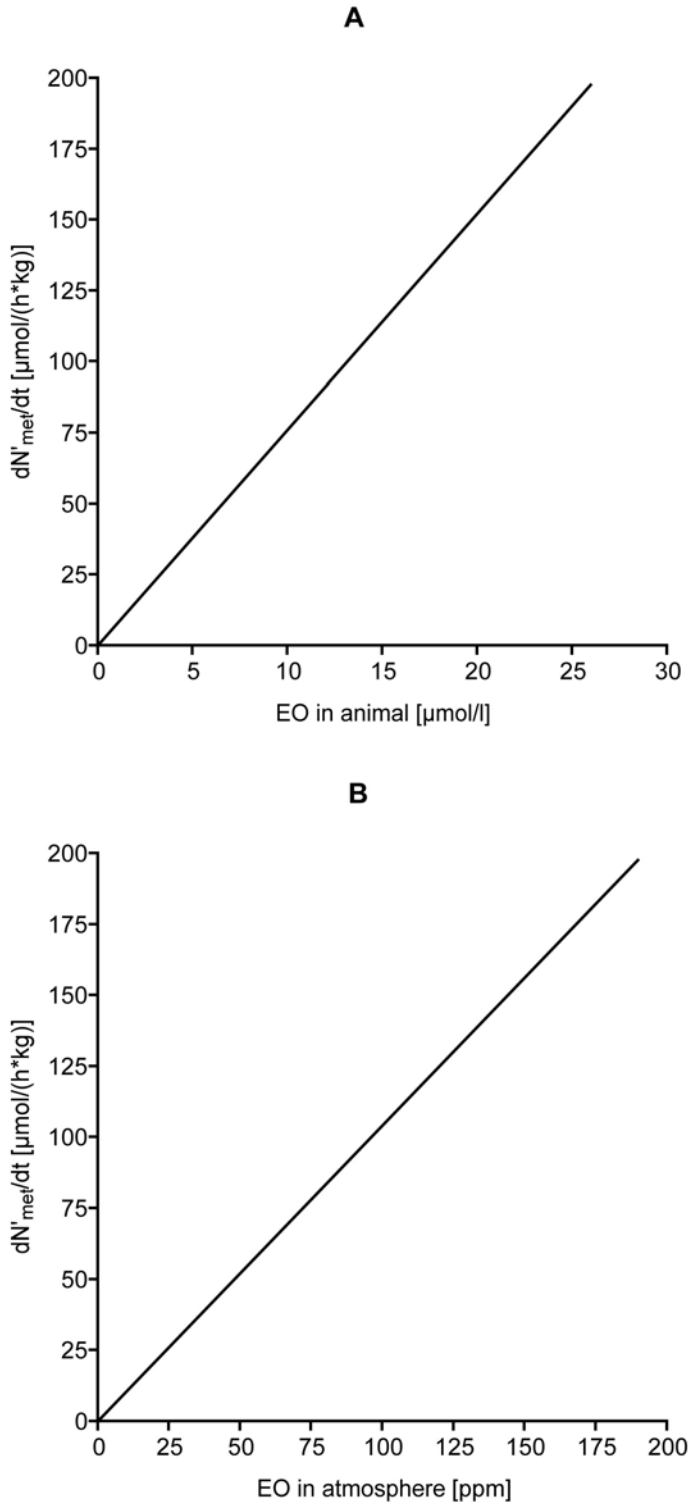


Figure 3.18 Dependence of the standardized metabolic elimination rate of EO (dN'_{met}/dt) in mice exposed to EO in an open system on EO concentrations. (**A**) dN'_{met}/dt versus the average EO concentration in the body. Line: model calculation. (**B**) dN'_{met}/dt versus the atmospheric EO exposure concentration at steady state. Line: calculated from **A** using K'_{st} and the ppm-to-micromolar conversion factor 25.13.

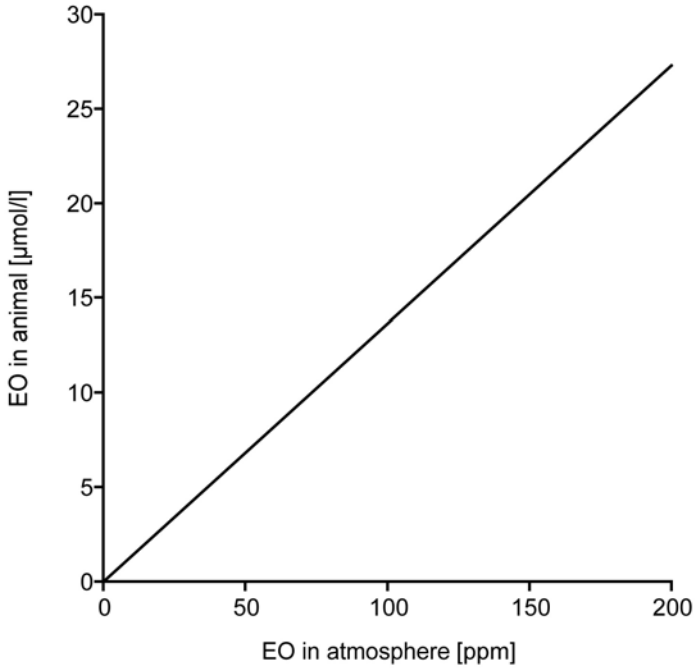


Figure 3.19 EO concentrations at steady state in a standard mouse of 25 g versus constant EO exposure concentrations in the atmosphere. Line: model calculation.

3.4 Exhalation of ethylene oxide during ethylene exposure

In these experiments, 5 mice each were exposed to a series of quasi-constant atmospheric ET concentrations covering an ET concentration range from 1 up to 10000 ppm. A typical concentration-time course of atmospheric ET at a low (1 ppm, A) and a relatively high concentration (1180 ppm, B) is given in Fig. 3.20. At concentrations <1000 ppm, loss of ET resulting from ET metabolism was compensated by repeatedly re-injecting ET gas into the chamber atmosphere. At concentrations ≥1000 ppm, ET was not compensated because its loss was very small (because of saturation of ET metabolism). ET concentrations did not deviate more than 20% from the mean value at ≤10 ppm ET and not more than 10% at ≥30 ppm ET.

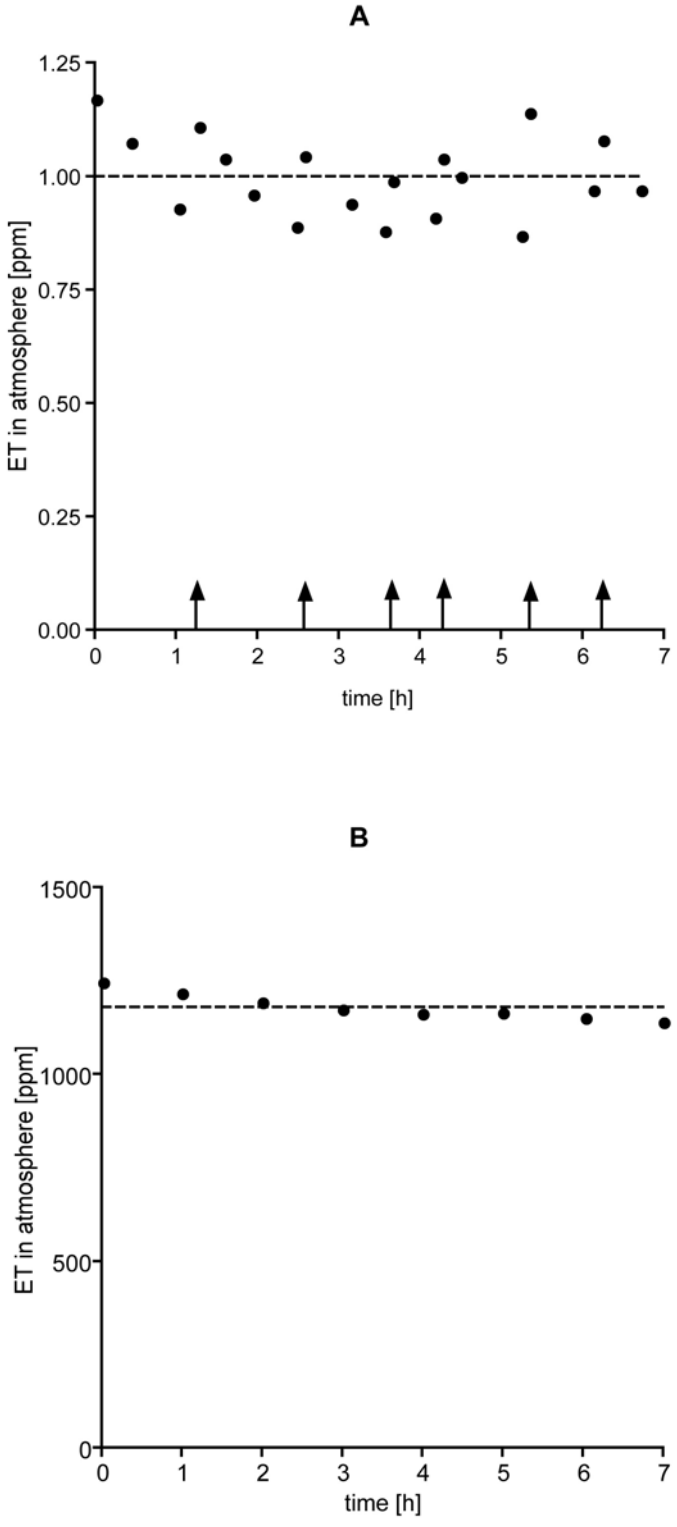


Figure 3.20 Concentration-time courses of ET in the atmosphere of closed chambers of 2.8 l each containing 5 mice exposed to (**A**) 1.0 ppm or (**B**) 1200 ppm ET. Symbols: measured data of single experiments; dashed lines: mean concentrations; arrows: time points of ET injections into the atmosphere in order to compensate for the ET loss due to metabolism.

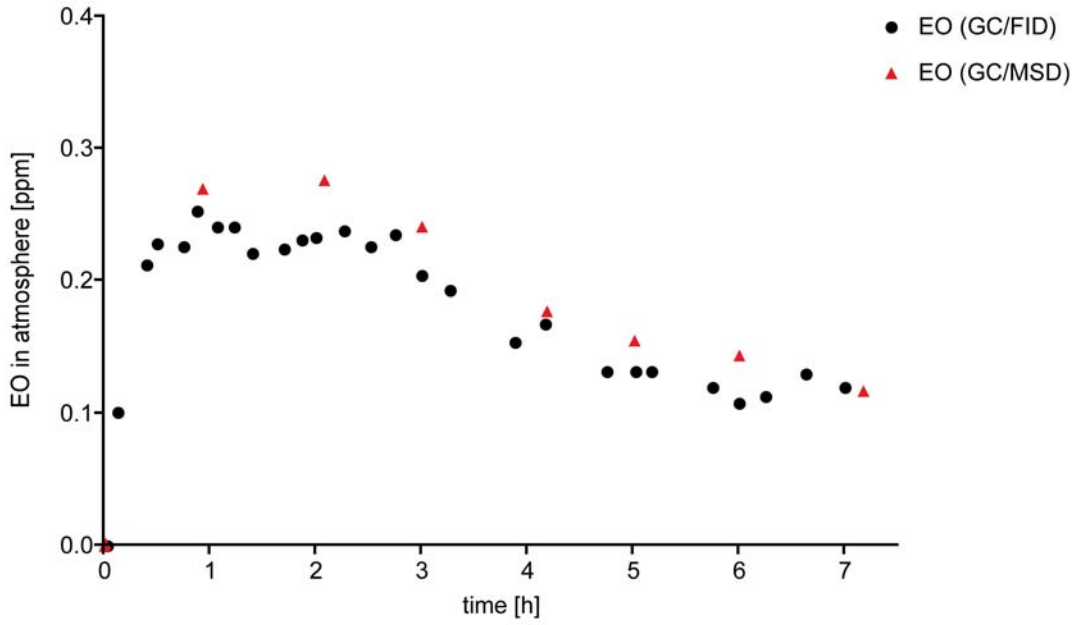


Figure 3.21 Comparison of the GC/FID and the GC/MSD method used for the determination of exhaled EO (one ET exposure experiment). Symbols: measured concentration-time course of atmospheric EO exhaled by 5 mice exposed together to 100 ppm ET in a closed chamber (2.8 l).

During exposure to ET, animals exhaled the metabolite EO that accumulated in the atmosphere of the closed chamber. The GC/FID method with on-column injection and the GC/MSD method were used to quantify EO at ET exposures ≥100 ppm and ≤100 ppm, respectively. Both methods agreed well as exemplified in Fig. 3.21 for an ET concentration of 100 ppm. No EO was detectable in the chamber atmosphere when ET was absent (detection limit 0.45 ppb).

The concentration-time courses of measured EO in chamber atmospheres upon ET exposures are summarized in Fig. 3.22. Up to 30 ppm ET, the EO concentrations increased continuously until reaching plateaus. At ET concentrations ≥100 ppm, the concentrations of metabolically formed EO showed early peaks in the chamber atmosphere. The peak heights increased with the ET concentrations reaching a maximum value of about 0.75 ppm EO.

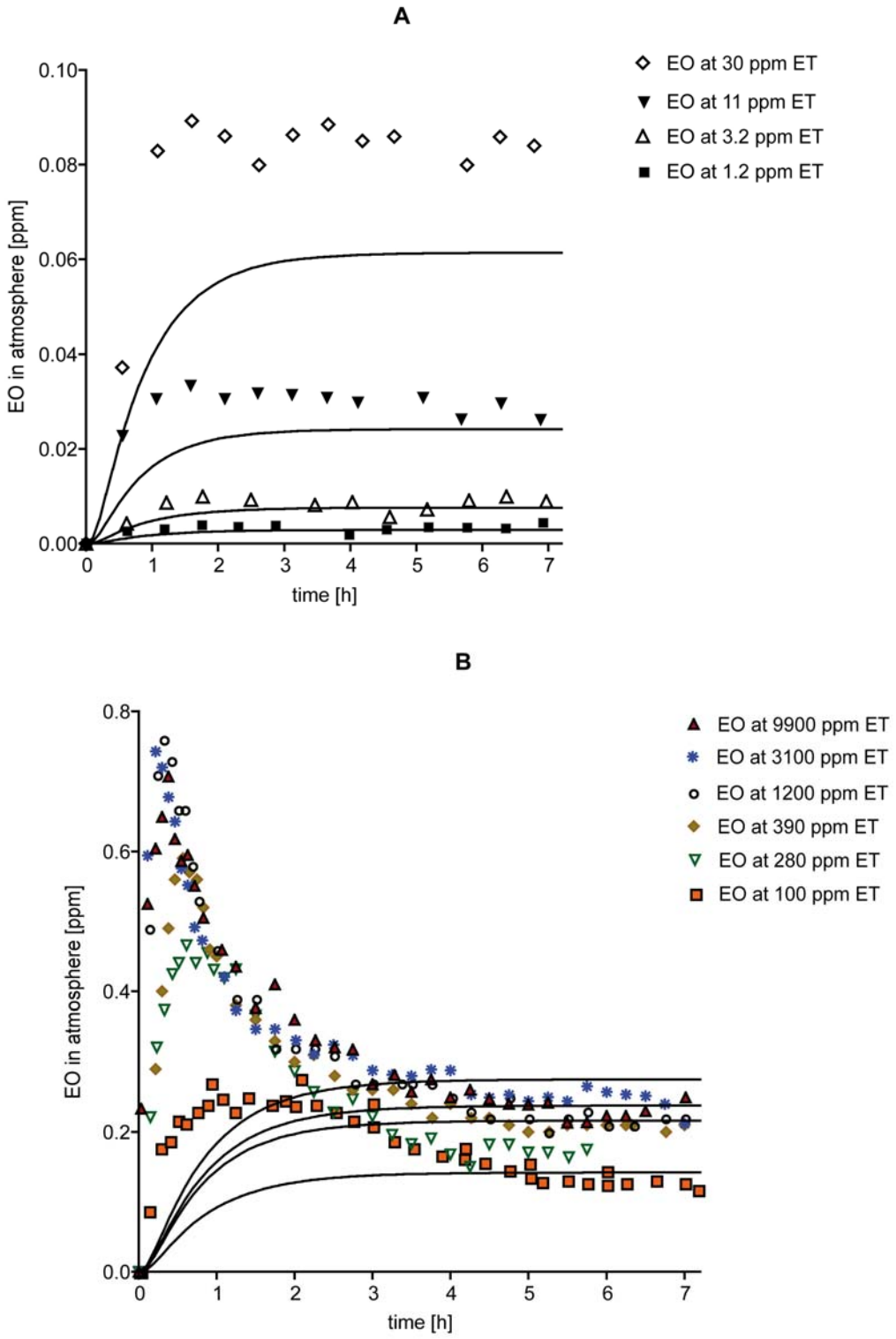


Figure 3.22 Concentration-time courses of EO in the atmosphere of closed chambers (2.8 l), each containing 5 mice exposed to constant ET concentrations. (**A**) low ET concentrations of ≤30 ppm. (**B**) High ET concentrations of ≥100 ppm. Symbols: data sets measured in single experiments; lines: model predicted EO concentration-time curves for ET exposure concentrations of 1, 3, 10, and 30 ppm (**A**) and of 100, 280, 400, and 1000 ppm (**B**) assuming for each ET exposure that 33% of metabolized ET would become bioavailable as EO.

The time to peak decreased from about 45 min at 100 ppm ET to about 20 min at ≥1000 ppm ET. After peaking, the EO concentrations fell to plateaus after 4-5 h of exposure. Up to an ET exposure concentration of about 1000 ppm, the EO concentrations at given time points and the heights of the plateaus increased with the ET concentration. Only marginal differences in the EO concentration-time courses were seen when increasing the ET concentration from 1000 up to 10000 ppm. The maximum plateau concentration of EO was 0.24 ppm.

At ET exposure concentrations ≥1000 ppm, the EO concentration-time courses became congruent. For the three concentration-time courses in the ET concentration range between 1000 and 10000 ppm, an AUC of 2.23 ± 0.05 ppm*h (mean ± SD) was calculated (see 2.2.3.1). Respecting only the maximum EO plateau concentration of 0.24 ppm, an AUC of 1.68 ppm*h was calculated (see 2.2.3.1). This means that during an ET exposures ≥1000 ppm for 7 h about 25% of the AUC resulted from the early EO peak.

The model parameters obtained for ET and for EO were used to predict time courses of exhaled EO during ET exposures (see 2.2.3, *Formation of EO from ET*). The predicted EO plateau concentrations were in average about 3 times higher than those measured (not shown). Reasonably approximations to the plateau concentrations were only obtained when drastically reducing the formation of bioavailable EO by introduction of a factor F which reflected the fraction of metabolized ET becoming bioavailable as EO (see Eq. 46). The lines shown in Fig. 3.22 gave the best approximation between measured and modeled plateau concentrations when using only one and the same F value of F = 0.33 for all of the 10 data sets given in Fig. 3.22. The factor F was considered to be independently of the ET exposure concentration. Only the curves for constant ET exposure concentrations of 1, 3, 10, 30, 100, 280, 400, and 1000 ppm are presented in Fig. 3.22A and B. The curves for the higher ET concentrations of 3000 an 10000 ppm were omitted because they differed only marginally from that of 1000 ppm ET. The early peaks of exhaled EO at high ET concentrations could not be predicted by the model since time-dependent changes in the ET metabolism were not observed in the ET inhalation experiments the model development was based on.

The plateau values represent steady-state concentrations of exhaled EO, i.e. equilibrium is reached between animal and atmosphere since the amount formed from ET equals the amount metabolized. Consequently, the plateau values can directly be used to calculate corresponding EO concentrations in the animal by multiplication with K_{eq}. It was shown by Filser et al. (2007) that the concentration of a volatile daughter metabolite (like EO) in the body at steady-state exposure to a parent compound (like ET), when determined in a closed exposure system, does differ by not more than 10% from the concentration that is reached when exposure occurs in an open system, provided $P_{blood/air}$ is higher than 50. Fig. 3.23 shows calculated EO concentrations in the body at steady state (i.e. at plateau) versus the ET exposure concentration in the atmosphere. In addition, a function (calculated by means of V'_{max} and K_m of ET given in Table 3.1) is plotted which describes the rate of ET metabolism (dN'_{met}/dt) in dependence of the ET exposure concentration in the air of an open system at steady state (see also Fig. 3.14). It is evident that the relationship between EO concentrations in the body and ET exposure concentrations follows the same curve as the function. This indicates that the EO formation (at plateau) is directly linked to the saturable metabolism of ET, the kinetics of which were obtained in the ET gas uptake experiments.

The concentration of EO in the animal resulting from endogenously produced ET was calculated to be 0.055 nmol/l. This value is four orders of magnitudes lower than the maximum plateau concentration that can be reached from exposure to ET.

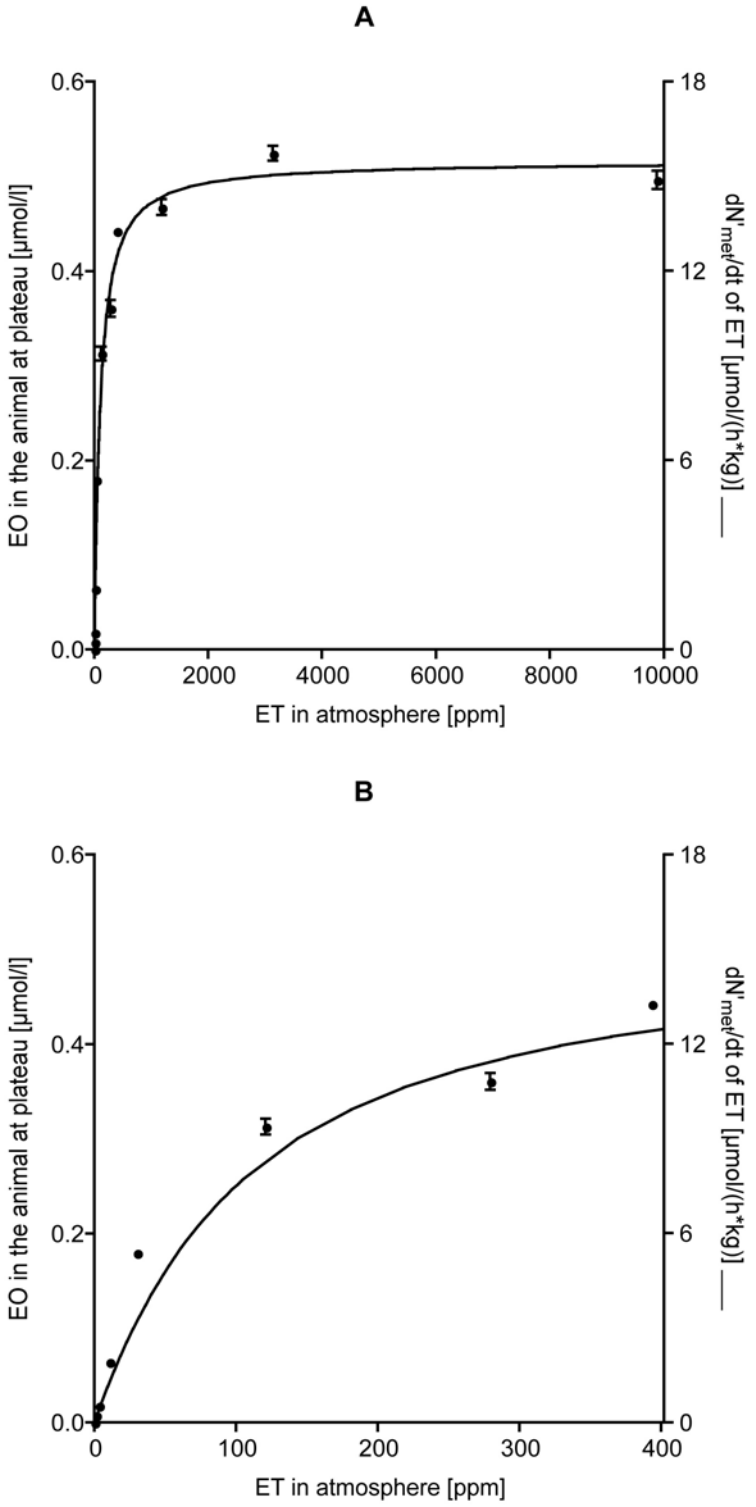


Figure 3.23 Concentrations of EO in animal at plateau concentrations in the air of closed chambers each containing 5 mice exposed to constant ET concentrations (compare Fig. 3.22) and function describing the rate of ET metabolism (dN'_{met}/dt) in dependence of the ET concentration in the air of an open exposure system (compare Fig. 3.14). (**A**) ET exposure concentrations up to 10000 ppm. (**B**) ET exposure concentrations up to 400 ppm. Symbols: means ± SD of measured data (n between 7 and 13). Line: function calculated using the toxicokinetic model.

4 Discussion

4.1 Analytical methods

Gas chromatographic methods with packed columns developed earlier in the working group for the determination of ET and EO (Filser, 1992; Filser et al., 1992) were adapted in the present study. When using sample loop injection of 5 ml gas samples, the ET method was sensitive enough to determine the exhalation of endogenous ET by rats kept in closed exposure systems (Filser, 1992). The detection limit of this method (7 ppb) was also achieved in the present study.

EO exhaled by mice during exposure to ET could be measured by GC/FID only at ET concentrations of 100 ppm and higher. At lower ET concentrations, exhaled EO was below the detection limit of 100 ppb and afforded a more sensitive method with a detection limit of below 1 ppb. Two methods for EO in air with such a detection limit were described in the literature. In the EO method number 49 of the U.S. Occupational Safety and Health Administration (OSHA, 1984), EO is desorbed from a “3M Ethylene Oxide Monitor 3551” with tetrahydrofurane and derivatized with heptafluorobutyric acid anhydride. A GC equipped with an electron capture detector is used to quantify the EO derivative. The detection limit is reported to be 0.7 ppb EO. Eklund et al. (2004) collected atmospheric EO in electropolished or silica-lined pre-evacuated stainless steel canisters before injecting 500 ml thereof via a cryogenic-trap into a GC/MSD. The detection limit of this method was as low as 0.25 ppb. However, both methods are not suitable for the direct and rapid measurement of EO in small air samples (<10 ml). In our laboratory, a GC/MSD method with cold trap injection and electron impact ionization fulfilling these criteria had been developed for the measurement of the EO homologue propylene oxide in exhaled air of rats (Filser et al., 2008). This method was adapted to the EO measurement, applying chemical instead of electron impact ionization. The resulting detection limit (0.45 ppb) was similar to that reported for propylene oxide (0.5 ppb) and allowed the analysis of exhaled EO at ET concentrations between 1 and 100 ppm.

4.2 Some general considerations on the inhalation kinetics of gases

For gases and vapors, it has been shown earlier that the rate of inhalation uptake of a compound depends on physiological processes in the respiratory tract as well as on the physicochemical characteristics of the compounds. According to Henderson and Haggard (1927), the rate of the inhalation uptake is determined by the alveolar ventilation Q_{alv}, the blood flow through the lung Q_{card}, and the compound specific $P_{blood/air}$. During the passage through the respiratory tract, a part of the gas is absorbed in the lining epithelium (Cander and Foster, 1959; Wigaeus et al., 1981) according to its partition coefficient "epithelium to air" (Schrikker et al., 1985). A part of this amount is exhaled again (wash-in-wash-out effect), another part can possibly be metabolized, and a further one enters the circulation (Morris, 2001). The non-absorbed portion of the inhaled gas reaches the alveoli from which it is taken up by the lung blood according to its $P_{blood/air}$. The portion not taken up by the lung blood is directly exhaled.

The clearance of inhalation uptake (Cl_{up}) with the dimension volume per time is a kinetic parameter which, when multiplied with the atmospheric concentration, gives the rate of inhalation uptake into the lung blood. Experimentally determined Cl_{up} values of 16 gases and vapors in rodents (rat 16, mouse 5) were about 60% of the values predicted with the respective Q_{alv} values (Johanson and Filser, 1992) demonstrating quantitatively the relevance of the wash-in-wash-out effect.

4.3 Inhalation kinetics of ethylene and ethylene oxide

In mice, toxicokinetics of ET have been investigated in this study for the first time. Comparable data are not available from the literature. However, toxicokinetic parameters of ET in male adult Sprague-Dawley rats have been gained earlier by means of the closed chamber technique and using a two-compartment analysis (Bolt et al., 1984, Filser, 1992). Allometric extrapolation of the parameters by the body surface factor "body weight$^{2/3}$" (Filser, 1992) enables an interspecies comparison of the present results obtained in mice with those published in rats. Since propylene is an ET homologue with similar

physicochemical characteristics as ET, the kinetic parameters of propylene determined by the same procedure in male B6C3F1 mice (Schmidbauer, 1997) are drawn on for comparison, too.

As for ET, toxicokinetics of inhaled EO in mice have been investigated for the first time by means of the closed chamber technique. In male Sprague-Dawley rats, corresponding parameters of inhaled EO were reported earlier (adult animals: Csanády et al., 2000; Filser and Bolt, 1984; growing animals: Denk, 1990) and are taken for comparison. Results of a kinetic study on the EO homologue propylene oxide in male B6C3F1 mice by means of the closed chamber technique (Schmidbauer, 1997) are also considered.

The species-specific inhalation characteristics of ET and EO determined in this study in mice and in earlier studies in male Sprague-Dawley rats (ET: Bolt et al., 1984; Csanády et al., 2000; EO: Denk, 1990; Filser and Bolt, 1984) were similar in both species because the inhalation uptake depends on physiological processes and physicochemical parameters. About 20% and 50% of inhaled ET and EO, respectively, reach the blood of rodents. In male B6C3F1 mice, the same percentage as for ET was reported for its homologue propylene (Filser et al., 2000). For the EO homologue propylene oxide a slightly higher percentage (59%) was determined (Schmidbauer, 1997). In mice and rats, about 80% of inhaled ET and about 50% of EO is exhaled again without becoming systematically available via the blood stream (mice, present work; rats, Bolt et al., 1984; Csanády et al., 2000; Denk, 1990; Filser and Bolt, 1984).

The slow uptake rates of ET result from its low blood solubility, since the inhalation uptake of compounds with such small $P_{blood/air}$ values as ET ($P_{blood/air}$= 0.48) is limited by the blood flow through the lung (e.g. Johanson and Filser, 1992). The solubility of atmospheric EO in blood is two orders of magnitude higher ($P_{blood/air}$= 61) than that of ET. However, Cl'_{up} of EO in mice, calculated from $P_{blood/air}$, the alveolar ventilation, and the lung blood flow (Eq. 21), is only 2.2fold higher than that of ET. This apparent discrepancy is explained by the findings of Johanson and Filser (1992) who showed in mice and rats that the maximum uptake by inhalation of volatile compounds at rest can generally not exceed a value of about $0.6 \cdot Q_{alv}$.

K_{eq}, the thermodynamic partition coefficient whole body-to-air is only dependent on physicochemical properties of the gaseous chemical and the tissues of the organism into which the chemical distributes. Therefore, it can be predicted from measured tissue-to-air partition coefficients (see Eq. 20).
The K_{eq} value of the lipophilic ET ($logP_{o/w}$ = 1.13) calculated by this method in the present work for the mouse (0.70) is somewhat higher than that calculated for the rat (0.65, Csanády et al., 2000). The difference is caused by the reported higher fat content of the mouse if compared to that of the rat (Arms and Travis, 1988). The K_{eq} of the ET homologue propylene in mice was slightly higher (0.94, Filser et al., 2000) in agreement with the higher $logP_{o/w}$ (1.77; Hansch and Leo, 1979). For EO, identical values of K_{eq} of 57 were calculated for both species (mouse, present work; rat, Csanády et al., 2000) because EO is amphiphilic as evidenced by its $logP_{o/w}$ of -0.30. For propylene oxide ($logP_{o/w}$ = 0.03, Sangster, 1989), a similar (mean) value of 50 was calculated based on $P^{i}_{tissue/air}$ values measured in mice, rats and humans (Schmidbauer, 1997).

4.4 Metabolism of ethylene and ethylene oxide

In the concentration ranges of first-order kinetics, 27% and 24% of systematically available ET was metabolically eliminated in mice (this study) and male Sprague-Dawley rats (Filser, 1992), respectively. Accordingly, 73% in mice and 76% in rats was exhaled unchanged. With increasing concentrations, ET metabolism showed saturation kinetics in both species. In male Fischer 344 rats (Andersen et al., 1980) and male Sprague-Dawley rats (Bolt et al., 1984), the maximum rate of metabolism V'_{max} of 8.5 µmol/(h*kg) reported is nearly half of that found in mice whereas K_m (5.1 µmol/l) in rats is twice the value obtained in mice. The atmospheric concentration at $V'_{max}/2$ in mice (105 ppm) is close to the lowest corresponding concentration published in rats (125 ppm, Csanády et al., 2000; 130 nl/ml tissue equivalent to 204 ppm, Bolt and Filser, 1987; 218 ppm, Andersen et al., 1980). Compared to ET, V'_{max} of propylene in male B6C3F1 mice is nearly one order of magnitude higher (110 µmol/(h*kg), Filser et al., 2000). The authors found for

propylene also a 2fold lower V'_{max} in rats as compared to mice. An about 2fold species difference in V'_{max} between mouse and rat has also been reported for other olefinic compounds that are metabolized by CYP to epoxides, e.g. 1,3-butadiene (male Sprague-Dawley rats 220 µmol/(h*kg), Bolt et al., 1984; male B6C3F1 mice 400 µmol/(h*kg), Kreiling et al., 1986), and styrene (male Sprague-Dawley rats 224, male B6C3F1 mice 480 µmol/(h*kg), Filser et al., 1993).

Analysis of gas uptake experiments with male Sprague-Dawley rats by the two-compartment model revealed that metabolic elimination of EO follows first-order kinetics up to an atmospheric concentration of about 300 ppm, i.e. no saturation of EO metabolism takes place in this concentration range (Filser and Bolt, 1984). Similar findings were obtained in the present study from the corresponding experiments in mice, in which EO metabolism followed first-order kinetics up to an atmospheric concentration of about 200 ppm. Linear correlations between EO exposure concentrations (up to 200 and 300 ppm in mice and rats, respectively) and EO concentrations in blood at steady state were also seen in whole-body exposed male B6C3F1 mice and male Fischer 344 rats (Brown et al., 1996) and in nose-only exposed male B6C3F1 mice (Brown et al., 1998). In both species, only a small percentage of EO taken up into the body is exhaled unchanged. In the range of linear kinetics, the overwhelming amount of systemically available EO was eliminated metabolically in male B6C3F1 mice (94%, present work) and in male Sprague-Dawley rats (95%, Filser and Bolt, 1984).

At concentrations of up to 200 ppm, half-life of EO elimination in the mouse was 5 min in the present work being in the range of the values published by other authors. From measurements of EO adducts to hemoglobin of male CBA mice, Ehrenberg et al. (1974) calculated a half-life of 9 min. Based on EO measurements in blood following a nose-only exposure to 100 ppm EO, Brown et al. (1996) reported a half-life of 3 min in male B6C3F1 mice. Half-lives of 9 min (Csanády et al., 2000) and 4.2 min (Fennell and Brown, 2001) were estimated using physiological toxicokinetic models. The half-life of propylene oxide is very similar to that of EO; it was reported to be 4.2 min in male B6C3F1 mice at PO concentrations of up to 140 ppm (Schmidbauer, 1997). In rats, published half-lives of EO are longer. Osterman-Golkar et al.

(1983) estimated for male Fischer 344 rats exposed to EO concentrations of between 0 and 100 ppm the half-life to be between 10 and 17 min on the basis of investigations on EO adduct formation with hemoglobin. From measurements of EO in blood following whole-body exposure of male Fischer 344 rats to 100 ppm EO, Brown et al. (1996) calculated a half-life of 14 min. In male Sprague-Dawley rats, the half-life was estimated by physiological modeling to be 19 min for EO exposure concentrations of up to 300 ppm, (Csanády et al., 2000). Allometric extrapolation of the present mouse value to the rat yielded a predicted half-life of 11 min.

The relationship between the EO concentrations in the blood of male B6C3F1 mice and in the atmosphere were supralinear at concentrations higher than 200 ppm (Brown et al., 1998). This observation is in agreement with the present kinetic findings. In the EO gas uptake studies, the EO concentration-time curves showed a deviation from linear kinetics when the initial EO concentrations in the chamber atmosphere were >1000 ppm. An almost identical picture was obtained in gas uptake studies with propylene oxide (Schmidbauer, 1997). Like EO, propylene oxide is metabolized by cytosolic GST (Faller et al., 2001). The deviation from linearity in the present EO experiments results most probably from a drastic depletion of GSH as has been demonstrated in male B6C3F1 mice exposed to propylene oxide concentrations of 2000 and 3000 ppm (Schmidbauer, 1997). Following continuous 4-h nose-only inhalation exposure of B6C3F1 mice to EO concentrations between 300 and 400 ppm, Brown et al. (1998) detected in livers and lungs a loss of water-soluble non-protein-thiol (surrogate for GSH) of 70-80% as compared to the control value.

4.5 Endogenous production of ethylene

In rats, Filser (1992) reported an endogenous production rate of ET of 11.2 nmol/(h*kg). The about 6fold difference to mice cannot be explained so far since quantitative relations of endogenous ET to possible sources in the bodies of both species have not yet been investigated.

Using a closed chamber, Lawrence and Cohen (1985) determined the exhalation of endogenous ET over 1 h in untreated male Swiss Webster mice to be 1.7 nmol/(h*kg). This rate agrees very nicely with the value that can be calculated based on the present toxicokinetic analysis: When multiplying the clearance of exhalation (Cl'_{ex}) with the endogenous body burden (y'_{2end}) and with the number of animals (40) per kg body weight, an exhalation rate of 1.3 nmol/(h*kg) is obtained (see Table 3.1).

4.6 Ethylene oxide in exhaled air and in blood during exposure to ethylene

During continuous (7 h) exposures of mice in closed chambers to constant ET concentrations of ≥100 ppm, the concentration-time courses of metabolically formed EO displayed a similar picture as was seen in male Fischer 344/N and also in male Sprague-Dawley rats at ET concentrations of ≥300 ppm (Erbach et al., 2005; Filser and Bolt, 1984). At a given ET concentration, shapes and heights of the early EO peaks depended on the size of the animals and the volumes of the closed exposure chambers; final steady-state concentrations were independent on the experimental conditions. The maximum plateau concentrations of EO in mice (about 0.2 ppm, present work) and in rats (about 0.3 ppm, Filser and Bolt, 1984) were similar. In the ET exposed rats, the EO concentration-time courses in exhaled air were reflected by the concentration-time courses in blood (Erbach et al., 2005). In blood of male F344 rats exposed to 600 ppm ET, the complex behaviour of EO (an early peak followed by a decrease to a lower plateau) was also found by Maples and Dahl (1993) and Fennell et al. (2004). Interestingly, also concentration-time courses of the propylene metabolite propylene oxide show similar characteristics in rats exposed to propylene concentrations ≥1000 ppm

(Filser et al., 2008). The complex behavior of metabolically produced EO was explained in part by an early inactivation of CYP2E1 because a significant loss of hepatic CYP activity to the substrate 4-nitrophenol was observed in rats exposed for 4 h to 300 ppm ET (Fennell et al., 2004). The authors speculated that ET was metabolized by CYP2E1, which was inactivated during ET metabolism but kept finally at a certain level as a result of CYP2E1 resynthesis. However, this theory can hardly explain the obvious normal saturation kinetics of ET in mice (this work) and rats (Andersen et al., 1980; Bolt et al., 1984), where no hint for an inhibition of ET metabolism was observed. Considering this, one could hypothesize the presence of at least two ET-metabolizing CYP isozymes one being inactivated during ET metabolism and a second one remaining unaffected. The long-lasting induction of ET metabolism in rats following pretreatment with Aroclor 1254, a mixture of polychlorinated biphenyls, hints to an ET oxidizing CYP isozyme different from CYP2E1 (Bolt et al., 1984). This view does not disagree with the present findings that only one third of the ET metabolism yields EO, an observation made in rats, too (30%, Filser and Bolt, 1984; 45%, Csanády et al., 2000). It was speculated that a considerable amount of ET was oxidized to a carbonyl compound (acetaldehyde). Alternatively, a large intrahepatic first-pass effect was discussed for EO when formed from ET (Filser and Bolt, 1984). Such a drastic intrahepatic first-pass metabolism was proven in livers of male B6C3F1 mice and male Sprague-Dawley rats perfused with the ET homologue 1,3-butadiene. Before leaving the liver, a major part of the direct daughter metabolite 1,2-epoxy-3-butene was already hydrolyzed to 3-butene-1,2-diol (Filser et al., 2010).

4.7 Equivalent exposure concentrations of ethylene and ethylene oxide

The modeled internal EO burden resulting from exposures to constant atmospheric concentrations of EO (Fig. 3.19) or ET (Fig. 3.23) was used to calculate equivalent exposure concentrations of EO or ET in the mouse at steady state (Table 4.1). In earlier studies, equivalent ET and EO exposure concentrations were calculated on the basis of covalent hydroxyethyl adducts

with macromolecules. Adducts of EO to the N7 of guanine in the DNA of several organs were used as measure of the internal EO dose upon 4 weeks of exposures (6 h per day) of male B6C3F1 mice to EO up to 100 ppm (Wu et al., 1999a) or to ET up to 3000 ppm (Walker et al., 2000). The reported equivalent concentrations (Walker et al., 2000) are also listed in Table 4.1. Exposure of male CBA mice to ^{14}C-labeled ET or EO gave rise to hydroxyethylations of nucleophilic sites in hemoglobin and DNA (Ehrenberg et al., 1977; Segerbäck, 1983). Using the degree of the alkylation of cysteine in hemoglobin as internal dose monitor, equivalent ET and EO concentrations (Table 4.1) were calculated by Segerbäck (1983) from single exposures of the mice to 9100 ppm ^{14}C-ET (Ehrenberg et al., 1977) and up to about 6 ppm ^{14}C-EO (Segerbäck, 1983). The data given in Table 4.1 demonstrate a quite consistent picture of equivalent ET and EO concentrations obtained by different experimental onsets and laboratories.

Table 4.1 Equivalent exposure concentrations of ethylene (ET) and ethylene oxide (EO) in the mouse.

ET concentration in air [ppm]	Equivalent EO concentration in air [ppm]		
	Present data	Walker et al., 2000*	Segerbäck, 1983**
40	2.0	3.0 - 3.7	-
1000	4.7	7.4 - 11.1	-
3000	5.2	6.7 - 12.7	-
9000	5.6	-	4.0

* DNA adducts of EO in spleen and brain
** Hemoglobin adducts of EO

In rats, similar equivalence concentrations were obtained. For male Sprague-Dawley rats, ET exposure concentrations of ≥1000 ppm (V_{max} conditions) were calculated by means of toxicokinetic data to be equivalent to an EO exposure concentration of 5.6 ppm (Bolt and Filser, 1987). Walker et al. (2000) reported for the same ET exposure conditions an EO equivalence concentration of 6.4 - 8.6 ppm EO, calculated from DNA-adduct data

determined in spleen and brain of male Fischer 344 rats exposed to ET or to EO.

The maximum equivalent EO concentration of 5.6 ppm achievable by an ET exposure of mice (present work) is one order of magnitude lower than the lowest EO exposure concentration (50 ppm) used in a long-term study with B6C3F1 mice (NTP, 1987). Considering the control incidences and the increased incidences of tumors at 50 ppm of EO (males: lungs: untreated 34%, treated 58%; Harderian gland: untreated: 2%, treated 20%; females: lungs: untreated 0%, treated 6%; Harderian gland: untreated: 2%, treated 13%; mammary: untreated 2%, treated 17%; uterus: untreated 0%, treated 2%), it can be derived according to Haseman (1984) that an exposure to 5 ppm of EO would lead to a non-significant increase only. This means that a long-term study with ET in mice can be expected to become negative. A negative study cannot be used to estimate the human tumor risk from ET. A long-term study performed with ET-exposed male F344 rats was also negative (Hamm et al., 1984) in agreement with a prediction based on the results of toxicokinetic studies with ET and EO in this species (Bolt and Filser, 1984; 1987).

Outlook

The enzymatic mechanism underlying the early EO peaks observed only at high ET exposure concentrations are currently investigated in our laboratory. Together with the toxicokinetic results obtained in the present study in mice, the outcome of these experiments will be used to refine a physiological toxicokinetic model that allows the calculation of the species-specific EO burdens in organs and tissues of mice, rats and humans upon inhalation exposure to various concentrations of ET or EO. The physiological toxicokinetic model will serve as a basis for the estimation of the human risk induced by exposure to ET.

5 Summary

Ethylene is one of the most important industrial chemicals. The gas is found ubiquitously in the environment as a result from its formation by combustion processes and by plants in which ethylene acts as ripening hormone. Also, mammals produce ethylene endogenously. In the mammalian organism, ethylene is metabolized by cytochrome P450 dependent monooxygenase to ethylene oxide. This epoxide alkylates DNA and is mutagenic and tumorigenic in rodents. In spite of its metabolism to ethylene oxide, ethylene was negative in a long-term carcinogenicity study in rats. From toxicokinetic data, it became evident that the ethylene oxide burden in ethylene-exposed rats had been too low to result in an increased tumor incidence. In mice, no long-term study had been conducted with ethylene. There are also no toxicokinetic data on ethylene and limited data on ethylene oxide in this species. Hence, the aim of the present thesis was to investigate the toxicokinetics of ethylene and ethylene oxide in male B6C3F1 mice in order to estimate whether a future carcinogenicity study with ethylene in this species could give reliable results.

Gas uptake experiments were done in closed all-glass chambers with initial ethylene and ethylene oxide concentrations ranging from 1 to 10000 and 10 to 3000 ppm, respectively. In exhalation experiments, mice were exposed for 7 h to constant ethylene concentrations of between 1 and 10000 ppm and the accumulation of exhaled ethylene oxide was determined in the atmosphere of the closed chambers.

Concentration-time courses were monitored by gas chromatography. Inhaled ethylene and inhaled ethylene oxide were analyzed using a flame ionization detector, exhaled ethylene oxide using both a flame ionization detector and a mass selective detector. The detection limits defined as three times the background noise were 10 ppb for ethylene and 0.45 ppb for ethylene oxide (mass selective detector). The obtained data were analyzed by means of a two-compartment model, describing for both compounds the processes of inhalation, exhalation, metabolic elimination, and enrichment in the organism.

Metabolism of inhaled ethylene follows first-order kinetics at atmospheric concentrations of below 10 ppm. Within this linear range, 21% of inhaled

ethylene is bioavailable. Hereof, 25% is metabolized whereas 75% is exhaled unchanged. Under steady-state conditions, the enrichment of ethylene in the body is 0.55 times the atmospheric exposure concentration. At higher concentrations, metabolism of ethylene follows saturation kinetics with a maximum rate V_{max} of 15.5 µmol/(h*kg). $V_{max}/2$ is reached at 105 ppm ethylene in the inhaled air. Pretreatment of the animals with the cytochrome P450 inhibitor diethyldithiocarbamate abolished the metabolism of ethylene completely.

Metabolism of inhaled ethylene oxide follows first-order kinetics at atmospheric concentrations of up to 200 ppm with about 46% of ethylene oxide inhaled being bioavailable. Hereof, 94% is metabolized and only 6% is exhaled unchanged. The enrichment of ethylene oxide in the body under steady-state conditions is 3.4 times its atmospheric concentration. The elimination half-life of ethylene oxide is 5 min. At ethylene oxide concentrations above 200 ppm, metabolic elimination of ethylene oxide is reduced, most probably due to depletion of reduced glutathione originating from conjugation of ethylene oxide with glutathione, the cofactor of the glutathione S-transferase.

As a measure of the ethylene oxide body burden resulting from ethylene exposure, exhaled ethylene oxide was determined in mice exposed in closed chambers for 7 h to constant concentrations of ethylene of between 1 and 10000 ppm. Up to 30 ppm ethylene, the concentrations of exhaled ethylene oxide in the chamber atmosphere increased exponentially to plateaus, which were dependent on the concentration of the parent ethylene. At higher ethylene concentrations, the time courses of exhaled ethylene oxide showed an early maximum followed by decreases to a plateau. This picture might in part result from inactivation of cytochrome P4502E1 by ethylene. Maximum peaks (area under the concentration-time curve) and plateau concentrations were reached at ethylene concentrations >1000 ppm. The corresponding maximum plateau concentration of ethylene oxide in the body was calculated to be 0.55 µmol/l.

The endogenous production of ethylene was investigated in exhalation experiment using naïve and diethyldithiocarbamate-pretreated mice. The mean endogenous production rate is 1.75 nmol/(h*kg) leading to an internal

ethylene oxide burden of 0.055 nmol/l. This burden is 4 orders of magnitude lower than the maximum possible burden resulting from exposure to external ethylene.

The internal ethylene oxide burden in mice exposed for 6 h to ethylene at exposure conditions leading to the maximum possible rate of ethylene oxide formation is calculated to be the same as that resulting from a 6-h exposure to 5.6 ppm ethylene oxide. From the outcome of an ethylene oxide long-term inhalation study in mice, it is estimated that this ethylene oxide burden is too low to result in a significant increase in the tumor incidence. Therefore, it is concluded that a study on the tumorigenicity of ethylene in mice would become negative and not suitable to estimate the human tumor risk from ethylene.

6 Abbreviations

AUC	area under concentration-time course
b.w.	body weight
C_a	desired atmospheric concentration
C_1, C_2, C_3	constants of exponential functions
CAS	Chemical Abstracts Service
Cl	clearance
$Cl^{'}$	clearance of a standard animal
Cl_{up}	clearance of inhalation uptake (related to the concentration in air)
Cl'_{up}	clearance of inhalation uptake (standard animal, related to the concentration in air)
Cl_{ex}	clearance of exhalation (related to the average concentration in animals)
Cl'_{ex}	clearance of exhalation (related to the average concentration in a standard animal)
Cl_{met}	clearance of metabolism (related to the average concentration in animals)
Cl'_{met}	clearance of metabolism (related to the average concentration in a standard animal)
Cl'_{metA}	clearance of metabolism (standard animal, related to the concentration in air)
CV	coefficient of variation
CYP	cytochrome P450-dependent monooxygenases
DEM	diethylmaleate
DFG	Deutsche Forschungsgemeinschaft
dithiocarb	diethyl dithiocarbamate
DNA	deoxyribonucleic acid
dN_{ex}/dt	rate of exhalation
dN_{met}/dt	rate of metabolism
dN'_{met}/dt	rate of metabolism (related to 1 kg b.w. representing 40 standard mice)

dN_{pr}/dt	rate of endogenous production
dN_{prEO}/dt	rate of endogenous EO production
dN_{prET}/dt	rate of endogenous ET production
$dN^{\tilde{O}}_{prET}/dt$	rate of endogenous ET production (related to 1 kg b.w. representing 40 standard mice)
dN_{up}/dt	rate of inhalation uptake
ET	ethylene
EO	ethylene oxide
F	fraction of metabolic elimination of ET which is metabolized to EO
GC	gas chromatograph
GC/FID	gas chromatograph with flame ionization detector
GC/MSD	gas chromatograph with mass selective detector
GSH	glutathione, reduced form
GST	glutathione S-transferase
IARC	International Agency for the Research on Cancer
IUPAC	International Union for Pure and Applied Chemistry
ID	inner diameter
λ_1, λ_2	slopes of exponential functions
k_{12}	rate constant of inhalation uptake
k'_{12}	rate constant of inhalation uptake of a standard animal
k_{21}	rate constant of exhalation
k'_{21}	rate constant of exhalation of a standard animal
K_{eq}	thermodynamic equilibrium constant whole body/air
K_m	apparent Michaelis constant
k_{met}	rate factor of metabolism
k'_{met}	rate factor of metabolism of a standard animal
K'_{st}	bioaccumulation factor at steady state of a standard animal
MAK	Maximale Arbeitsplatzkonzentration
MSD	mass selective detector
n	number of animals
$P_{blood/air}$	partition coefficient blood/air

$P^{i}_{tissue/air}$	partition coefficient tissue i/air
Q_{alv}	alveolar ventilation
Q'_{alv}	alveolar ventilation of a standard animal
Q_{card}	cardiac output
Q'_{card}	cardiac output of a standard animal
R'_{alv}	alveolar ventilation of a standard animal
SD	standard deviation
SIM	Single Ion Monitoring
$t'_{1/2}$	half-life in a standard animal
TCT	thermal desorption cold trap
V_a	volume of one animal
V_{desc}	volume of desiccator
V_{gas}	required volume of pure gas to be injected into desiccator
V_i	tissue volume
V_{max}	maximum rate of metabolism
V'_{max}	maximum rate of metabolism (related to 1 kg b.w. representing 40 standard mice)
V_1	volume of atmosphere (compartment 1)
V_2	volume of animals (compartment 2)
V'_2	volume of a standard animal
$\bar{x}$	mean
y_1	atmospheric concentration (compartment 1)
y_2	average concentration in animals (compartment 2)
y_{1end}	atmospheric ET concentration (compartment 1) resulting from endogenous ET production in the case of $k_{met} = 0$

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