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The toxicokinetics of styrene and styrene oxide

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THE TOXICOKINETICS OF STYRENE AND STYRENE OXIDE

by

Crispin Hayes Pierce

DISSERTATION

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Preface

Comments on the Integration of Pharmacology and Toxicology

(C H Pierce, *Reg Tox Pharm* ; 16: 213-214; 1992)

All drugs are toxicants, at higher doses. The techniques of kinetic analysis of drugs can be an invaluble tool in the analyses of the effects of toxic substances. Symbiotically, toxicodynamic approaches can add to available methods to understand and protect against adverse effects from otherwise therapeutic agents.

We as scientists have allowed overspecialization in toxicology and pharmacology to hinder our ability to address critical research and safety evaluation issues. Recognizing our common frontiers and roots in biochemistry, we must integrate these fields to forge a unified approach to solve biomedical problems. Consider the following examples.

An 81-year old man with a 20-year history of recurring hiccups is admitted to the hospital after four weeks of almost continuous hiccuping. He has lost twenty pounds and his heart is enlarged and beating hard. Previous pharmacologic interventions included codeine, phenobarbital, belladonna, quinidine, Dramamine, pentylenetetrazol, chlorpromazine, nikethamide, promethazine, meperidine, and CO_2 and O_2 inhalation, none of which provides more than temporary relief. An exasperated but undaunted physician pauses to review the patient's history, and decides to suspend the administration of sulfamethiazole, periodically used to treat

recurring urinary tract infections. After twenty years, the hiccups abruptly and permanently cease. (Berton Roueche´, The Medical Detectives, v.2, 1986).

In another situation, *a distraught father calls the poison control center. His three-year old daughter is flushed, excitable, and has an elevated heart rate after ingesting the remainder of her theophylline tablets. After determining the maximum possible dose that she received, the pharmacist answering the phone assures the father that she will fully recover within several hours.*

These examples were chosen to illustrate how caregivers in these situations used an understanding of both the pharmacologic and toxicologic effects of exogenous agents. Can we as researchers similarly integrate approaches available to us in both pharmacology and toxicology?

In pharmacology I learned about receptor theory, agonists, antagonists, and secondary messenger systems. While the properties of nicotinic, muscarinic, cholinergic, adrenergic, and opioid drugs suffused my memory, I gained insightful knowledge regarding pharmacologic intervention in disease states. My introduction to toxicology, on the other hand, included LD_{50}s, heavy metal poisoning, and the multistage cancer model. In which discipline, then, does the administration of antisense oligonucleotides to "kill" tumor cell expression fit in? What about the infusion of antibody-toxin conjugates to selectively eliminate overabundant cell types? Perhaps overspecialization has led to the notion that the therapeutic index "between" pharmacologic and toxicologic approaches is indeed a deep

chasm. Methods of investigation using fundamentals of both pharmacology and toxicology are needed.

As "pharmaco/toxicologists," we share roots in biochemistry as well as numerous research frontiers, including, for example, the effects of placebos. I challenge pharmacologists to explain the use of placebos to successfully treat postoperative pain, rheumatoid arthritis, and motion sickness; and toxicologists to explain placebo-induced erythematous maculopapular rash, diarrhea, and hearing loss (op. cit.).

As we have seen in the examples of the undaunted physician and the experienced pharmacist, the joining of toxicology and pharmacology increases our ability to solve problems. These fields can be synergistically blended to provide a context for accurate and meaningful evaluation of the effects of xenobiotic substances. It is time to strengthen and expand our understanding of critical research and safety evaluation issues by selecting the most powerful tool, based on a thorough understanding of all the tools available.

In this dissertation, I have attempted to integrate the powerful tools made available to me in toxicology and pharmacology. The kinetics of styrene and styrene oxide, like those of all exogenous substances, can be accurately described using techniques developed to quantify drug disposition. The short- and long-term adverse effects of styrene exposure can be defined in approaches developed to protect workers and the public from adverse effects of toxicants.

The Use of Animals in Research

Through this investigation I have become more aware of the complexities of animal experimentation. I have come to have a greater respect and understanding for the contribution of animal models to my research. As was suggested to me by Drs. Joseph Spinelli and Jan Wyrick, the rats I worked with comprised the most complex aspect of my work. It is my firm conviction that all animals used in research must be treated with respect and dignity. Anxiety and discomfort should be minimized, and suffering must not play a role in any part of the experiment. End-of-experiment killing must be done with a minimum of pain to the animal; the method used should be rapid and reliable. Great care must be employed during all contact with research animals, not only for the respect of another living species, but also to provide reproducible results that provide as much accurate physiological response information as possible.

Several difficult ethical issues arose in the course of this research. In kinetic studies, it is desirable to have the animal in as much of a "normal" state as possible, to provide information about normal physiologic function. And yet, a fully awake rat will feel the pain of toxicant administration (IV injection into the penile vein), and blood sampling (tail vein incision). After experimenting with light ether anesthesia, I chose a recommended dose for intraperitoneal adminstration of sodium pentobarbital, which usually provided an adequate level of desensitization. Several rats, however, needed more pentobarbital (as judged by hind leg and tail pinch tests). Although most of these rats became fully anesthetized with

this additional dose, one animal died as a result. An additional two rats died upon the IV injection of the dose of styrene or styrene oxide in ethanol. These deaths were assumed to be due to the combined central nervous depressant effects of the pentobarbital, ethanol, and styrene or styrene oxide. A scaling down of administered dose, and a longer delivery time aided in reducing this source of mortality. Nonetheless, it was difficult to maintain adequate anesthesia without increasing the risk of overdose.

A second issue encountered was the reuse of animals. In the course of this work, I used five rats twice. This was done on the premise that the expermental procedures were not overly demanding on the rats, and that reuse of these animals would reduce the total number to be sacrificed. (A sidelight area of interest was whether induction caused by repeat injections would affect kinetic measurements.) Opinions even within the animal care staff at UC San Francisco varied on this issue, from a strong conviction against the reuse of animals, to acceptance of reuse if the rat was deemed healthy following the first use. I have become sympathetic to both points of view, and feel that this kind of judgement must be done on a case-by-case basis. The pivotal question that must be answered by investigator and animal care committee alike, is whether the specific procedures used in an experiment cause sufficient pain and anxiety so as to warrant not using that animal again, and thereby requiring another life.

While techniques such as physiologic modeling can reduce the number of animals used in research, animal models nonetheless play an indispensable role in our evaluation of pharmacologic and toxic

substances. The course of this work has greatly increased my sensitivity and respect for the sentient beings that have so greatly added to my knowledge.

Acknowledgments

First and foremost, I would like to thank my research advisor, Dr. Thomas Tozer, the richest and most generous man I have ever known. Tom possesses a deep knowledge of seemingly endless aspects of pharmaceutical sciences, is world renowned in the area of pharmacokinetics, is a gifted teacher, and has earned the respect of thousands of pharmacy students, graduate students, and senior scientists alike. He has provided me with a role model for an outstanding pharmaceutical scientist. I am still amazed at his modesty and selflessness. He was not offended by my frequent upstart challenges, and was uncondescendingly patient as I found my way through graduate school. He spent hours upon hours conveying his knowledge and supporting my research progress. His wife Margaret was equally generous with her warmth and hospitality.

The Department of Pharmacy at UC San Francisco is a very special place to work and learn. Les Benet is an extraordinary chairman who continues to demonstrate his support of graduate students. In addition to Les, Wolfgang Sadee and Sue Hawkes kept their doors open.

Svein Oie and Betty Hoener, in addition to their academic support over the last five years, provided invaluable comments on this dissertation.

To my wife Becky - thank you for your patience and understanding during all those late nights in the lab. Jack, Ilana, and Jane - I am very happy to be a part of the Mayeri family, and appreciate your support of my work.

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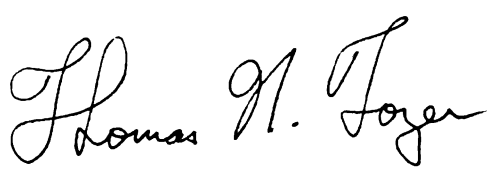
Abstract

The Toxicokinetics of Styrene and Styrene Oxide

Crispin H. Pierce

Styrene (S), an aromatic hydrocarbon with widespread exposure, causes CNS disturbances and chromosome aberrations. An obligate reactive intermediate, styrene oxide (SO), is carcinogenic in animals and humans, while S is only marginally so. The objectives of this research were to examine the toxicokinetics of S and SO, to develop physiologically-based models to summarize experimental observations, and to explore how these models can be used to assess risks from exposure to S and SO. Analysis of S in blood was carried out by headspace sampling, and in adipose tissue by CS_2 extraction, with subsequent gas chromatography (GC) - flame ionization detection. SO and SG were analyzed by derivatization and GC - electron capture detection. Physiologically-based toxicokinetic models were created to simulate the disposition of S in humans and S and SO in rats. Data in human volunteers suggested half-lives of 0.5, 45, and 3,220 min, consistent with a blood clearance approaching hepatic blood flow. Analysis of literature data suggested that CNS toxicity is associated with simulated S brain concentrations above 5 μg/ml. Styrene levels of 1 μg/g were measured in the adipose tissue of nonoccupationally exposed persons, suggesting undiscovered sources of exposure, and/or slower elimination of S from adipose tissue than previously thought. Physiologic damping played a role in mediating CNS toxicity from different exposure frequencies. Work

load was found to be a pivotal consideration in a suggested redefinition of occupational exposure standards. A chronic exposure standard of 100 ppm-years was suggested to protect workers from chromosome aberrations. Results of S and SO i.v. dosing to rats were in accord with model predictions of a high clearance of SO, but differed in regards to S, for which the model predicted a high, and the data suggested a low, clearance of S. The difference in carcinogenic risk from S and SO may be explained by the degree of systemic exposure to SO, which is much less following S administration because of rapid sequential metabolism. The ratio of tissue-specific enzyme activities for forming and degrading SO are also determinants of carcinogenic risk from S exposure.



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Chapter 1
Introduction, Overview and Objectives

Styrene as a Model For Drug and Toxicant Exposure.

Styrene (S) (Fig. 1-1) is a useful model toxicant because of the wealth of information on its toxicologic and metabolic properties, its lipophilic nature, and its occupational toxicity. Styrene is among the 50 most widely-produced chemicals, with fifteen million metric tons[1] produced yearly in the manufacturing of boats, containers, toys, packaging and insulation materials, and synthetic rubber. More than 300,000 workers worldwide are exposed in the rubber and plastics manufacturing industries[2]. Because it is lipophilic, S is concentrated during chronic exposure in a particular tissue (adipose), much like the intravenous anesthetic thiopental[3], and the toxicants DDT, dioxin, polychlorinated biphenyls (PCBs), and dieldrin[4].

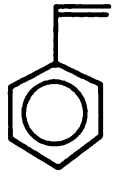


Figure 1-1: Styrene

Styrene-7,8-oxide, commonly called styrene oxide (SO), is an obligate metabolite in mammalian metabolism of S (Fig. 1-2). It is used industrially as an intermediate in the production of styrene glycol (SG), and as a reactive diluent in epoxy resins. It is also used in the preparation of agricultural products, cosmetics, surface coatings, and textiles and fabrics[5].

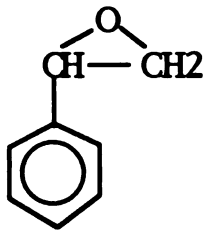


Figure 1-2: Styrene oxide.

Occupational Exposure.

Due to its high oil/water partition coefficient, S is absorbed through the skin[6]. Inter individual differences in work load, duration and intensity of exposure, and alcohol consumption have been shown to affect the internal dose.

Some of the highest exposures occur in the boat-building industry, where polymerized S is used to strengthen the fiberglass sheets comprising boat hulls (30% of a luxury yacht is S). As much as 10% of S used in lamination processes can become airborne by evaporation in occupational settings, and 60-70% of the amount inspired is retained. Intraday exposure can vary widely, from high-level exposure (150-300 ppm) during a hull-spraying operation for 30 minutes, to relatively low-level exposure (< 10 ppm) during other activities for several hours. The current federal 8-hour exposure standard is 50 ppm in air. An estimated daily dose of 200 mg S can be calculated using a typical average 8-hour exposure to 10 ppm, a minute volume (total volume of air inhaled per minute) of 10 l/min, a retention of 65%, and a conversion factor of 1 ppm = 4.25 mg/m^3.

Environmental Exposure.

The sources of non-occupational exposure to S are widespread, including cigarette smoke, car exhaust, and the use of Styrofoam® containers and insulating materials. Leaching of S into yogurt, water and 50% ethanol from polystyrene and Styrofoam® containers has been demonstrated by Withey[7], who found ingested doses of up to 33 μg per container. Wallace et al.[8] measured a delivery of 6 μg S from smoking a cigarette. Some individuals may be exposed to increased breathing zone air concentrations of S during hobby model construction and painting, and exposure to plastics plants, dye plants, degreasing, and metal work[8].

Metabolism.

Like many toxic and pharmacologic agents, including acetaminophen (Fig. 1-3), S is converted in vivo to SO, a more biologically toxic form. The SO formed (Fig. 1-4) can then bind to cellular macromolecules (including DNA). While the parent substance is associated with central nervous system depression, the intermediate metabolite SO is presumed to be responsible for more severe forms of toxicity, including mutation, binding to macromolecules, and cancer. For the latter toxicity, the activation of S is similar to that of vinyl chloride (Fig. 1-5) and benzo[*a*]pyrene. Other selected exogenous agents which are similarly "bioactivated" are listed in Table 1-1.

Table 1-1: Selected Examples of Bioactivation

Parent Compound	Toxic Metabolite	Toxicity
Aflatoxin B_1	Aflatoxin-2,3-epoxide	Hepatic cancer
Carbon tetrachloride	Trichloromethane free radical	Hepatic necrosis and cancer
Cyclamate	Cyclohexylamine	Bladder cancer
Isoniazid	Metabolite of acetylhydrazine	Hepatic necrosis
Thalidomide	Phthalyl-L-glutamic acid	Teratogenesis
Urethane	N-Hydroxyurethane	Cancers, cytotoxicity

Source: Adapted from [4]

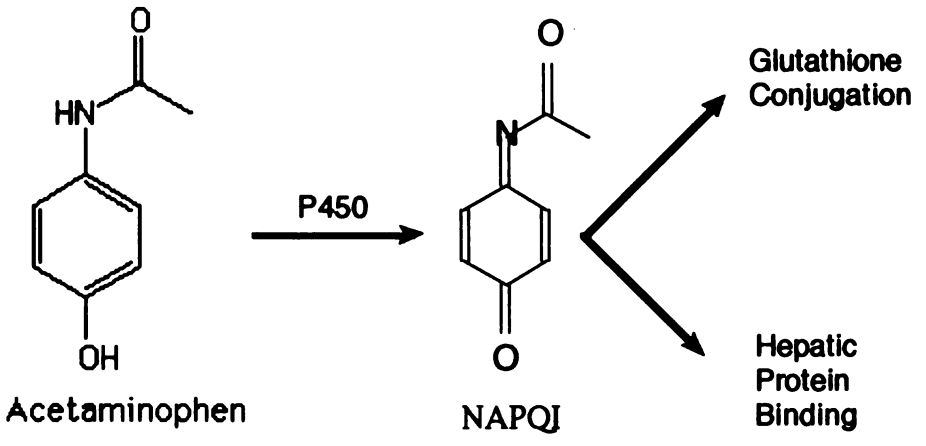


Figure 1-3: Bioactivation of acetaminophen (paracetamol) to an active (toxic) species, NAPQI (N-acetyl-p-benzoquinoneimine).

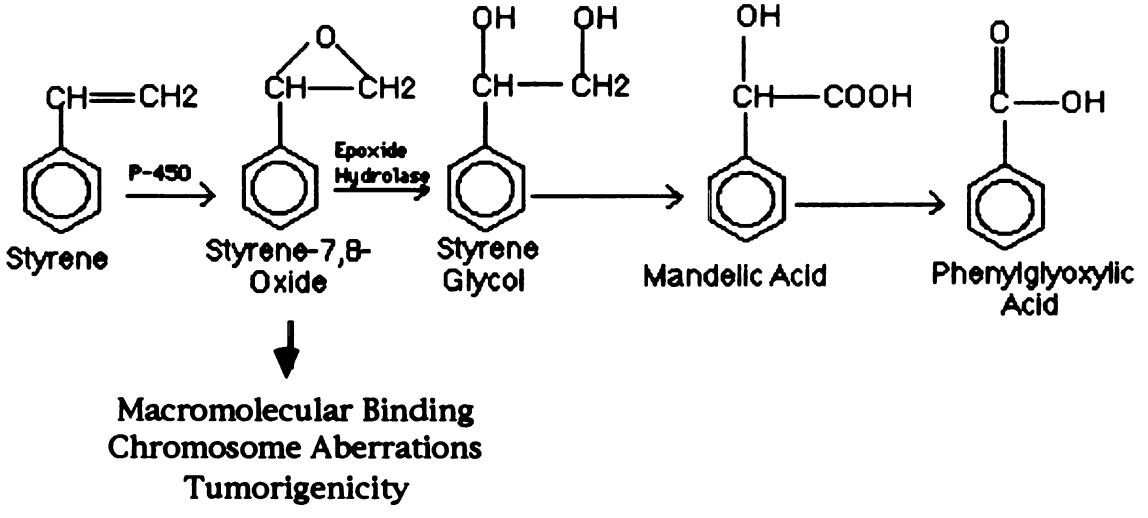


Figure 1-4: Principal pathway of styrene metabolism in humans

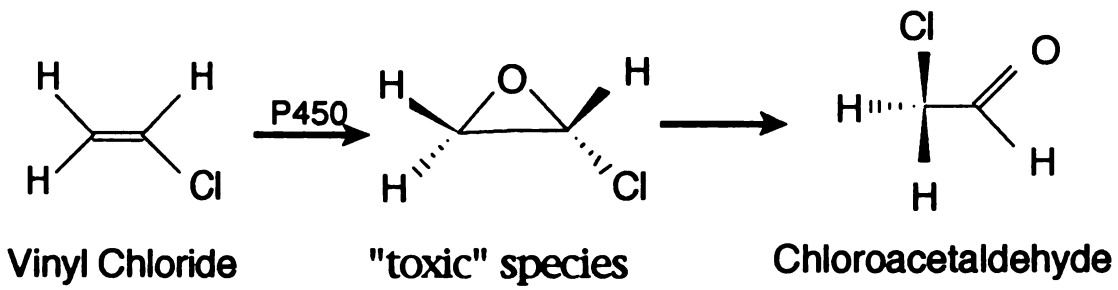


Figure 1-5: Bioactivation of vinyl chloride to the active (toxic) metabolite.

The S metabolite SO is hydrolyzed by epoxide hydrolase to SG, a relatively innocuous diol that is further oxidized to mandelic and phenylglyoxylic acids (Fig. 1-4), which are, in turn, excreted in the urine. Mandelic and phenylglyoxylic acids in the urine of rats account for 51 and 25% of the S dose administered, respectively. These two metabolites account for ca. 90% of the administered dose in humans[9]. The difference in metabolism in these two species can be explained by glutathione conjugation of SO playing an important role in metabolism in rats[10].

Many drugs and toxicants demonstrate a process which is called *facilitated high-extraction sequential metabolism.* The process may be briefly defined as at least two consecutive metabolic steps facilitated by the proximate location of the responsible enzymes. The final step is a high-extraction step. This process has been extensively explored by Pang[11], who found that acetaminophen was more rapidly sulfated when formed from its precursor phenacetin than when given itself. An investigation of whether S undergoes sequential metabolism is presented in chapter 7.

Toxicokinetics.

Styrene presents a number of interesting toxicokinetic issues. In contrast to its obligate intermediate, S is not considered to be an established human carcinogen. There is, however, some evidence of carcinogenicity in rodents following S administration. A decreased proportion of plasma protein and liver DNA alkylation at higher S

doses in animals suggests saturation of the bioactivation pathway (P-450 oxidation). An increased proportion of DNA adduct formation observed at higher SO doses suggests saturation of detoxification routes (hydrolysis, glutathione conjugation, glucuronidation). Adduct formation, and presumably the associated carcinogenicity, are therefore regulated by a balance of the rates of two saturable enzymatic processes.

Styrene Toxicity.

Exposure to S is of concern because of evidence in animal studies that it is mutagenic, carcinogenic, teratogenic, and can cause lung and nervous system toxicity. The dominant primary metabolite, styrene-7,8-oxide (SO), forms covalent bonds with DNA, plasma proteins, and hemoglobin, and is mutagenic and carcinogenic in animals. Human acute toxicity includes mucous membrane irritation and CNS disturbances, and chronic toxicity is evidenced by chromosome aberrations, increased spontaneous abortions, and some indication of carcinogenicity (specific findings discussed below).

Styrene is a colorless to yellowish liquid with a sweet and pleasant odor at low concentrations (threshold 0.1 ppm), and disagreeable odor at higher concentrations. Physical properties of S are presented in Table 1-1.

Table 1-2: Physical properties of styrene.

Boiling Point: 145.2°C

Freezing Point: -30.63°C

Color: Colorless

Odor: Sweet & pleasant at

low conc., disagreeable at

high conc. Odor threshold: 0.1 ppm

Specific Gravity: 0.9045

Molecular Weight: 104.14

Solubility in Water: 0.03%

Animal Exposure

The evaluation of S toxicity in animals has been focused on its carcinogenic potential, with equivocal results. In vitro and in vivo tests suggest that SO rather than the parent compound is responsible for macromolecular binding.

In vitro Results.

Styrene has shown negative results in bone marrow mutagenicity tests but positive sister chromatid exchange results. Onion root cells (*Allium cepa*) showed chromosome damage after incubation with S in vitro[12].

Six studies found negative results in tests for mutagenicity using Salmonella typhimurium or Escherichia coli while two studies found a weakly positive result in Salmonella strains TA 100, TA 1530, and TA 1535, following metabolic activation[13]. Styrene became mutagenic in one bacterial system after depletion of epoxide

hydrolase[14]. Given that SO is a potent mutagen[15], these findings suggest that activation of S to the oxide is necessary for mutation to occur.

Mammalian cell culture observations mirror those in bacterial systems. Styrene does not induce sister chromatid exchanges (SCEs) in Chinese Hamster Ovary (CHO) cells, but does cause SCEs in human whole blood lymphocyte cultures[13]. The varied results of S mutagenicity in different in vitro and in vivo test systems [9] may be explained by the ratio of SO forming (P-450) and detoxifying (epoxide hydrolase, glutathione transferase) activities in various tissues.

In vivo Results.

The acute oral LD_{50} for S is about 5 g/kg[16]. There is some evidence of mutagenicity and chromosome damage[17]. Styrene causes recessive lethal mutations in Drosophila[10]. Increased embryotoxicity in mice and Chinese hamsters has been observed. While a number of studies have shown no increase in tumors[17], two studies suggest carcinogenic potential. Following chronic inhalation exposure to 500 ppm, female rats had increased lymphoid and hematopoetic tumors[17]. Male B6C3F1 mice exposed to 78 weeks of gavage dosing (150 mg/kg) showed an increase in alveolar/bronchiolar adenomas and carcinomas[14]. In another study, there was a small statistically significant increase of lymphomas in female mice, and hepatocellular carcinomas of male mice[18]. Increased appearance of lung tumors in male and female mouse offspring was observed after S dosing of dams[14]. Following

chronic inhalation exposure of up to 300 ppm S, male and female Sprague-Dawley rats demonstrated an increase in benign and malignant mammary tumors[18]. These findings were confirmed by Conti et al.[17], who found increased malignant mammary tumors as a result of chronic inhalation of 25-300 ppm; it is notable that gavage, intraperitoneal and subcutaneous injections of similar size doses (50-250 mg/kg) did not increase tumor appearance[17]. There is also evidence for the appearance of leukemias and lymphosarcomas in rats following inhalation exposure[18]. However, mice treated either for 4 days on a daily dose of 500 mg/kg or 70 days of 200 mg/kg did not develop chromosome aberrations in bone marrow cells[18].

Human Exposure

Controlled Studies.

Controlled experimental studies have been undertaken to determine conditions under which mucous membrane irritation and CNS disturbances occur. Styrene exposure can cause eye, nose, throat, and respiratory tract irritation[19, 20]. Short-term exposure can produce mucous membrane irritation and central nervous system (CNS) disturbances, such as changes in reaction time[21], perceptual ability[22, 23], and balance[19, 24]. Additional acute CNS disturbances include decreased performance on coordination and eye-tracking tests, headache, and nausea[19].

Edling and Ekberg[25] found no changes in reaction time for 12 workers exposed to 11.5 ppm, although the lapse between end of exposure and testing was not stated. Given that systemic levels of S fall rapidly after cessation of exposure, this factor is crucial in assessing acute CNS effects. Similar findings were reported by Triebig et al. [20] who found no changes in 36 workers between pre- and post-shift results in a battery of neurobehavioral tests with a mean exposure of 36 ppm (range 13-136).

Occupational Exposure

Occupational exposure causes both short-term and chronic neurologic changes. A form of CNS intoxication termed "styrene sickness," including balance impairment, headache, and nausea, is common after short-term high-level (>100 ppm) exposure. Acute effects (during which S is present in brain tissue based on extrapolation from rat brain levels) appear to be related to CNS function, whereas subchronic and chronic effects (observable even after S has left brain tissue) appear to be manifested in the peripheral nervous system.

Cases of peripheral neuropathy and degeneration in the autonomic nervous system[26] have been diagnosed in workers. Harkonen et al.[23] found that 30% of workers occupationally exposed to 30 ppm or greater had abnormal electroencephalograms. Pronounced decrements in visuomotor and psychomotor performance were observed in workers exposed to 55 ppm. As evidence of chronic autonomic nervous system toxicity, decrease in

variability in EKG R-R intervals, sensory nerve conduction velocity, and distribution of nerve conduction velocities (as measured between finger and elbow) were observed in 11 S workers exposed to a mean of 22 ppm [26].

Long-term exposure is associated with chromosomal abnormalities[13, 27-31], and micronuclei[28]. Styrene-hemoglobin adducts have been detected in workers. Brenner et al.[32] found statistically elevated levels of micronuclei, single-strand breaks, and N-acetoxy-2-acetylaminofluorene-induced DNA binding in 14 fiberglass-reinforced plastics boatbuilders, compared to 9 unexposed controls. Human lymphocytes showed chromosome damage after incubation with S in vitro[12].

Evidence of Carcinogenicity

Hodgson and Jones[33] reported an increase in the incidence of lymphoma deaths in 622 S-exposed workers (with no control for smoking or exposure to other workplace toxicants). Ott et al.[34] found an increase in the expected numbers of lymphatic leukemias in 2,904 workers exposed to S. In a cohort study of 622 exposed workers, there were excesses in the numbers of non-Hodgkin's lymphoma, lymphatic leukemia, and laryngeal cancer[14]. Epidemiological investigations have generally found no correlation between exposure and cancer, and found some link (as detailed above) with leukemia and lymphoma appearance[2]. Styrene is placed in the International Agency for Research on Cancer carcinogenicity Group 2B, based on sufficient animal evidence and inadequate human evidence of carcinogenicity[14]. Based on the

inconsistency of reports of carcinogenicity in animal models and exposed human populations[10], the federal Occupational Safety and Health Administration (OSHA), has classified S as a narcotic[24].

Other Toxicity

Elevated albuminuria and lysozymuria in humans have also been found, as well as an increase in spontaneous abortions[30]. Occupational exposure to S has also been found to depress δ-aminolevulinate dehydratase (ALA-D) levels in erythrocytes[35]. These authors suggest that the obligate reactive intermediate, styrene-7,8-oxide, may react with ALA-D or a precursor in the bone marrow. The presence of decreased ALA-D concentrations in bone marrow and erythrocytes, but not in hepatic tissue, suggests that rapid sequential metabolism of S through the oxide to S glycol occurs more efficiently in hepatic vs. bone marrow tissue.

Styrene Oxide Toxicity

Styrene oxide is a reactive epoxide that is mutagenic in many test systems, and forms covalent bonds with macromolecules in vitro and in vivo. Like benzo[*a*]pyrene diol epoxide, it is considered an animal and human carcinogen and is regulated as such[36]. Physical properties of SO are presented in Table 1-2.

Table 1-3: Physical Properties of Styrene Oxide[a]

Boiling Point: 194°C

Freezing Point: -37°C

Color: Colorless

Vapor Density: 4.14

Specific Gravity: 1.054

Molecular Weight: 120.14

Solubility in Water: 0.3%

[a]Data from [37].

Animal Data

In vitro.

SO has been found to be mutagenic in *Salmonella typhimurium* strains TA 100 and TA 1535, E. Coli K12, the yeasts *Sacromyces pombe* and *cerevisiae,* Chinese hamster V79 cells[2, 15], and *Drosphila*[10]. There is evidence in *S. typhimurium* that the R enantiomer of SO is more mutagenic than the S form[37]. (Styrene is epoxidized to R and S forms in the ratio 1:1.3 in rat liver microsomes. Styrene oxide is hydrolyzed in a regiospecific manner in microsomes such that the ratio of R to S hydrolysis is 1:4[1].) Like S, SO caused the appearance of micronuclei and chromosome breaks in *Allium cepa* root tip cells[12]. Styrene oxide also binds to cysteine-containing polyamino acids[37], free amino acids, human serum proteins[38], hepatic proteins and nucleic acids[37, 39] in vitro.

In vivo.

The oral LD_{50} in rats has been reported as 2, 3, and 4.29 g/kg[5, 37]. Treatment of male and female Sprague-Dawley rats with 50 or 250 mg/kg daily by gavage results in a dose-dependent increase in the incidence of squamous-cell carcinomas and papillomas of the forestomach[17]. Increased forestomach tumors were similarly found in BDIV rats dosed orally with 100-150 mg/kg from birth[40]. However, skin-painting studies in C3H and Swiss ICR/Ha mice did not show an increase in tumors[37]. Nor was there an observed increase in chromosome aberrations or sister chromatid exchanges (SCEs) in the bone-marrow cells of male Chinese hamsters following inhalation exposure to 25-100 ppm up to 21 days[37]. Administration of SO to mice results in DNA alkylation in brain, lung, and liver tissues[10]. In vivo perfusion of the liver of up to 500 μmol/liver resulted in DNA, RNA, and protein binding; a sharp increase in protein binding was associated with hepatic glutathione depletion at the 500 μmol dose[2].

Doses of 0.5-5 μmol injected into fertilized eggs from White Leghorn chickens caused embryolethality and malformations. Addition of the epoxide hydrolase inhibitor trichloropropylene oxide augmented these effects[37]. Exposure of New Zealand white rabbits to 15 or 50 ppm SO for seven hours/day during days 1-24 of gestation resulted in dose-dependent increases in maternal mortality and fetal resorption[5]. Similar exposure to Sprague-Dawley rats (to 100 ppm) caused extensive maternal mortality, and increased preimplantation losses and increased ossification defects of embryos[5].

Human Toxicity

Styrene oxide is extremely destructive to mucous membrane and upper respiratory tract tissue[41]. Acute SO exposure causes eye and skin irritation[37]. SO causes unscheduled DNA synthesis in EUE human cells in vitro (a DNA repair test)[15]. Clastogenicity and micronucleus appearance in cultured human lymphocytes was seen after incubation with SO[12].

Animal mutation and carcinogenicity studies are sufficiently strong to classify SO as a human carcinogen[36, 41]. There are no known cohorts of workers exposed to high levels of SO, although S workers also receive exposure to low levels of SO (ca. 0.1% of S level[37]).

Objectives.

The overall objectives of this dissertation research are to examine the toxicokinetics of S and SO, to develop physiologically-based models to summarize experimental observations, and to explore how the models can be used to assess and predict risks from exposure to S. The central hypothesis to be tested is that the relative rates of SO formation and elimination determine species-specific and tissue-specific toxicity. The specific objectives of this work were to:

1) Develop reproducible assays to measure S, SO, and SG in blood and adipose tissues.

2) Develop physiologic toxicokinetic models to simulate S exposure and disposition in humans and rodents.

3) Administer known airborne concentrations (5-200 ppm) of S to volunteers, and measure consequent blood levels of S and SG.

4) Administer S and SO i.v. to rats, and measure consequent blood concentrations of S, SO, and SG.

5) Compare the model-predicted and measured levels of S, SO, and SG.

6) Measure S in human adipose tissue samples and use this information to estimate exposure.

7) Incorporate in vitro enzyme activity measurements into the physiologic models and to examine the utility of the models in providing kinetic explanations for observed toxicity in the literature.

The next chapter (chapter 2) discusses the use of models to understand toxicokinetic and toxicodynamic observations (objective 2). The results of human subject exposure to S and accompanying modeling efforts are presented in chapter 3 (objectives 1, 2, 3). Chapter 4 details the evaluation of arteriovenous differences, physiologic damping, and of the sensitivity of predicted blood concentrations of S to changes in model parameters (objective 2). In chapter 5, measurements of S in human adipose tissue and an estimation of nonoccupational exposure are discussed (objective 6). Chapter 6 describes the reevaluation of current S occupational standards (objective 7), and chapter 7 presents the results of S and

SO administration to rats, and summarizes the implications of sequential metabolism of S in understanding observed toxicity (objectives 1, 4, 5 and 7).

Chapter 2
Modeling Approaches

Early pharmacologic, and traditional toxicologic investigations essentially regarded test animal and human physiologic systems as "black boxes," in which nothing was known about the functioning of the organism and in which the effects of drug or toxicant delivery are measured only by external events such as pharmacologic or toxicologic endpoints. A schematic representation of this approach as applied to risk assessment, including interspecies scaling, is presented in Figure 2-1. This approach traditionally involves the administration of a test substance to an animal model, quantification of toxicity (e.g., LD_{50}, tumor appearance, teratogenic effects), and extrapolation of these results to predict human risk.

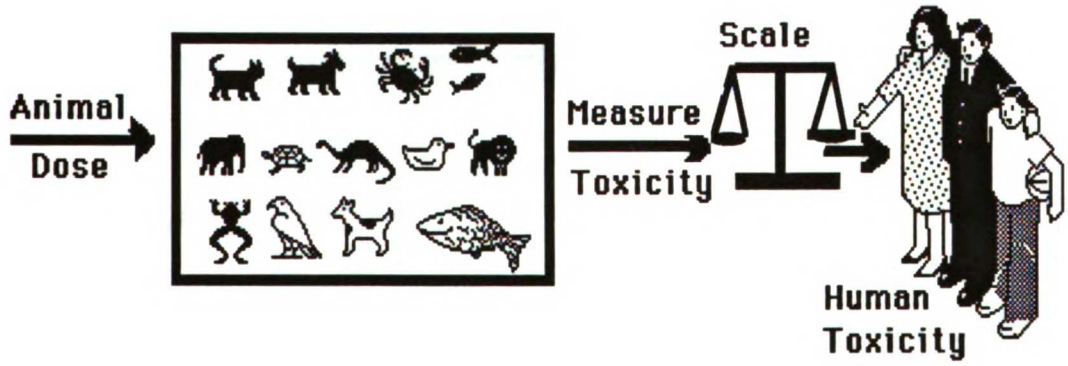


Figure 2-1: Schematic representation of the traditional "black box" approach and interspecies scaling, as used in risk assessment. Depiction of the test species as a black box does not address absorption, distribution, metabolism or excretion within that species.

Essential information gained in animal models must be accurately interpreted to predict human response. Clewell and Andersen[42] succinctly state the requirements for methods to extrapolate animal toxicity data to humans.

> *(There are requirements for) (1) predicting the low dose response in experimental animals based on observed responses at very much higher doses, (2) predicting the response in the human population based on results in the test species, (3) predicting the risk associated with the anticipated human exposure route based on the toxicity with the particular route of exposure used in the animal toxicity studies and (4) predicting human response at realistic discontinuous environmental exposures based on animal results in well-controlled, much more easily characterized exposures.*

Kinetic Approaches.

Kinetic approaches can prove invaluable as a tool for understanding the disposition of exogenous agents. (*Pharmacokinetics* and *toxicokinetics* refer to the absorption, distribution, metabolism, and excretion of therapeutic and toxic substances, respectively.)

As a major improvement to the simple "black box" model of risk assessment, mathematical models involving scaling doses by metabolic rates, often proportional to body surface area, have been useful.

Noncompartmental pharmacokinetic methods have been used to model uptake, residence, and elimination of exogenous drugs and toxicants. Parameters used in this approach include volume of distribution, mean residence time, clearance, and half-life. Interpretation of these parameters requires the assumption of a mammillary model in which input, sampling, and elimination all occur from a central compartment.

Compartmental kinetic methods define theoretical compartments between which toxic or pharmacologic substances transfer. Rate constants are defined that describe substance flux into and out of each compartment. The parameters of multiexponential equations (usually one to three corresponding to the number of compartments) are fit to experimental data. The parameters of a specific compartmental model (consistent with the multiexponential model) are then calculated.

Physiologic Models.

The physiologically-based pharmacokinetic model is an attempt to mathematically simulate the absorption, distribution, and elimination of drugs, toxicants, and their metabolites based on the anatomy and physiology of the body. The animal or human body is

represented as a series of physiologic compartments with individual tissue volumes, blood flows, tissue/blood partition coefficients, and enzyme activities (Fig. 2-2). The network of compartments forms a model with which kinetic predictions of drug/toxicant metabolism can be made. A schematic view of a physiologic model is presented in Figure 2-3.

Construction of Physiologically-Based Pharmacokinetic (PBPK) and Physiologically-Based Toxicokinetic (PBTK) models, subsequently called physiologic models, is carried out using a number of logical guidelines. While there are many potential compartments within the body, organs and organ systems can be logically grouped. In general, the physiologic model with the fewest tissue groups that fits the data and is biologically feasible should be used. The model must be based on an understanding of whether delivery of substance to a tissue is dependent on blood flow to that tissue, or diffusion across membranes or other biologic barriers. Given that the model is an attempt to simulate physiologic processes, parameter values must fall within measured biologic values. Finally, physiologic models must be validated in animal models as well as in human trials (where feasible).

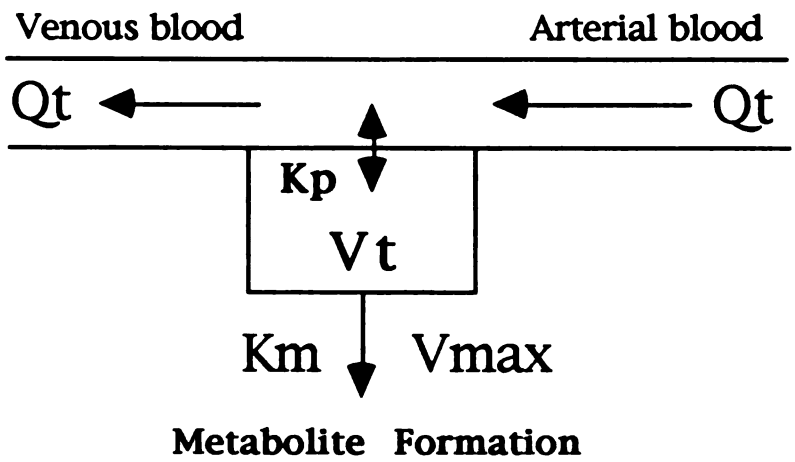


Figure 2-2: Schematic view of a physiologic compartment, where Q_t is tissue blood flow, V_t is tissue volume, K_p is the tissue/blood partition coefficient, and K_m and V_{max} are Michaelis-Menten parameters of metabolite formation.

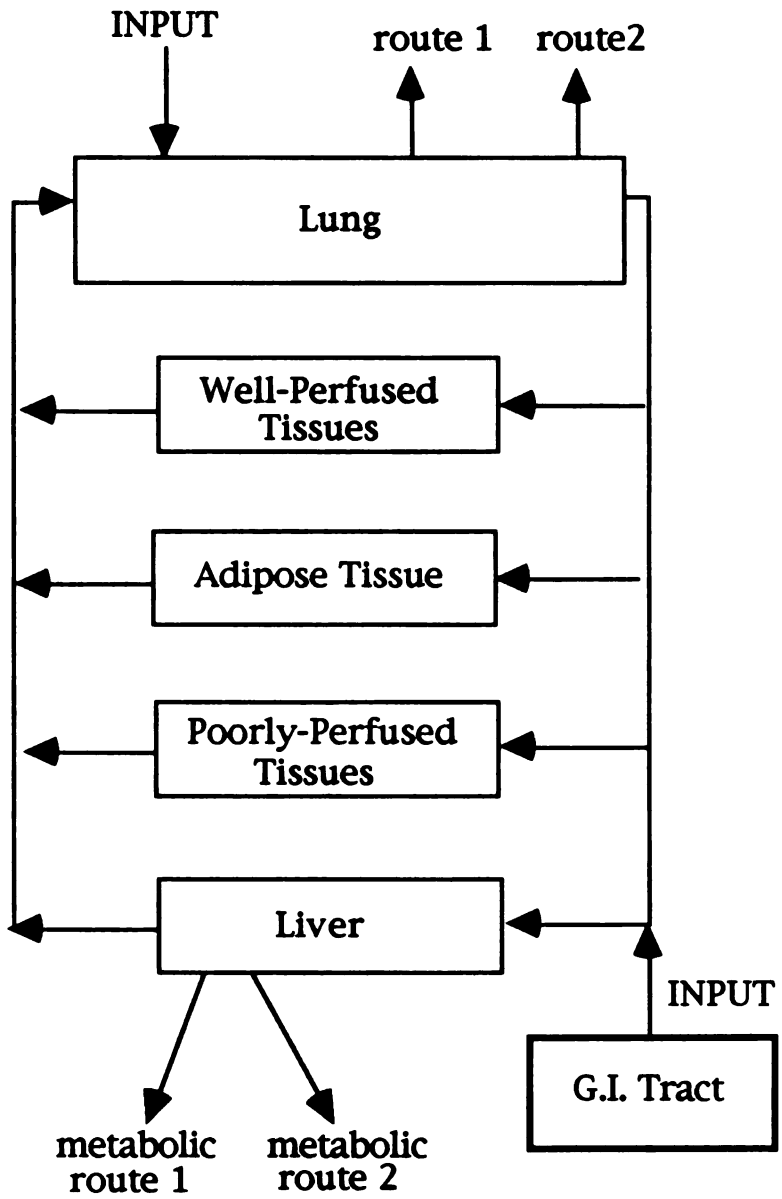


Figure 2-3: Schematic view of a physiologic model with input via the G.I. tract and lung , and elimination in the lungs and liver by multiple routes (adapted from Krewski et al.[43]).

Models based on physiologic parameters have been successfully used to scale across species and exposure routes[44-46]. Another important use is in the modeling of target tissue toxicokinetics. Factors which affect the toxicant concentration at a target site include tissue blood flow, tissue/blood partitioning, and the differing abilities of tissues to metabolize parent toxicant and/or metabolite. Physiologic models have also been linked to toxicodynamic models of mutation and cancer[47], and have included multiple compartments to represent gastrointestinal absorption and lymph flow[48]. Examples of models used to investigate drugs and toxicants are presented in Table 2-1.

Kinetic investigations using physiologic models provide an important inroad into questions of metabolism-induced therapeutic activity and toxicity. However, there are a number of disadvantages, to wit:

1) This kind of approach is ultimately only a **model**, and as such can be verified to be consistent with observations, but never proved.

Table 2-1: Various applications of physiologic models

Substance (Reference)	Exposure Route	Model Details
Styrene[44]	Inhalation	Scale-up, rodents to human.
Formaldehyde [43]	Inhalation	Carcinogenic risk studied.
Vinyl Chloride[43]	Inhalation	Carcinogenic risk studied.
Thiopental[48]	Intravenous	Adipose tissue, protein binding included; membrane-limited delivery.
Methotrexate [48]	Oral	Multiple G.I. lumen and lymph compartments.
Ethylene Dichloride[49]	Oral	Saturable clearance.
Diethyl Ether[49]	Inhalation	Early (1924) model, incorporated ventilation rate.
Benzene[49]	Oral	Found blood and bone marrow concentrations to be parallel; found that ingestion pattern greatly affected tissue exposure.
Non-genotoxic carcinogens [47]	Inhalation	Links physiologic model with models of mutation and cancer.
Ozone[50]	Inhalation	Showed potential application to NO_2; used mathematical model of the respiratory tract and included the effects of exercise.
Carbon Tetrachloride [51]	Inhalation	Included accumulation in adipose tissue and 3 metabolic elimination routes.

2) Results are generally only applicable within the experimental dose range from which data are available. For example, Peterson[52] stated for dichloromethane that:

Careful note should be made of the fact that the [results] *presented here should be used only for interpolation and not for extrapolation. That is, these* [results] *may not be valid for exposures to* [toxicant] *concentrations below 50 or above 500 ppm or for durations less than one hr or more than 7.5 hr, or for more than five successive days of exposure.*

3) Similar to the "black box" risk assessment model, pharmacokinetic investigations can only model the output, rather than probe the mechanisms of metabolic transformations (although some distinction between metabolic pathways is possible).

4) Physiologic models are normally based on average physiologic parameters (tissue composition and volume, blood flow, minute volume and metabolic activity), and as such are not sensitive to interindividual differences.

5) The large uncertainty associated with extrapolating high animal test doses down to probable human exposure doses in cancer risk assessment is usually not addressed, and must be evaluated using other approaches (e.g., the multi-hit model of carcinogenesis). Additional factors such as species longevity and tumor latency periods must also be considered.

Chapter 3

Exposure Chamber Studies, Physiologic Modeling of Styrene, and Evaluation of CNS Toxicity

Measurable neurologic changes as a result of S exposure have been difficult to quantify. As described in the introduction to S toxicity (chapter 1), a variety of CNS disturbances are associated with S exposure. Electroencephalograms, neurotransmitter levels, neuropsychologic performance, reaction time, balance tests, and evoked potentials have all been used to study S neurotoxicity[19, 24, 53, 54], and have often been in disagreement with respect to the exposure required to produce a given response. Murata et al. [26] stated that "Such apparent disagreement [among studies] may reflect complex dose-response relationships between S exposure and neurotoxicity...". We hypothesized that S levels in the brain would correlate well with its CNS toxicity. To accomplish this end, a physiologic model for styrene was constructed, and using data for inhaled S concentrations and minute volume (from our studies and previous studies), predictions of S level in venous blood were made (without data fitting). To validate the model, predictions were then compared with measured values. We then used the model to determine the effect of different exposure regimens on S kinetics and CNS toxicity, and to seek to determine if there is a relationship between modeled brain concentration and acute CNS effects.

Materials and Methods.

Subjects.

Four healthy male volunteers aged 26-30 were selected as a preliminary test group. Candidates were excluded if they had a history of solvent exposure, smoked cigarettes, took any medications, or drank more than 18 g of alcohol per day. No alcohol or caffeine was permitted three days before or during the two days of testing. Volunteers were given a screening physical examination prior to exposure. This research protocol was approved by the Committee on Human Research at the University of California, San Francisco.

Exposure Chamber.

The walls, floor, and ceiling of a 13.8 m^3 exposure chamber (WOO-3R Norlake, Inc., Hudson, WI, USA) were covered with sheets of stainless steel. Glass panels in the door allowed for visual contact with the subjects. The chamber had independent ventilation with banks of activated charcoal filters for removal of contaminants from both the air supply and the exhaust. The complete volume of air in the chamber was replaced every 90 seconds. Relative humidity and temperature were controlled electronically. Openings in the wall allowed routing of cables for monitoring equipment and passage of blood samples. The chamber was operated under slightly negative pressure to prevent leakage.

Airborne S concentrations were generated by dripping S onto a hot plate at a rate controlled by an HPLC pump. Styrene

concentrations inside the chamber were continuously monitored using a Miran 1B infrared spectrophotometer (Foxboro, MA, USA). The signal of the monitored concentration was sent to a computer equipped with a data acquisition converter package that compared the measured to targeted S concentrations every 30 seconds. Deviations from desired concentrations changed the voltage supplied to the pump, thereby automatically adjusting the rate of S delivery into the chamber. A full description of this research protocol has been described by Petreas et al.[55].

Two groups of two subjects each were exposed to S while seated, with occasional standing to perform selected tasks. Four exposure regimens were used to represent different exposure conditions: Day 1 - three sequential 100-minute periods of 15, 32.5 and 50 ppm; Day 2 (next day) - three sequential 100-minute periods of 50, 75 and 99 ppm; Day 3 (several weeks later) - a rapidly fluctuating exposure (10-150 ppm) over four hours; and Day 4 (next day) - a relatively constant exposure of about 10 ppm, followed by a highly variable autocorrelated[56] exposure of 5-200 ppm, over 4 hours.

Sample Analysis.

An indwelling catheter (Angiocath®) with obturator was placed in the forearm. Blood samples were taken before and at selected times during S exposure. Blood (4 ml), drawn from each subject into heparinized Monovette (Sarstedt, Germany) syringes, was immediately passed out of the chamber through a small port. The

blood was then transferred to 7-ml glass vials containing 100 μl of ethyl benzene as internal standard. After addition of 1 ml pentane and capping with a Teflon® seal, the mixture was vortexed for 30 seconds and frozen at -70 °C for subsequent analysis.

Analysis was performed by injecting a sample (1 μl) of the pentane phase into a Hewlett-Packard Model 5890 gas chromatograph (Palo Alto, CA., USA) with a split-splitless capillary injection port, silanized splitless liner, flame ionization detector and a Hewlett-Packard Model 3396A integrator. An Ultra-2 (Hewlett-Packard, Palo Alto, CA., USA) column (25 m x 0.32 mm I.D., 0.52 μm film thickness) was used to separate components. Helium carrier gas head pressure was 83 kPa. Split sampling was performed with a ratio of 10:1. Split vent and septum purge flow rates were 30 and 3 ml/min, respectively. Injection port, column, and detector temperatures were 250, 65, and 300 °C. The column was operated isothermally for 5 minutes; retention times were 3.64 and 4.35 minutes for the internal standard, ethyl benzene, and S, respectively.

To validate the accuracy of the method, S blood samples in the concentration range of 0.1-1 mg/l were prepared by the laboratory of Dr. Steven Rappaport (School of Public Health, University of California at Berkeley) and assayed in a single blind study. There was a good correlation between prepared and measured blood concentrations of S (r^2 = 0.998, slope = 1.037).

Physiologic Modeling.

Scheme of physiologic model.

A physiologically-based toxicokinetic (PBTK) model, similar to those used by others[44-46], and introduced in Chapter 2, was used to mathematically simulate the absorption, distribution, and elimination of S. A minimum number of compartments (six) was chosen to simplify the model (Fig. 3-1). A brain tissue compartment was needed to correlate with CNS effects; muscle tissue was isolated due to the changes in blood flow accompanying increased exertion; adipose tissue was added as it is a prominent storage site; the liver was assumed to be the site of all metabolism. The other well-perfused tissues represented the rest of the body considered in this model; the kidneys were incorporated into this compartment, because less than 2% of S dose is excreted unchanged in the urine[10].

A unique aspect of the simulation of venous blood concentrations in this model was the use of combined flow from two tissue groups. Venous blood, depending on where it is sampled, is expected to have very different S concentrations. For example, blood leaving the liver (under non-saturating conditions) and leaving adipose tissue (well before steady state has been reached) has much lower S levels than that leaving a "non-extracting" tissue such as muscle. Thus, the actual venous S concentrations measured are expected to be highly dependent on the sampling site. In this model, venous blood was assumed to represent drainage from adipose (15%)

and muscle tissue (85%). A schematic representation of the model is presented in Figure 3-1.

An adipose tissue/blood partition coefficient of 106 was determined by equilibrating rat adipose tissue with human blood and comparing the concentration of S in this blood to that of an identical sample without adipose tissue. (Human adipose tissue was found to have measurable background levels of S [chapter 5] and so was not used for this purpose.) The value of 106 is similar to the coefficient of 84 that was found by Andersen et al.[57].

Model equations.

Styrene dosing rate was given by

$$\text{Dosing Rate} = \text{(Concentration in Air) (Minute Volume) (Retention Fraction)}$$

where minute volume is the volume of air inspired per minute, and the retention fraction is (1 - [S conc. in exhaled air/S conc. in inhaled air]).

Mass transfers within the PBTK model are governed by a series of simultaneous differential equations. Changes in the amount of S in non-metabolizing tissues (brain, other well-perfused tissues group, muscle, and adipose tissue) are given by

$$\frac{dA_t}{dt} = Q_t C_a - \frac{Q_t A_t}{K_p V_t}$$

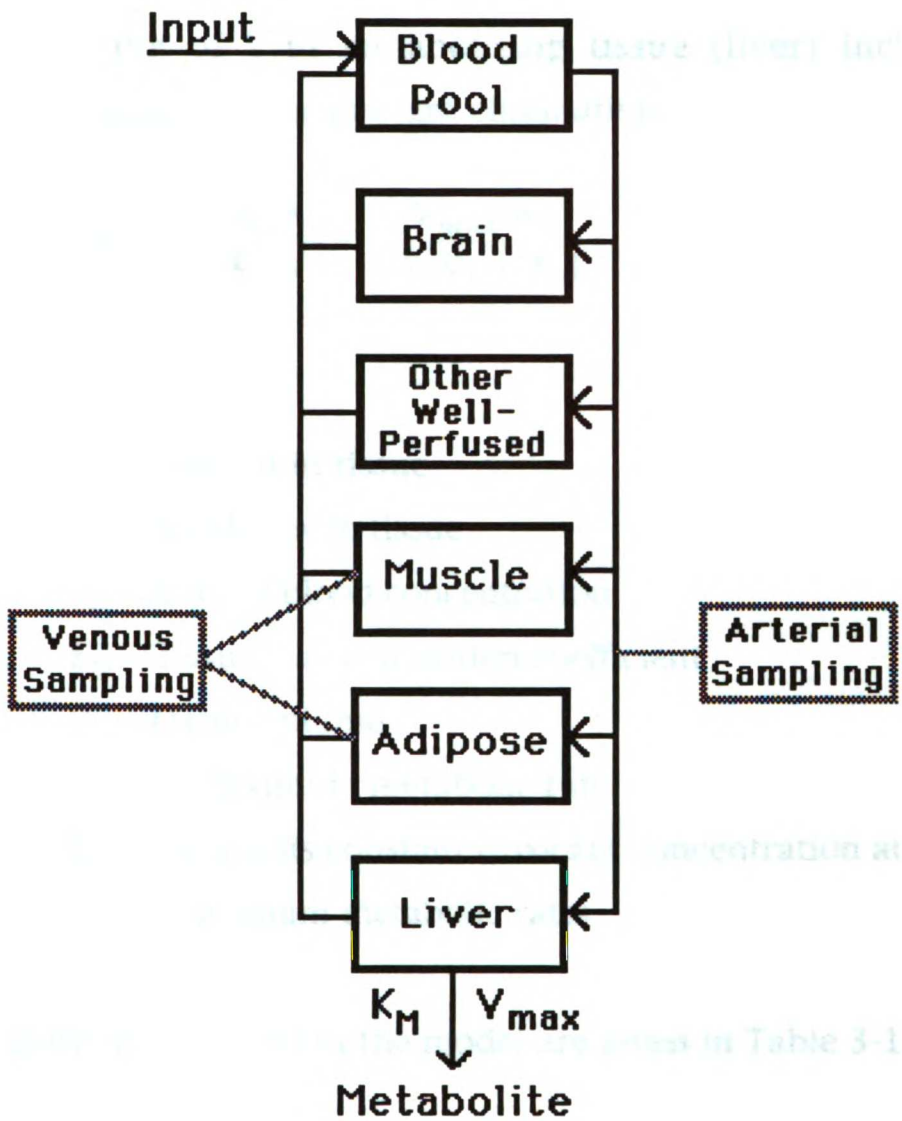


Figure 3-1: Physiologically-Based Toxicokinetic (PBTK) model used to predict arterial and venous concentrations of S after various inhalation exposures (input). Elimination occurs by Michaelis-Menten metabolism in the liver with the metabolic parameters K_m and V_{max}.

Disposition of S in metabolizing tissue (liver) includes a Michaelis-Menten term for toxicant elimination,

$$\frac{dA_t}{dt} = Q_t C_a - \frac{Q_t A_t}{K_p V_t} - \frac{V_{max} A_t}{(K_m V_t + A_t)}$$

where:

A_t = Amount in tissue

Q_t = Blood flow to tissue

C_a = Arterial blood concentration

K_p = Tissue/blood partition coefficient

V_t = Tissue volume

V_{max} = Maximum metabolic rate

K_m = Michaelis constant (toxicant concentration at half-maximum metabolic rate)

Specific values used in the model are given in Table 3-1.

In this model equilibration and/or metabolism of S in tissues is assumed to be limited by blood flow to the tissue. Styrene, a small lipophilic molecule, is assumed to rapidly diffuse across tissue membranes. Bone tissue (volume = 11.2 liters, Q_t = 0.25 liters/min) was excluded, as it was assumed to have a negligible effect on S disposition. The STELLA software program (High Performance Systems, Inc., Hanover, NH) was used for model simulations.

To fully test the model's ability to simulate important arterial-venous differences, S levels were predicted based on the experimental protocols of 21 previous studies[19, 58-61]. These predicted and the measured arterial and venous blood concentrations were then compared. These studies included exposures from 50 to 150 ppm over 30 minutes to 7 hours, under steady-state and nonsteady-state conditions. Minute volume and muscle blood flow also varied across these studies as described by the authors. The measured and model-predicted blood concentrations are shown in Figure 3-2. The predicted levels were 35% and 28% below measured arterial and venous concentrations, respectively. This difference is likely due to an underestimation of the minute volumes of the varying experimental exposure conditions, which were difficult to exactly simulate because the study conditions ranged from resting to hard work activity.

Human Clearance.

Clearance in the model was determined both by blood flow to the liver (under non-saturating conditions) and the metabolic parameters K_m and V_{max} (under saturating conditions). Clearance in humans under non-saturating conditions was determined in two ways: 1) from CL = Dose/Area under the blood concentration curve (AUC) after either a simulated bolus intravenous (i.v.) dose of 1 mg, or after an inhalation exposure of 25 ppm for 500 minutes at a minute volume of 30 L/min; and 2) from the steady-state blood

concentration (C_{SS}) following a constant inhalation exposure to 50 ppm, using the relationship CL = Dosing Rate/C_{SS}.

Half-lives.

The apparent half-life ($t_{1/2}$) in blood is defined as the time for one-half of S in the body to be eliminated. The half-life also determines how quickly steady-state is achieved during constant exposure. To examine apparent $t_{1/2}$ changes with time, the fractional rate of decline ([dC/dt]/C), where C is the concentration of S in blood) was calculated every 0.1 min following a simulated i.v. bolus dose of 1 mg.

Neurologic Determinations.

Because S has a unique odor which is difficult to disguise even at low levels (<1 ppm), subjective tests of neurologic outcomes are difficult to control (although olfactory fatigue rapidly masks the subjects' perception). The P300 wave, a human brain wave with a frequency of 300 milliseconds, is measurable using electrodes attached to the scalp and neck. The P300 latency and amplitude are objective measurements that have been shown to be sensitive indicators of subtle cognitive deficits[65]. An auditory "odd-ball" paradigm was used to obtain the P300 potential. The auditory stimulus consisted of a pseudorandom sequence of two tones with

Table 3-1: Blood flows, partition coefficients, and organ volumes used in the physiologically-based toxicokinetic (PBTK) model

Tissue	Q_t (L/min)[a]	K_p[b]	V_t (l)[a]
Adipose	0.42	106[c]	14[d]
Blood	-	1	4.9
Brain	0.7	2.1[e]	1.4
Liver	1.35	2.7	1.61
Muscle	0.75[f]	1	29.4
Other Well-Perfused[g]	1.51	5.7	6.76
Minute Volume: 7.4 Liters/min[f]		Retention Fraction: 0.65[h]	
V_{max}: 3.1 mg/min[b]		K_M: 0.36 mg/L[b]	

Table 3-1 notes: [a]Values from Rowland and Tozer[3] unless otherwise noted. [b]Values from Ramsey and Andersen[44] unless otherwise noted. [c]Determined as described in text. [d]Using 20% body fat. [e]Data in rats[62]. [f]Resting conditions: Qmuscle[3]; volume[63]. [g]Includes heart, kidneys, lungs, adrenal and thyroid glands, and skin (without subcutaneous fat). [h]Approximately 65% of inhaled styrene is retained[58, 59, 62, 64].

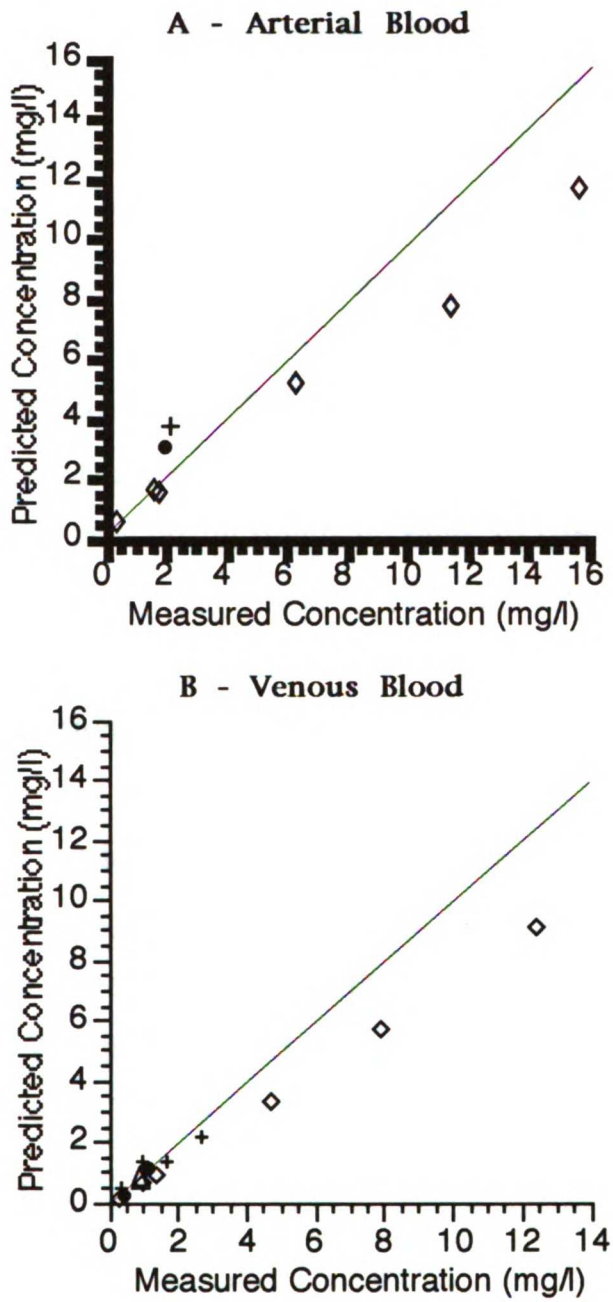


Figure 3-2: Measured vs. predicted concentrations of styrene from previous studies. A: Arterial concentrations from 8 studies. Key: ◊: [60]; •: [58]; +: [59]. B: Venous concentrations from 13 studies. Key: ◊: [60]; •: 50, 100 ppm[19]; +: 200, 375 ppm[19]; ∆: [61]. The thin line indicates the exact correspondence of measured and predicted concentrations.

frequencies of 1,000 hz and 2,000 hz. Subjects were asked to count the total number of the 2,000 hz (target) tones, which comprised ca. 15% of the generated tones. Their EEG was recorded from the scalp vertex, referred to linked mastoid, and was averaged separately for the 2 tones. A minimum of 40 trials of target tones was obtained during each test. The difference in response to target and nontarget tones was used for analysis. The latency was measured as the time between tone and peak of the P300 wave, and the amplitude was measured from the preceding negative peak to the P300 peak.

The 2 and 7 digit recognition test has been used successfully to assess neurotoxicity[66]. This test consists of rapidly identifying 2's and 7's from a series of random digits in several consecutive 15-second trials.

Neurologic testing was performed on each subject during the first two days of exposure to S. On both days, subjects were evaluated prior to exposure (both tests), after 35 minutes of exposure (2 and 7 digit recognition test), and at the end of each 100-minute exposure (P300 test). Styrene blood levels were then compared to P300 event-related potentials and scores on the 2 and 7 test.

Statistical Methods.

A partial correlation coefficient test using a t statistic was used to measure association between exposure level and performance on

the 2 and 7 digit recognition test. The Kendall Rank Correlation test was used to evaluate the relationships between observed toxicity and four potential predictors of toxicity. Three of these were exposure measures which have been shown to be good predictors of toxicologic effects: the peak exposure level, the duration of exposure, and the product of average exposure level and duration (total dose). A fourth potential predictor tested was the peak brain concentration as simulated in the model.

Results

Predicted Versus Measured Blood Styrene Concentrations.

The model-predicted blood levels were much more sensitive to minute volume (directly proportional) and hepatic blood flow (inversely proportional, while under nonsaturating conditions) than any of the other parameters. This sensitivity was expected for a toxicant with both a high blood/air partition ratio[67] and a high hepatic extraction-ratio[3]. To better fit the observed data, minute volume was adjusted by up to 50%. This parameter was chosen for adjustment because of the wide range of values, a 1,000% range, from about 7 L/min to 70 L/min[60, 63], depending on the level of exertion. Measured vs. predicted values for Days 1 and 2 are presented in Figure 3-3, where simulated arteriovenous differences are readily apparent in the two lines plotted.

Measured S concentrations and model predictions in subjects A and D under the variable exposure regimens are presented in Figure 3-4.

Human Toxicokinetic Parameters.

Clearance in humans.

Clearance, as determined by model simulations, was determined in several ways. The model was run with an exposure to 25 ppm for 500 minutes and a minute volume of 30 L/min, and the AUC was "collected" for 15,000 minutes (10.4 days). Clearance was then calculated as CL = Dose (1,038.67 mg)/AUC (841.05 mg-min/L) = 1.23 L/min. The second determination of clearance, entailing the measurement of AUC following a bolus dose of 1 mg, produced an AUC of 0.8 mg-min/L and thus a clearance value of 1.25 L/min Simulation of exposure to 50 ppm at a resting minute volume of 7.4 L/min yielded a steady-state blood concentration of 0.9 mg/L. Clearance, as measured by CL = Dosing Rate/C_{SS}, was calculated as 1.025 mg/min / 0.9 mg/L= 1.14 L/min.

Half-lives.

Three relatively constant apparent half-lives were observed, 0.5, 45 and 3220 minutes. Changes in S blood level and apparent half-life with time after an i.v. bolus dose are presented in Figure 3-5.

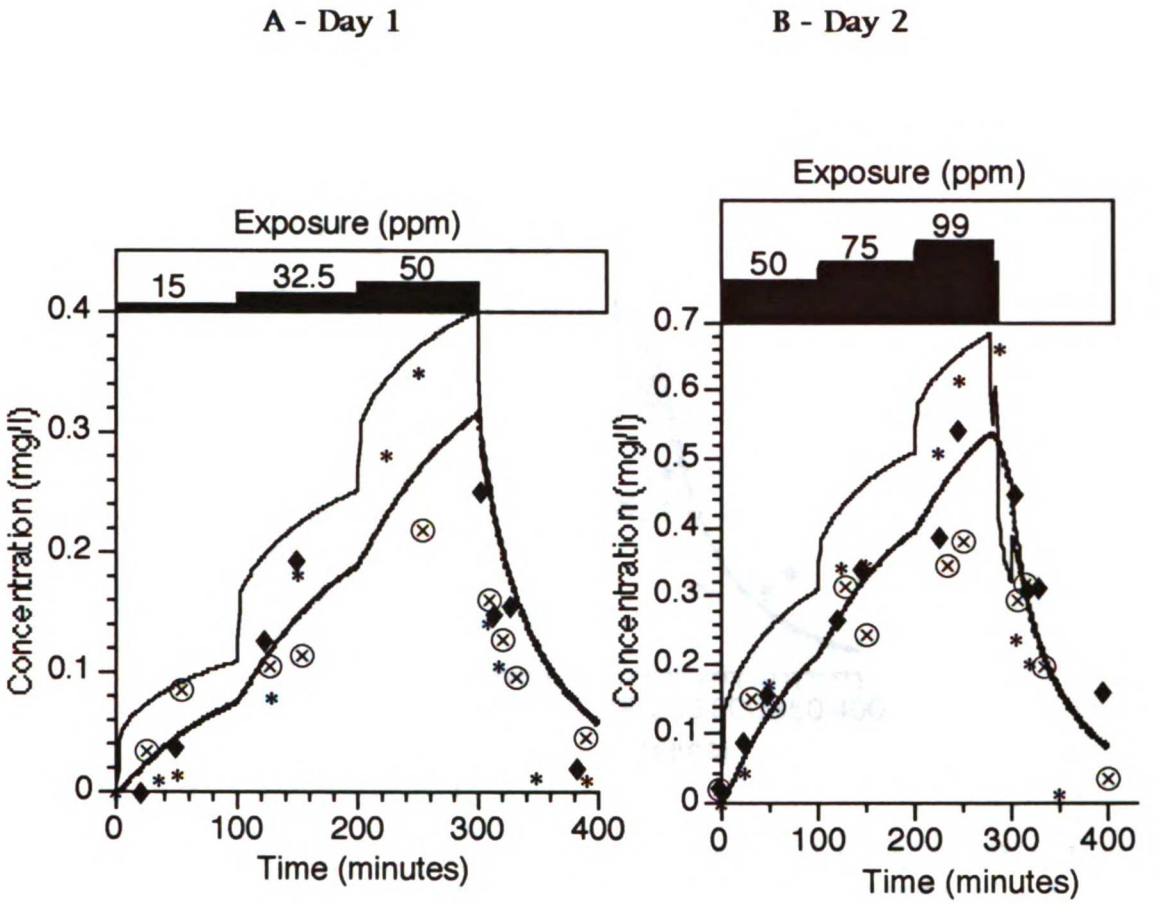


Figure 3-3: Inhaled styrene concentrations (upper portion of each graph) and comparison of simulated concentrations in arterial (upper) and venous (lower) blood with measured venous blood concentrations in subjects A (♦), B (⊗), and C (*). A: (Day 1) Minute volume was decreased by 30% from nominal value (Table 1). B: (Day 2) Minute volume was decreased by 40% from nominal value (Table 1).

A - Day 3

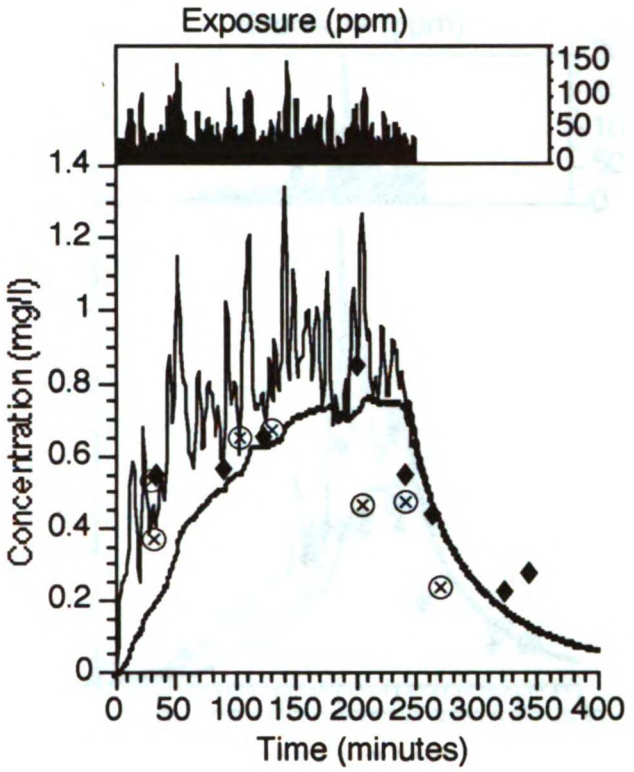
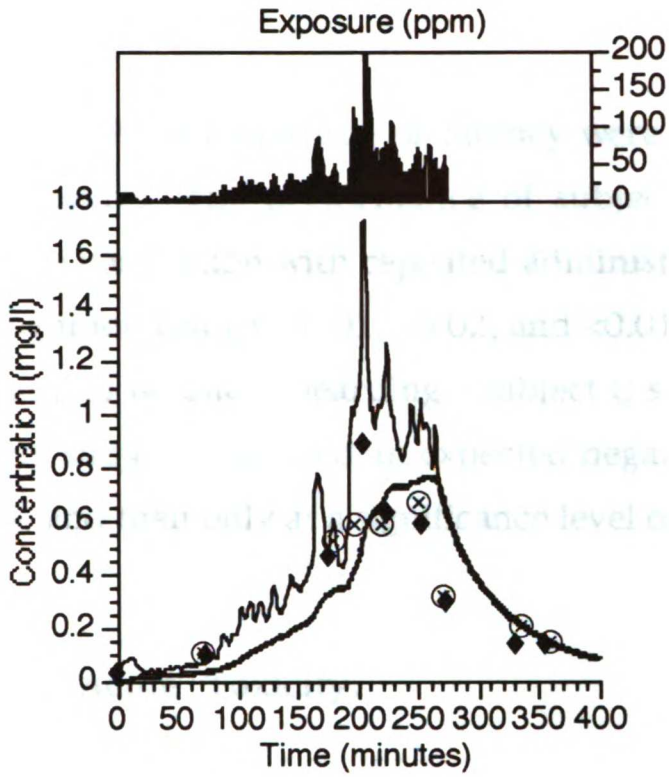


Figure 3-4: Styrene exposure and comparison of simulated concentrations in arterial (upper) and venous (lower) blood with the venous concentrations observed in subjects A (♦) and D (⊗). Minute ventilation was increased by 50% from nominal value (Table 1). A: (Day 3) . B: (Day 4) (on next page).

B - Day 4


Exposure (ppm)
200
150
100
50
0
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Concentration (mg/l)
0 50 100 150 200 250 300 350 400
Time (minutes)

Neurologic Tests.

No change in P300 amplitude or latency were observed with increased S exposure. The performance of subjects A, B, and D showed a positive correlation with repeated administration of the 2 and 7 digit recognition test ($p < 0.01$, <0.02, and <0.01, respectively). This was assumed to be due to learning. Subject C showed no such correlation. Only subject D showed an expected negative correlation with S exposure, and then only at a significance level of $p < 0.1$.

Predictors of Acute Toxicity.

Although the 2 & 7 test, and particularly the P300 test, were expected to be sensitive indicators, based on previous uses of these tests [65, 66], no evidence of acute neurologic effect was found. This led to cessation of the volunteer exposures, and to a more thorough examination of previous studies that documented CNS toxicity from S exposure. In fifteen exposures described in 5 studies[19, 24, 53, 54] including our own, a variety of toxicity measurements were used, including changes in balance, visual acuity, and eye-hand coordination. Observed toxicity from these studies was classified as *none*, *some*, or *all*, with respect to the portion of subjects exhibiting toxicity in each study. Using the physiologic model, the stated inhaled S concentration, duration of exposure, and minute volume in each of these fifteen studies were simulated to determine the peak

brain concentration of S during each exposure. The exposures, observed CNS toxicity, and peak brain level of S for these studies are summarized in Table 3-2.

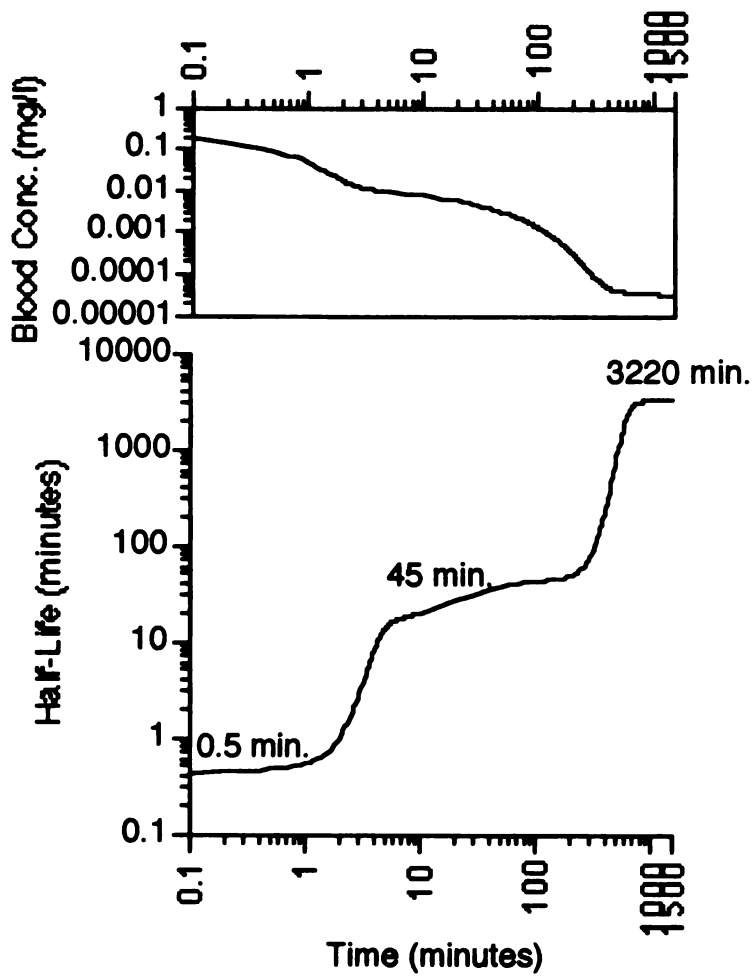


Figure 3-5: Blood concentration (top graph) and apparent styrene half-life (lower graph) vs. time following a simulated i.v. bolus dose of 1 mg. Note that both axes are logarithmic scales to emphasize model-determined half-lives.

Table 3-2: Acute neurotoxicity studies used in the testing of potential predictors of toxicity.

Exposure (ppm)	Model Peak Brain Level (mg/L)	Toxicity	Refer -ence
376 for 0.25 hour	2.25	No CNS toxicity.	[53]
50, 150, 250, 350 for 0.5 hour each	6	All subjects had prolonged simple reaction times in last 30 minutes.	[54]
100 for 7 hours	2.5	Three of six subjects reported balancing difficulty, though no objective confirmation.	
300 for 1 hour w/50W load on bicycle ergometer	20	Impairment in "eye-tracking ability," but no nystagmus, "eye movement," or balance impairment.	
52 for 1 hour	0.9	No balance, dexterity, or coordination changes in 3 subjects.	[19]
117 for 2 hours	2.6	No balance, dexterity, or coordination changes in 1 subject.	
216 for 1 hour	3.6	No balance, dexterity, or coordination changes in 3 subjects.	
376 for 1 hour	6.5	Abnormal neurologic findings and headache in 5 subjects; balance impairment in 1/5; decreased dexterity in 2/5; decreased coordination in 3/5; nausea in 1/5.	

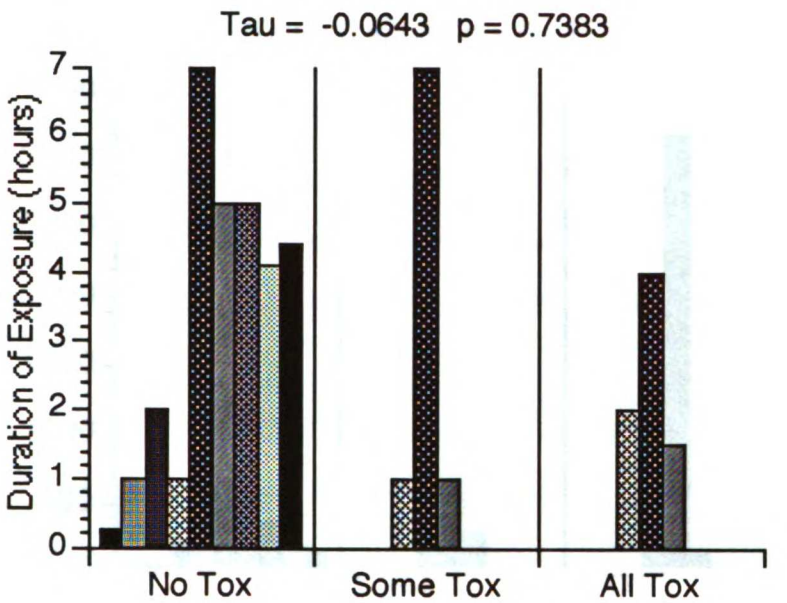
Exposure (ppm)	Model Peak Brain Level (mg/L)	Toxicity	Refer -ence
99 for 3.5 hours, 0.5 hour rest, 99 for 3.5 hours	2.5	No nausea, headache, or impairment of coordination, dexterity, or balance.	
800 for 4 hours	20	Listlessness, drowsiness and impaired balance.	[24]
100 for 1.5 hours	2	All three subjects showed slower reaction times.	
25 for 1.7 hours, 37.5 for 1.7 hours, 50 for 1.7 hours	1.2	No CNS toxicity as measured by P300 transit times and "2s and 7s" identification score.	This work.
50 for 1.7 hours, 75 for 1.7 hours, 99 for 1.7 hours	2.4	No CNS toxicity as measured by P300 transit times and "2s and 7s" identification score.	
Variable exposure, peak=153, time = 4.10 hours, AUC = 202 ppm x hours	1.3	No CNS toxicity as measured by P300 transit times and "2s and 7s" identification score.	
Variable exposure, peak = 200, time = 4.43 hours, AUC = 142.5 ppm x hours	1.51	No CNS toxicity as measured by P300 transit times and "2s and 7s" identification score.	

The predictive values of the four measures tested - peak inhaled concentration, duration of exposure, average exposure•duration, and simulated peak brain level - are summarized in Figure 3-6.

Figure 3-6: A. Duration, B. Peak exposure level, C. Average exposure level•duration, and D. Simulated peak brain level, as predictors of CNS toxicity, where each bar represents a separate study. "None" "Some" and "All" refer to the portion of subjects exhibiting CNS toxicity. The value tau is the Kendall Rank Correlation coefficient, and p is the probability that there is no association between the parameter and toxicity.

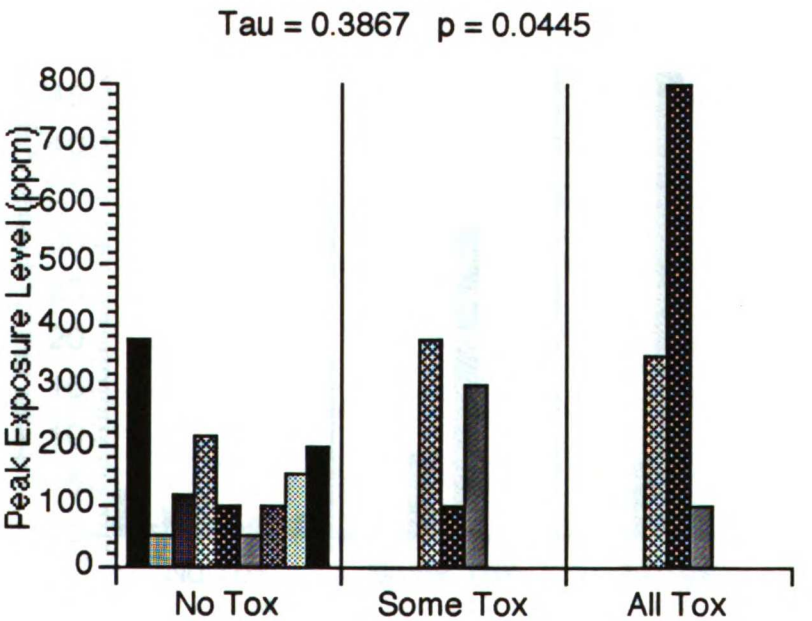
A

Duration of Exposure



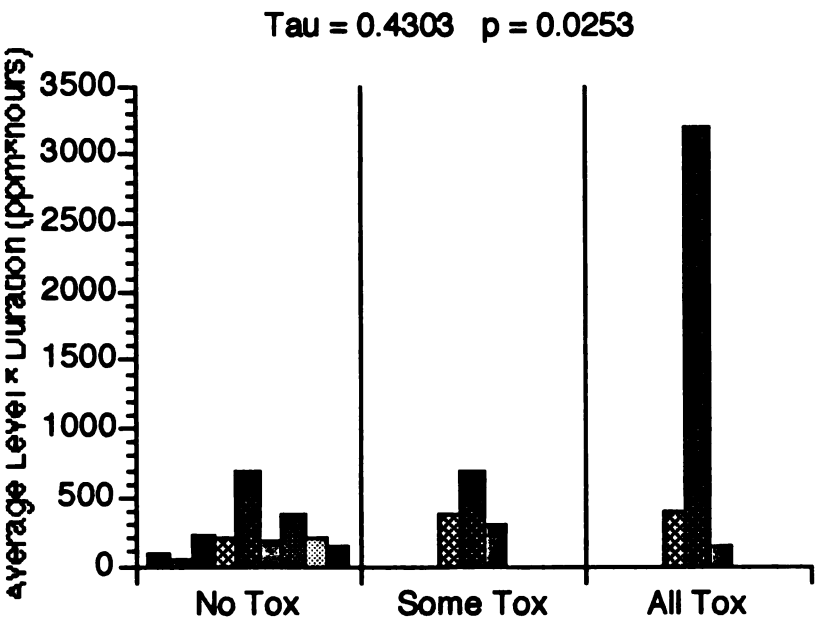
B

Peak Exposure Level



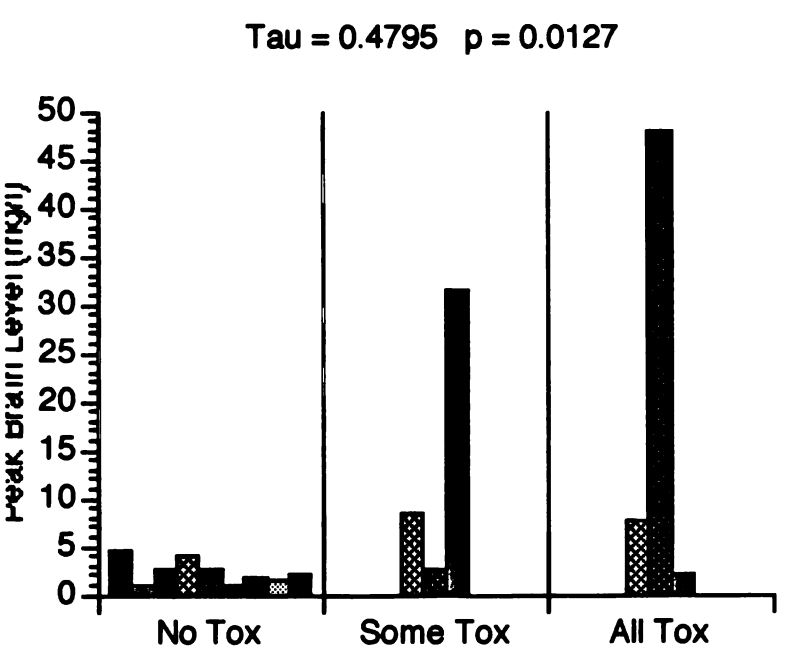
C

Average Exposure • Duration



D

Peak Brain Concentration



Discussion

Physiologic Model.

Minute volume and hepatic blood flow had the greatest influence on predicted blood concentrations. The former was adjusted by up to 50% to fit observations in the four subjects. This physiologic parameter commonly varies by 1,000%, from about 7 L/min to 70 L/min[60, 63], depending on the level of exertion. Hepatic blood flow, conversely, varies comparatively much less[68, 69]. Ramsey and Andersen[44], by scaling up from the rat, used a minute volume similar to our lowest value, but also used physiologically unrealistic values[3] for adipose and muscle volumes (9% and 73% of body volume, respectively). Moreover, these authors adjusted adipose tissue blood flow and partition coefficient to better fit observed values; no such adjustment was made in our model. After adjustment of minute volume, the PBTK model gave good qualitative predictions of measured venous levels during and following exposures.

One of the disadvantages of the physiologic model is its use of average parameters (organ volumes, blood flows, partition coefficients, and metabolic values) to predict values in given individuals. In these studies, minute volume has been varied to account for discrepancies between data and model predictions.

Toxicokinetics.

Clearance.

Using different methods of assessing clearance in humans, the physiologic model predicted values of 1.14, 1.23, 1.25 (mean = 1.21) L/min, which approach the rate of hepatic blood flow of 1.35 L/min (Table 3-1). Given that all metabolic elimination occurred in the liver, this result indicates that S is among numerous toxicants and drugs that are termed "high extraction ratio." Styrene metabolism is thus governed by the rate of S delivery to metabolizing tissue (i.e., liver).

The mean value in this study of 1.21 L/min is near the clearance of 1.7 ± 0.3 L/min found by Wigaeus et al.[58], and a value of 1.75 L/min found by Lof et al.[59] in non-occupationally exposed subjects, but is further from the value of 2.33 L/min found by the latter group in occupationally exposed persons. Lof et al. suggested that "metabolic adaptation" was responsible for the increased clearance they observed. However, given that S has a high blood clearance, and is primarily metabolized in the liver, its elimination should not be affected by enzyme induction[3]. It is possible that extrahepatic metabolism, induced by repeated exposure, however, could lead to an increased clearance. In addition, there is some limited evidence that increased hepatic blood flow and clearance of drugs can result from pretreatment with the inducing agent phenobarbital[70]. The discrepancy could also be due to some unknown artifact of the studies.

The model revealed 3 time periods of relatively stable half-lives (0.5, 45, and 3220 minutes, Fig. 3-7), providing an explanation for previously reported values of 2 minutes to 5.2 days (Fig. 3-2). Each half-life, strictly speaking, results from both elimination and distribution between tissues. However, a predominant process may be associated with each half-life. The shortest half-life (0.5 min), appears to primarily represent the distribution of blood S into well-perfused tissues. This conclusion is supported by the observation that this half-life is apparent only for the first two minutes (Fig. 3-5, lower graph), a time in which S is dispersing into blood and being circulated to well-perfused organs. The intermediate half-life (45 min) probably represents the elimination and redistribution of S from well-perfused tissues, a contention supported by relatively large area under the blood-concentration curve (Fig. 3-5, upper graph). The longest half-life (3220 min or 2.24 days), likely represents the desorption of S from adipose tissue and its subsequent elimination. This final half-life, seen 800 min post-dosing, approaches the half-life determined during studies with adipose tissue (chapter 5).

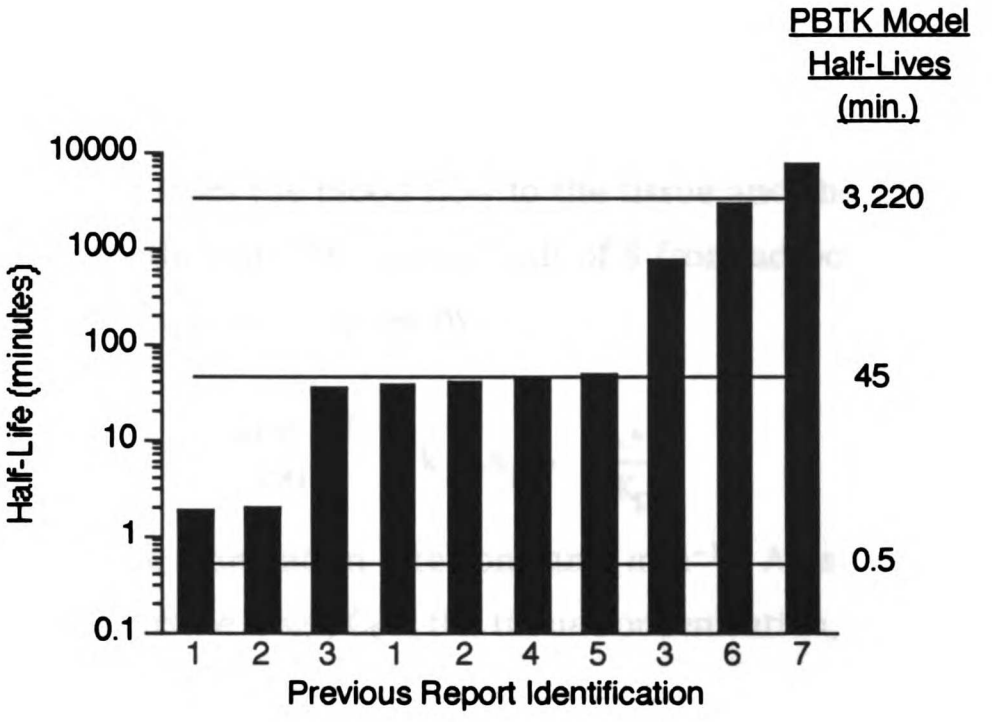


Figure 3-7: Previously reported half-lives (vertical bars), and PBTK model-predicted half lives (horizontal lines) of styrene. Key to sources of half-lives: 1:[59]; 2:[58]; 3:[44]; 4:[18]; 5:[19]; 6:[71]; 7:[72].

Predicted terminal half-life.

The half-life associated with S measured in adipose tissue can be estimated from the blood flow to the tissue and the tissue/blood partition coefficient. The rate of exit of S from adipose tissue after cessation of exposure is given by:

$$\text{Rate of Exit} = k \cdot A_t = \frac{C_t \cdot Q}{K_p}$$

where k is the elimination rate constant (min^{-1}), A_t is the amount of S in adipose tissue (mg), C_t is the tissue concentration, Q is the blood flow (ml/min), and K_p is the tissue/blood partition coefficient. This relationship assumes that $C_v = C_t/K_p$ (where C_v is the venous concentration leaving tissue).

Since $A_t = C_t \times V_t$, where V_t is the volume of tissue (liters),

$$k = \frac{Q/V_t}{K_p}$$

Perfusion (Q/V_t) of adipose tissue is about 0.03 ml/min-ml of tissue[3], and the adipose tissue/blood partition coefficient has been measured as 106 as described in Chapter 2. An elimination rate constant (k) of 2.8×10^{-4} min^{-1} is calculated from these values. However, the observed terminal half-life of S in adipose tissue is a function of both venous loss *and* arterial return to adipose tissue. As discussed in Chapter 4, virtually all the S in the body during this terminal phase is in the adipose tissue. Assuming a steady-state condition, and given that virtually all S is eliminated in the liver, the

rate of loss from the body during the terminal elimination phase is approximated by

$$\text{Rate of Loss} = Q_a C_V - Q_a C_A = Q_h C_A$$

where: Q_a = Blood flow to adipose tissue.

C_V = Venous concentration of S leaving adipose tissue.

C_A = Arterial concentration of S.

Q_h = Blood flow to liver.

Rearrangement of this equation and substitution with values for adipose tissue and liver blood flow (0.42, 1.35 L/min[44]) yields the expression $C_A/C_V = 0.24$. This indicates that about 24% of S "leaving" adipose tissue is returned through arterial blood. The actual rate constant describing loss of S in the terminal elimination phase is then $2.8 \times 10^{-4}\ min^{-1} \times (1 - 0.24) = 2.2 \times 10^{-4}\ min^{-1}$, corresponding to a half-life of 2.23 days (where $t_{1/2} = \ln 2/k$). This method of estimating half-life in adipose tissue is in good agreement with the 2.24 day half-life predicted by the PBTK model, and in agreement with the range of 2-5.2 days measured in workers by Engstrom et al.[71, 72].

Model-simulated values of human clearance agree with previous literature values, and the half-lives of the three major phases of decline help to explain the widespread values of S half-lives in humans previously reported.

CNS Toxicity.

In this study, an exposure of young, nonoccupationally-exposed subjects to about 100 ppm for 2 hours under resting conditions was

not sufficient to measure CNS changes as reflected in the P300 or 2 and 7 digit recognition tests. However, similar exposure of the mixed-age cohort of 300,000 S-exposed workers[2] may result in observable toxicity in particularly sensitive individuals.

The mechanism of S-induced CNS toxicity has not yet been identified. Mutti et al.[22, 73, 74] have suggested that phenylglyoxylic acid (PGA), a major metabolite of S, is responsible for acute neurotoxicity in one of two ways. PGA may be aminated to phenylglycine and compete with dopamine for vesicular storage; or PGA may react with dopamine, rendering this neurotransmitter ineffective. However, these suggestions have not been widely adopted, and the mechanism of toxicity may be a CNS intoxication based on the Ferguson principle of structurally nonspecific CNS depression[75].

Predictors of Toxicity.

Because we did not find evidence of CNS toxicity in either the P300 or "2s and 7s" tests, we examined previous studies in which toxicity was observed. Exposures from these studies were simulated and significant ($p<0.05$) correlations between CNS toxicity and peak exposure level, average level•duration of exposure, and peak brain level were found. The strongest association was between the average level•duration of exposure and toxicity. CNS toxicity began to appear at a simulated peak brain concentration of about 5 mg/l (Fig. 3-5).

These results suggest that the California Occupational Health and Safety Administration (Cal-OSHA) and Federal OSHA

occupational standards (100 ppm, and 50 ppm, over an 8-hour working day, respectively) are protective against acute CNS toxicity at a resting breathing rate. However, exposure at these levels during conditions of increased respiration due to work activity may lead to CNS toxicity. This issue is more fully developed in Chapter 6.

Chapter 4

Arteriovenous Differences, Physiologic Damping, and Parameter Sensitivity in the Physiologic Model

As described in chapter 3, physiologic models allow a number of investigations that were not previously possible, such as the continuous simulations of S levels in the brain. In addition to the ability to model target site kinetics, these models can be used to evaluate arteriovenous differences, physiologic damping, and sensitivity of selected kinetic parameters, the subjects of this chapter. Arteriovenous differences in drug and toxicant exposure can be pronounced. Chiou[76] highlights up to 3,240-fold differences in arterial and venous griseofulvin levels following i.v. dosing, and suggests that drug metabolism and uptake in poorly-perfused sampling tissues can produce such differences. Physiologic damping, defined as the relative fluctuations in tissue concentrations compared to exposure levels, plays an important role in modulating toxicant exposure to critical target site tissues. As will be seen, evaluation of these kinetic aspects of drug and toxicant disposition provides further insight into toxicodynamics.

Methods.

Arteriovenous Differences.

In contrast to most other authors using physiologic models (chapter 2), the model developed in this research incorporates

arteriovenous differences across a non-metabolizing tissue, the forearm. Using the model (described in chapter 3), differences in arterial and venous blood concentrations of S were examined by simulating exposure to 50 ppm at a light work minute volume (volume of air inhaled per minute) of 30 L/min Exposure was simulated for 300 minutes and arteriovenous differences were examined during and after exposure.

Physiologic Damping.

Physiologic damping plays a major role in how a chosen sampling site reflects toxicant concentrations in other parts of the body, in how toxicokinetics is linked to toxicodynamics, and in how measurements in different tissues can provide exposure information. For example, S in blood provides a picture of very recent exposure whereas S in adipose tissue provides a long-term description of exposure. Damping was qualitatively assessed by comparing the relative fluctuations in S tissue levels to those in exposure concentrations. In order to simulate occupational exposure conditions to S, where exposure cycles can fluctuate rapidly in, for example, a boat-building factory (chapter 1), a sine wave with an amplitude of 25 ppm and a period of 15 minutes was used to simulate exposure concentrations. Corresponding changes in blood, brain, and adipose tissue levels were then examined.

Sensitivity of Kinetic Parameters.

Three tests of model sensitivity to changes in kinetic parameters were performed: (1) Model values, including minute volume, hepatic blood flow, tissue/blood partition coefficients, and tissue volumes, were varied to determine which parameters would have the greatest effect on predicted blood concentrations of S. (2) The maximum rate of metabolism, V_{max}, was increased over a ten-fold range (3.1 - 31 mg/min) to observe any changes in clearance and in S blood levels. (3) The inhaled dose of S was increased to test for saturation of metabolism.

Results

Arteriovenous Differences.

Differences in arterial and venous S concentrations during and following a simulated 300-minute exposure to 50 ppm at a minute volume of 30 L/min are evident in Figure 4-1. It was not until 225 minutes into the exposure that venous concentration was within 15% of arterial concentration. Similarly, it was not until 208 minutes after the cessation of exposure that arterial concentration dropped below 85% that of venous levels. From 700 min to the end of exposure (2,500 min), arterial levels were 31% lower than venous levels.

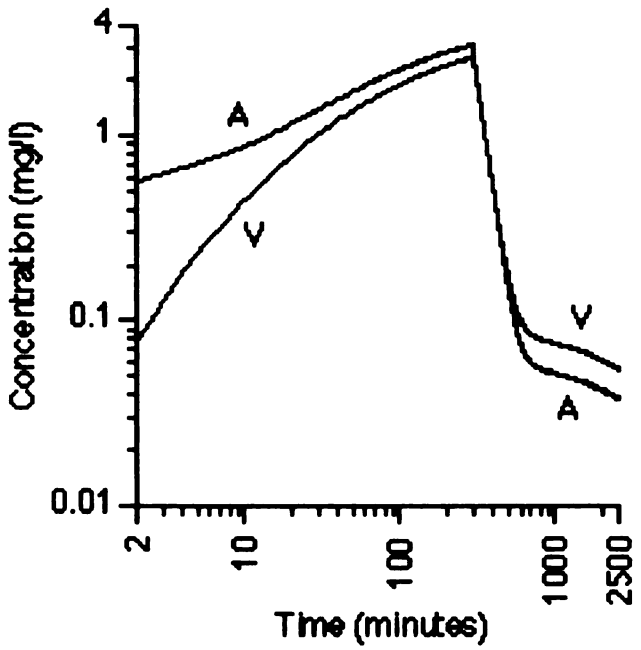


Figure 4-1 : Simulated arterial (A) and venous (V) levels of styrene during and following a 300-minute exposure to 50 ppm at a minute volume of 30 L/min Both axes are plotted on logarithmic scales to more easily visualize arteriovenous differences with time.

The constant arteriovenous difference in the terminal phase can be explained in terms of mass balance of S. As discussed in chapter 3, assuming a high extraction of S in the liver, the rate of loss from the body during the terminal elimination phase is approximated by

$$\text{Rate of Loss} = Q_a C_V - Q_a C_A = Q_h C_A$$

where: Q_a = Blood flow to adipose tissue.

C_V = Venous concentration of S leaving adipose tissue.

C_A = Arterial concentration of S.

Q_h = Blood flow to liver.

Rearrangement of this equation and substitution with values for adipose tissue and liver blood flow (0.42, 1.35 L/min[44]) yields the expression $C_A/C_V = 0.24$. This indicates that about 24% of S "leaving" adipose tissue is returned through arterial blood during the terminal phase. Since venous blood is simulated using drainage from a tissue that is 15% adipose tissue and 85% muscle, arterial blood concentrations as a fraction of venous levels are $0.24/(0.15 + 0.85 \cdot 0.24) = 0.68$, or 32% lower, in agreement with the 31% observed in the simulation.

Arteriovenous differences in S concentration are most pronounced at the onset of constant exposure, and gradually decrease as steady-state is approached. Under these conditions, venous blood sampling underestimates arterial levels (perfusing target tissues) until steady-state is reached. Upon cessation of exposure, arterial levels fall more rapidly than venous levels (Fig. 4-1) with arterial levels up to 31% lower.

Reexamination of the simulated arterial and venous blood levels from the human exposure chamber studies (chapter 3) reveals arteriovenous differences, particularly at early time points (Fig. 4-2).

Physiologic Damping.

Styrene concentrations in inhaled air, blood, brain, and adipose tissue from a sine wave-generated exposure are presented in Figure 4-3. Physiologic damping is also evident in Figure 4-2 in a comparison of fluctuations of inhaled air concentrations (top graph) vs. simulated venous blood levels of S (lower graph).

Physiologic damping at steady-state has been defined as the ratio of the coefficients of variation of a substance measured in blood to that in inspired air[56]. Damping increases with membrane transit time, toxicant affinity for tissue, and tissue volume, and decreases with tissue perfusion rate. Styrene levels in the brain are damped relative to inhaled concentrations (particularly when the latter are highly variable). Given the high lipophilicity of S (adipose tissue/blood partition coefficient of 106), and the low rate of tissue perfusion (0.03 ml/min-ml of tissue[3]), adipose tissue is the most highly damped tissue. As such, measurement of S in adipose tissue can be used as a measurement of chronic, slowly-fluctuating exposure. This approach is further discussed in chapter 5.

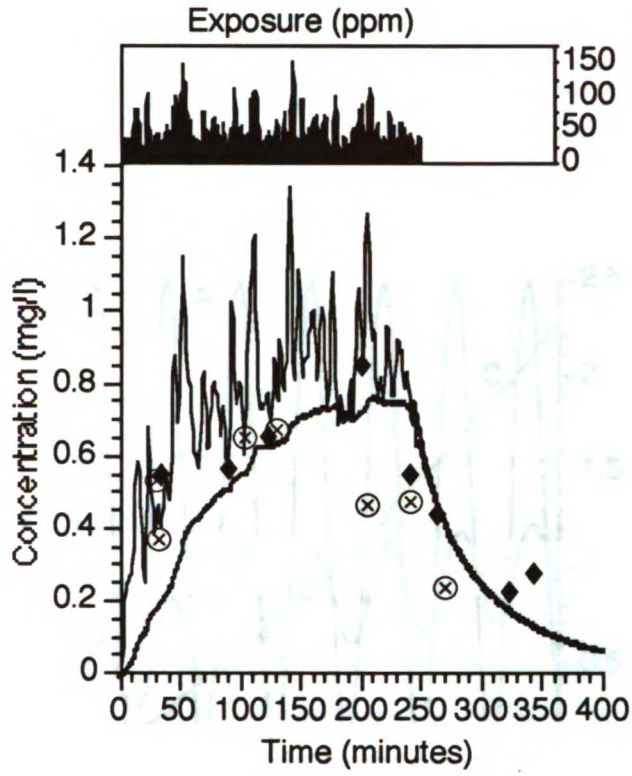


Figure 4-2: Styrene exposure (Day 3) and comparison of simulated concentrations in arterial (upper) and venous (lower) blood with those observed in subjects A (♦) and D (⊗).

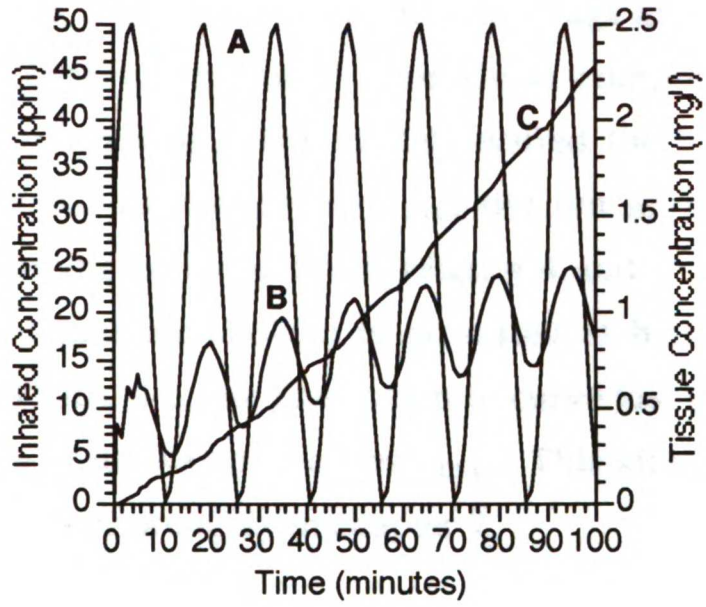


Figure 4-3: Relative damping of inhaled styrene fluctuations with time in arterial blood and adipose tissue. A widely fluctuating inhaled concentration (A), using a sine wave with a 15-minute period, is reflected by smaller oscillations in arterial blood concentration (B) and by a steadily increasing adipose tissue concentration (C) (during the onset of exposure).

Kinetic Parameters.

Drug or toxicant elimination in the liver is described by hepatic clearance (CL) in this model. Clearance is described by CL = $Q_{hepatic} \cdot ER$; where Q_H is the hepatic blood flow and ER, the extraction ratio, is the fraction of toxicant entering the liver that is metabolized. As we have seen, S is among the numerous drugs and toxicants that are labeled "highly-extracted." For these compounds, hepatic clearance approaches Q_H. The model verified the high extraction ratio (in humans) as changes in V_{max} did not appreciably affect clearance or predicted blood levels (Figs. 4-4 and 4-5). The model also suggested that metabolism is saturated at higher doses. As illustrated in Figure 4-6, the lines begin to curve just above 30 ppm, where the rate of intake approaches V_{max}. This kinetic observation is discussed more thoroughly in chapter 7.

Discussion

There are several characteristics of S that have been incorporated into the physiologic model. These include a high lipid solubility, retention of about 65% of inhaled S, distribution into brain tissue (to account for observed CNS effects), and metabolic elimination (principally in the liver). While demonstrating that these characteristics must be included, the model also suggested that significant arteriovenous differences and physiologic damping of S

can occur. Model simulations also showed that changes in V_{max} had little impact on blood concentration of S.

It was found that arterial and venous concentrations at the onset of inhalation exposure, before the tissues are fully equilibrated, can be different by an order of a magnitude (Fig. 4-1) and the arterial and venous concentrations also differ (31% in the model above) following cessation of exposure, as the S is cleared from the adipose tissue. Physiologic damping can lead to very different fluctuations in exposure vs. tissue levels of S (Fig. 4-3). As expected for a substance with a high hepatic extraction ratio, changes in V_{max} did not affect either clearance or simulated blood levels (Figs. 4-4 and 4-5). Testing of the effect of varying input rate showed that S metabolism would be saturated above 30 ppm following a two-hour exposure at a moderate work breathing rate of 50 L/min. This rate of intake is equivalent to a constant exposure of about 200 ppm at a resting breathing rate of 7.4 l/min.

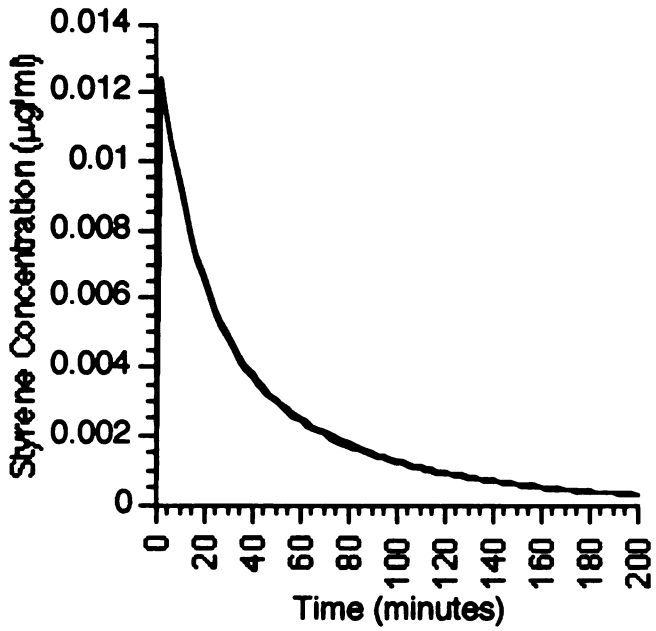


Figure 4-4: Simulated styrene concentration in blood following a 1 mg bolus dose. The simulated curves for five V_{max} values over the range 3.1 to 31 mg/min were observed to superimpose.

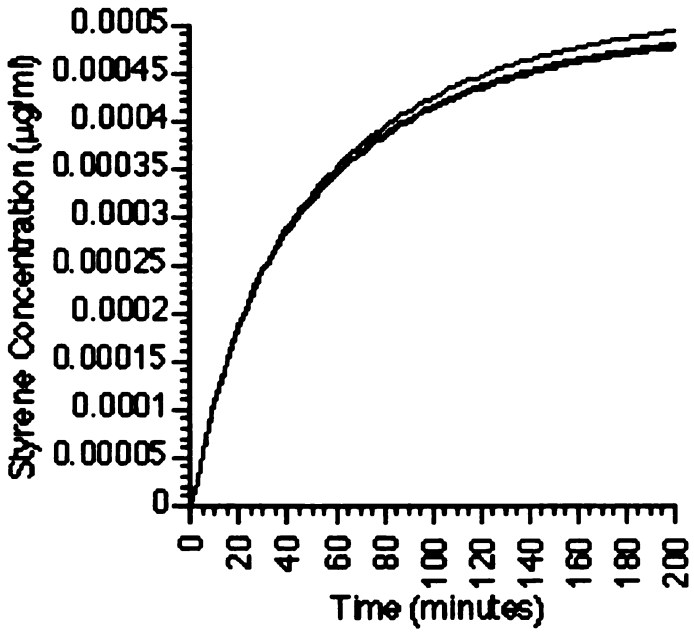


Figure 4-5: Simulated styrene concentration in blood during a 0.6 mg/min infusion. V_{max} was varied from 3.1 to 31 mg/min. Only at the extreme differences in V_{max} and at later time points were the simulated concentration-time curves not observed to be virtually superimposed.

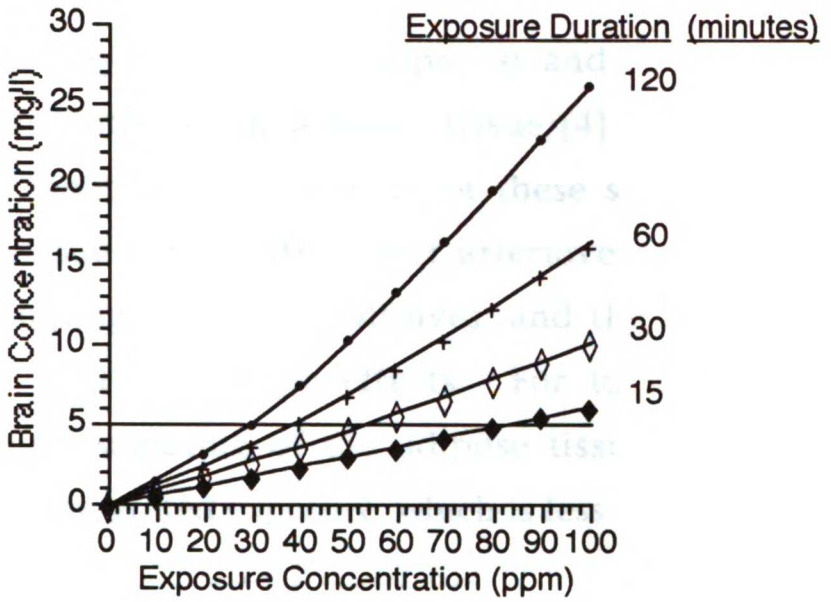


Figure 4-6: PBTK model-simulated brain concentrations of styrene at exposures of 10 to 100 ppm, where the minute volume is 50 l/min (moderate work) Four durations of exposure are represented: ♦, 15 minutes; ◊, 30 minutes; +, 60 minutes; •, 120 minutes. The solid line indicates a brain concentration of 5 mg/l, where toxicity begins to appear (chapter 3). (The nominal value of V_{max} was used [Table 3-1]).

Chapter 5
Styrene in Adipose Tissue

Storage of drugs and toxicants in specific tissues (e.g., lead in bone, iodine in thyroid tissue, thiopental and DDT [dichloro-diphenyl-trichloroethane] in adipose tissue)[4] has pronounced effects on the kinetics and dynamics of these substances. High affinity for a tissue can greatly affect arteriovenous differences, physiologic damping, apparent half-lives, and the time course of pharmacologic and toxicologic effects. For toxicants that are sequestered here, sampling of the adipose tissue can provide a measurement of integrated exposure, which is less subject to varying input levels than the blood. Due to the more slowly fluctuating concentrations, adipose tissue can also provide a better medium than blood in which to determine the kinetic parameters relevant to chronic exposure.

The goal of this investigation was to measure the level of styrene in the adipose tissue of non-occupationally exposed persons, and to use this information to assess background rates of environmental exposure.

Introduction.

A number of lipid-soluble environmental toxicants have been measured in the adipose tissue of occupationally and non-occupationally exposed individuals. Occupational exposures to

several volatile, lipid-soluble organic agents, including vinyl chloride, DDT, and PCBs (polychlorinated biphenyls) after known occupational-level exposure have produced measurable adipose tissue concentrations[77, 78]. Adeshina and Todd[79] found measurable levels of the pesticides DDT, oxychlordane, and heptachlor epoxide in the adipose tissue of nonoccupationally exposed North Texas residents. Mussalo-Rauhamaa et al.[80] measured a median of 0.002 μg/g for both tetrachlorophenol and pentachlorophenol in 58 persons living in Finland. PCBs have been found at concentrations up to 2 μg/g in 688 samples from the US. general population[77]. Styrene has also been found in adipose tissue following airborne exposure at occupational levels (typically 5-50 ppm)[71, 72, 77, 81]. Styrene was detected for up to 13 days following exposure to 50 ppm for four periods of thirty minutes each of resting, and 50 watt, 100 watt, and 150 watt intensity exercise on a stationary bicycle[71].

Despite evidence that smokers have 1.5 to 2 times the styrene concentration in the breathing zone or indoor air as compared to non-smokers[8, 82-84], differences in styrene levels in blood between the two groups have not been apparent[85]. This observation may be explained by the short blood half-life and highly variable blood styrene concentrations, and/or by an insignificant contribution of smoking to the total styrene dose. Because S has a high tissue/blood partition coefficient (chapter 3)[44] and the low perfusion rate of adipose tissue, this tissue may provide a useful physiologically-damped internal marker of dose. The intent of this study was to determine adipose tissue levels of S in nonoccupationally exposed individuals. Given the previously-

reported half-lives in adipose tissue of 2-5.2 days[71, 72], the styrene concentration in nonoccupationally exposed individuals was assumed to be at "steady-state".

Materials and Methods.

Portions (1-2 g) of adipose tissue normally discarded after surgery were obtained from three surgical patients and frozen for subsequent analysis. Another five samples of adipose tissue were obtained postmortem with limited donor information (gender and age). In addition, one cow sample was tested to represent different exposure conditions (the cow adipose tissue was a random sample from a northern California slaughterhouse). To minimize bacterial contamination, frozen samples were analyzed within one month; 30-mg portions used for analysis were cut from the center of several grams of adipose tissue from each subject. In a modification of a procedure by Wolff et al.[81], weighed adipose tissue samples were vortexed for 30 seconds with 90 μl cold (0-5 °C) CS_2 and 10 μl of the internal standard ethyl benzene (1.67 μg/ml in CS_2) in 1.5 ml glass vials capped with Teflon®-faced septa. Assuming analytical similarities with human tissue, rat adipose tissue with no detectable levels of styrene was handled identically to the human specimens and used for blank and standard curve samples. Standards and samples were allowed to equilibrate for 30 min at room temperature. Vials were then vortexed for 30 s and 1 μl of the CS_2 phase was injected into the gas chromatograph (GC).

GC analyses were carried out by the procedure described in chapter 3. To confirm the presence of styrene and ethyl benzene, the two highest samples (#2, #7) were analyzed by gas chromatography-mass spectrometry (GCMS) in selected ion monitoring mode (SIMS). Column retention times for ethyl benzene and styrene were 3.50 and 4.31 minutes, respectively. Mass/charge (m/z) ratios of 78 and 104 were used for styrene, and 78 and 106 for ethyl benzene. Blank samples showed no contamination, and mass ratios were the same for a styrene standard and the two samples.

The limit of detection was 50 ng/g (3 times standard deviation of background signal). Five of the seven human tissue samples with styrene (and the bovine sample) had measurable levels of approximately 0.2 μg/g ethyl benzene, which were small compared to the amount added. This background contribution was corrected for the determination of styrene/internal standard peak ratios. Conversion factors were 1 ppm (styrene in air) = 4.11 mg/m^3 = 39.4 nmol/liter, and 1 ppm (styrene in tissue) = 1 mg/kg = 9.58 μmol/kg.

Results

Seven of the eight human adipose tissue samples showed measurable levels of styrene. The range of concentrations was 0.053-2.92 μg/g, with a mean of 1.12 ± 1.06 μg/g. These data are illustrated in Figure 5-1. In comparison, the bovine tissue had a concentration of 0.99 μg/g.

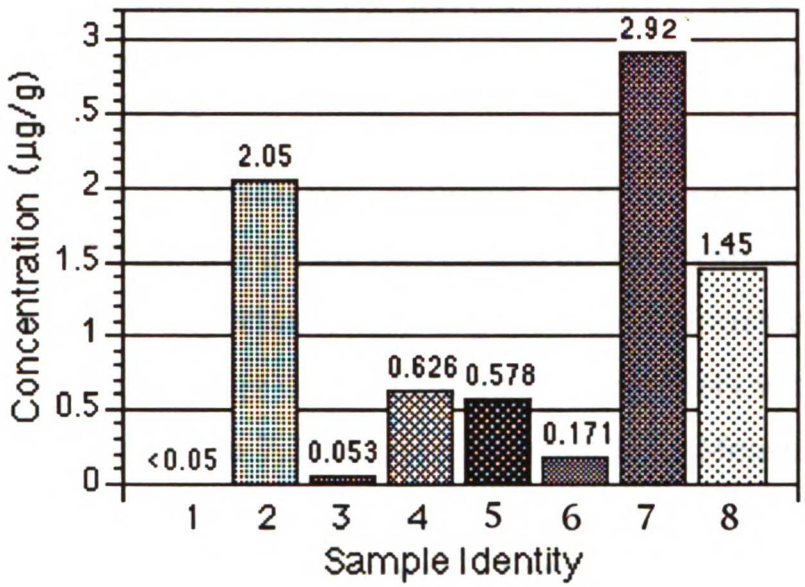


Figure 5-1: Styrene content of human adipose tissue samples.

Discussion

This is the first report, to our knowledge, of measured levels of styrene in adipose tissue of nonoccupationally exposed persons. The three surgical patients (samples #1-3) were interviewed and found to have no occupational sources of styrene exposure; the similarity in the mean level for these samples (0.70 μg/g) and those of the post-mortem samples (1.15 μg/g), strongly suggests that these subjects were also not occupationally exposed. The measured mean concentration of 1.12 μg/g can be compared to a range of 0.3-4.9 μg/g in volunteers experimentally exposed to 2 hours of 50 ppm styrene[71], and to a range of 2.8-11.6 μg/g in workers with ongoing occupational exposure to 7.5-20 ppm styrene[72]. Measurement of styrene in adipose tissue provides a physiologically-damped measure of average exposure. Because the tissue half-life is ca 2-5.2 days[71, 72], short-term exposures are expected to be damped, so that tissue levels represent ongoing sources of exposure.

Inhaled vs. Tissue Concentrations

The concept of "apparent clearance" of styrene can be useful in relating measured tissue concentrations to exposure levels. An expression for apparent clearance from adipose tissue can be derived by first examining the disposition of styrene in blood. Under steady-state conditions:

$$\text{Rate of Input} = CL \times C_{ss}$$

where **Rate of Input** is the average rate of styrene delivery to blood (mg/hour); **CL** is the clearance of styrene from blood (liters/hour); and C_{ss} is the styrene concentration in blood at steady state (ng/g).

The adipose (C_t, ng/g) to blood concentration ratio is defined by the partition coefficient K_p as follows:

$$K_p = \frac{C_t}{C_{ss}}$$

Therefore,

$$\text{Rate of Input} = CL \times \frac{C_t}{K_p}$$

An apparent clearance of styrene, defined as the ratio of blood clearance and partition coefficient, can be measured from the ratio of input rate and tissue concentration:

$$\text{Apparent Clearance} = \frac{\text{Rate of Input}}{C_t} = \frac{CL}{K_p} \qquad (1)$$

Rate of input to the blood from inhalation can be approximated as follows:

$$\text{Rate of Input} = \begin{pmatrix}\text{Inhaled}\\ \text{Concentration}\end{pmatrix} \times \begin{pmatrix}\text{Minute}\\ \text{Volume}\end{pmatrix} \times \begin{pmatrix}\text{Retention}\\ \text{Fraction}\end{pmatrix}$$

Using a "light work" minute volume of 29 liters/min[63], and a retention fraction of 0.65[86], this equation becomes

$$\text{Rate of Input} = \left(\begin{matrix}\text{Inhaled} \\ \text{Concentration}\end{matrix}\right) \times \left(19 \frac{\text{liters}}{\text{minute}}\right) \qquad (2)$$

Assuming that occupationally exposed individuals have reached steady-state (although a decrease in tissue concentration of ca. 33% between Friday afternoon and Monday morning samples has been observed[72]), an apparent clearance of S from adipose tissue can be derived from known inhaled and tissue concentrations of styrene. Engstrom et al.[72], measured levels of 4.7, 7.7, and 11.6 μg/g in Friday afternoon samples of three polymerization plant workers with estimated mean daily uptake values of 193, 558, and 343 mg/day, respectively. Using eq. (1), these data yield a mean apparent clearance of 33.17 ± 15.3 ml/min. Using the Ramsey and Anderson partition coefficient of 39[44], the calculated blood clearance is 1,291.67 ml/min, a value expected for a toxicant that is highly cleared by the liver (blood flow of ca. 1,350 ml/min), and a value in agreement with a calculated clearance of 1,166.7 ml/min from metabolic parameters V_{max} and K_m[44]. Alternatively, use of the adipose tissue/blood partition coefficient of 106 yields a blood clearance of 3,533 ml/min, which is suggestive of high hepatic and extrahepatic extraction of S.

Calculated Daily Dose

Using the apparent clearance value of 33.17 ml/min and the measured tissue concentration of 1.12 μg/g, the rate of input of 2.23 mg/hour, and an "inhaled concentration" of 1.96 mg/m^3 (476 ppb)

can be calculated using Eqs. 1 & 2. This value is two to three orders of magnitude higher than expected from typical inhaled air levels. Wallace has measured breathing zone air concentrations of 0.14-0.94 ppb[82, 83]. Styrene has been measured nearly ubiquitously in indoor air at about 0.24 ppb from three areas in California(Los Angeles-urban, Contra Costa county-rural, Woodland-medium-size agricultural)[87]. Exposure to glues, paint, mothballs, and petroleum products can produce exposures up to 3 ppb[87]. Styrene is an environmental contaminant, with half-lives of 2-4 weeks and 4-30 weeks in soil and subsurface water, respectively[88]. We have measured styrene in coffee leached from a Styrofoam® cup, indicating an ingested dose of ca. 3.5 μg styrene per medium cup of coffee (250 ml). At ca. 10 μg per food portion from a plastic container[7, 8], 6 μg per cigarette[8], and inhalation of about 1 ppb, these sources contribute to styrene dose, but do not fully account for the 2.23 mg/hour rate of input. Indeed, as also reflected in the measurement of styrene in cow adipose tissue, there are many undiscovered sources of environmental exposure to styrene.

Alternatives

There are four potential explanations for these findings. Potential analytical errors were minimized by use of a known method, duplicate analysis of each sample, identical treatment of standards and samples, and mass spectrometry confirmation of S and internal standard peaks. Large error in the measurement of S in

urban air seems unlikely given independent reports producing similar measurements[82, 83, 87]. A third explanation suggests that adipose tissue from differing sites is perfused at different rates. Thus, the gluteal samples used by Engstrom et al.[72] may represent tissue that is more highly perfused, whereas the abdominal samples analyzed in this research may have been less richly perfused. This difference in perfusion rate would lead to differing half-lives of S in adipose tissue, and as such an overestimation of calculated dose based on tissue with a longer S half-life. The fourth possible explanation is that there are major sources of exposure that have not yet been measured. These could include foodstuffs, particularly meats and animal products with high adipose content, where bioconcentration may have increased S content. This explanation is supported by the measurement of 0.99 μg/g S in the bovine tissue sample.

Conclusions

Styrene has been measured in the adipose tissue of non-occupationally exposed individuals. Using the apparent clearance concept, these measured levels correspond to an intake which is two to three orders of magnitude higher than that expected from typical breathing zone concentrations, indicating additional undiscovered sources of exposure to styrene.

Chapter 6
Evaluation of Occupational Exposure Standards

Ideally, evaluation of occupational (and environmental) exposure standards includes sufficient data to quantify the dose/response relationships for all toxicities of concern. An understanding of target site toxicokinetics and toxicodynamics greatly strengthens the basis on which standards can be promulgated. Just as a 15-minute exposure ceiling would be inappropriate in regulating exposure to a carcinogen, a lifetime dose limit may not help protect against short-term CNS depression from a volatile hydrocarbon.

The physiologic model allows simulation of the kinetic disposition of pharmacons and toxicons (and their metabolites) at sites throughout the body, including many locations not normally accessible for tissue sampling. A virtually unlimited number of different exposures (varying doses, exposure routes, physiologic conditions, and test species) can be tested to predict target site toxicokinetics (chapters 3 and 4).

Current occupational exposure standards were challenged by using the physiologic model to evaluate the effects of exposure frequency and minute volume on brain concentration of S. In addition, peak exposure level, duration of exposure, and the product of these factors were tested for the ability to predict the appearance of chromosomal aberrations in chronically exposed workers.

Methods.

Frequency of Exposure.

In occupational exposure, S dosing varies, depending upon ambient S concentration, duration of exposure, and degree of physical exertion. In order to address the variability in ambient occupational levels and its effect on simulated peak brain concentration of S, a sine wave of amplitude 50 ppm (equal to the US 8-hour occupational exposure standard), and a mean of 50 ppm was used in the model (described in chapter 3), to simulate S exposures of 15 and 240 minute sine-wave periods. Both exposures used a minute volume of 30 L/min.

Minute Volume, Exposure Level, Duration and Simulated Brain Concentrations.

To examine the effect of minute volume, exposure level, and duration on the simulated level of S in the brain, a variety of exposure scenarios were simulated using the physiologic model. The rate of S input is given by

$$\text{Dosing Rate} = (\text{Concentration in Air})\,(\text{Minute Volume})\,(\text{Retention Fraction})$$

Retention fraction has been found to be constant over a wide range of inspired concentrations[58, 59, 62, 64], whereas minute volume varies with work load. To measure resulting effects on peak brain concentration of S, simulations included exposures of 10 to 100 ppm and exposure durations of 15, 30, 60 and 120 minutes. The nominal values of minute volume (in L/min) used in these

simulations to represent varying working conditions, (with previous values used in parentheses) were: **Resting** - 7.4 (7.4[63], 9[89], 12.3[60]); **Light Work** - 30 (16.5[89], 29[63], 34.6[60]); **Moderate Work** - 50 (33[89], 50.8[60]); **Hard Work** - 70 (54[89], 60[63], 74.4[60]).

Chromosomal Damage.

Potential predictors of chromosomal aberrations were tested by examining eleven studies contained within six previous reports on S-exposed workers[27, 31, 90-93]. Average exposure level, number of years of exposure, and the product of these two variables, were tested for their abilities to predict chromosomal aberrations, using the Kendall Rank Correlation Coefficient.

Results.

Changes in exposure frequency and resulting increases in brain levels are summarized in Table 6-1; a representative graph of this relationship for a minute volume of 30 L/min is illustrated in Figure 6-1.

Results of the predicted brain concentrations from exposures of 10 to 100 ppm and durations of 15, 30, 60 or 120 minutes, are presented for minute volumes of 7.4, 30, 50 and 70 L/min in Figure 6-2. Figure 6-2 includes a line indicating a brain concentration of 5 mg/L, the apparent threshold above which CNS toxicity begins to appear, as discussed in chapter 3. The apparent discontinuity in

Figure 6-2 B (between 60 and 70 ppm for the 120 and 60 min durations) is likely due to the time interval dt that allows for fluctuation in the calculation of blood concentration.

Table 6-1: Simulated sine wave exposures (mean = 50 ppm, amplitude = 50 ppm) with frequencies of 15 and 240 minutes, and resulting increase in peak brain concentrations.

Condition	Minute volume (L/min)	Sine Wave Period (min)	Peak Brain Level (μg/g)	% Increase in Brain Level[a]
Resting	7.4	15	1.45	
		240	1.89	30%
Light Work	30	15	6.62	
		240	9.92	50%
Moderate Work	50	15	14.79	
		240	19.78	33%
Hard Work	70	15	24.13	
		240	30.06	25%

[a] (Peak with 240 min period - Peak with 15 min period)/ (Peak with 15 min period) • 100%.

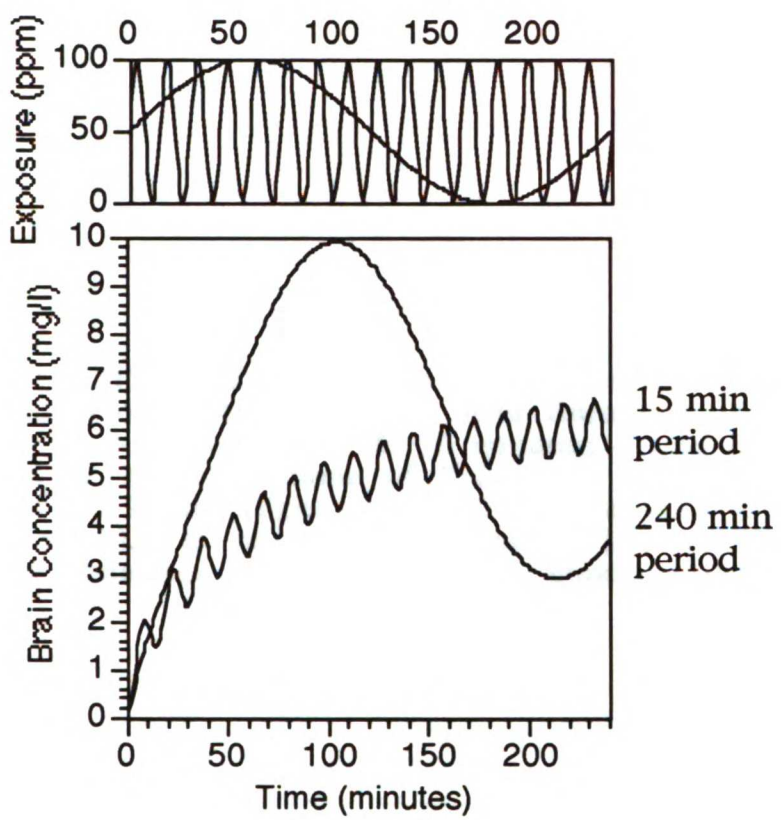
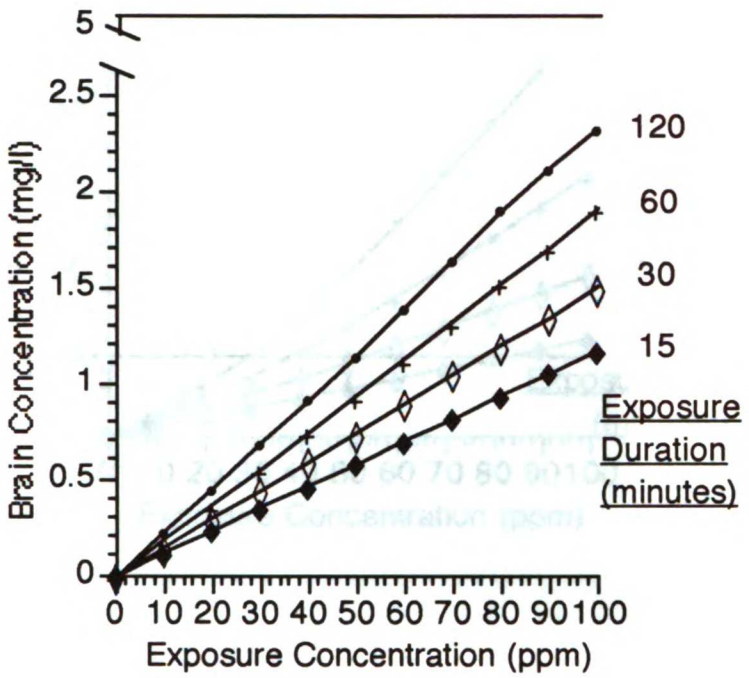


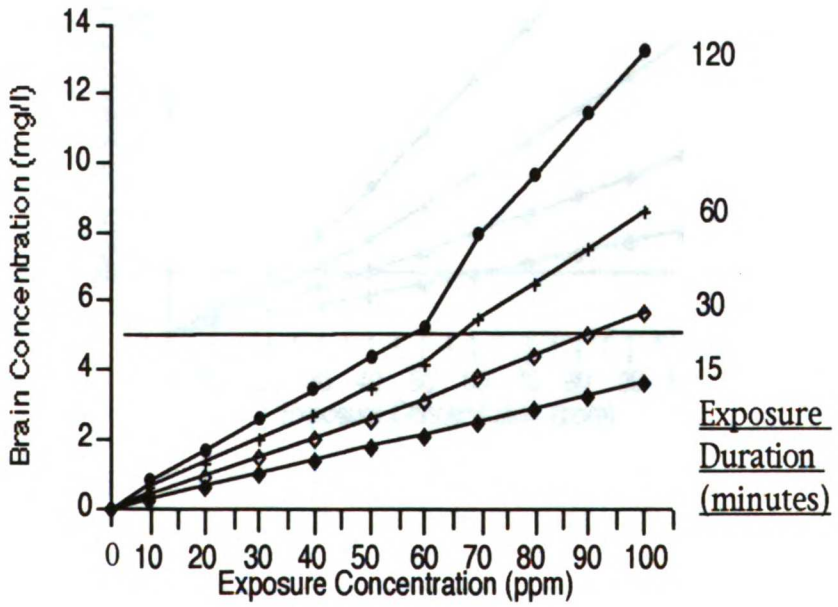
Figure 6-1: Simulated sine wave exposures to styrene (periods of 15 and 240 min) over 240 min (upper graph) and resulting brain concentrations (lower graph) under light work conditions (minute volume = 30 L/min).

Figure 6-2: PBTK model-simulated brain concentrations of styrene at exposures of 10 to 100 ppm. Four durations of exposure are represented: ♦ : 15 minutes; ◊: 30 minutes; + : 60 minutes; • : 120 minutes. A: Minute volume of 7.4 L/min (resting); B. Minute volume of 30 L/min (light work); C: Minute volume of 50 L/min (moderate work). D. Minute volume of 70 L/min (hard work). The solid line indicates a simulated brain concentration of 5 mg/L.

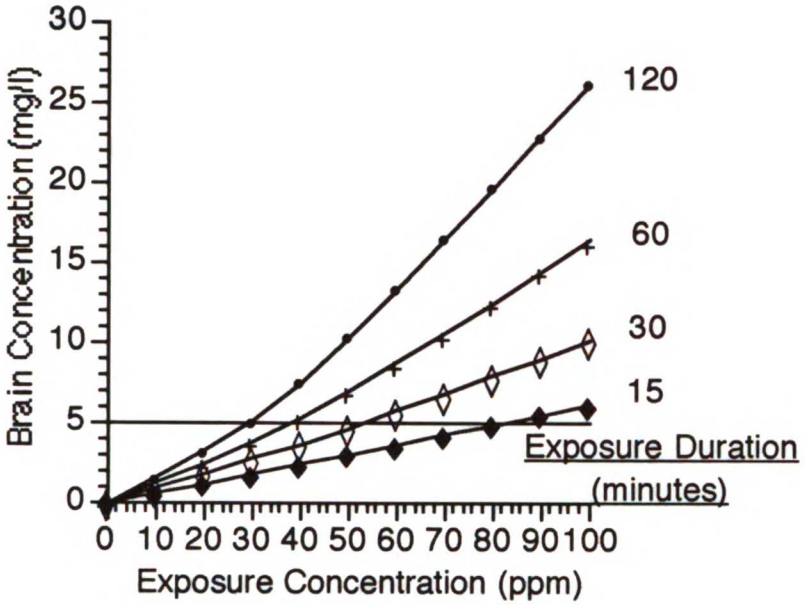
A - Resting



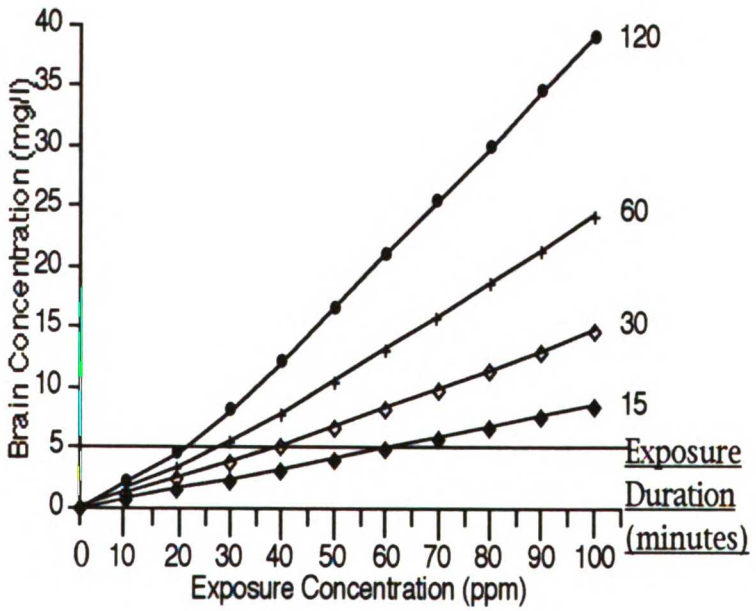
B - Light Work



C - Moderate Work



D - Hard Work



Chromosome Aberrations.

The results of the correlation of average daily exposure level, duration (years) of exposure, and average exposure•duration with the presence of chromosome aberrations are presented in Figure 6-3.

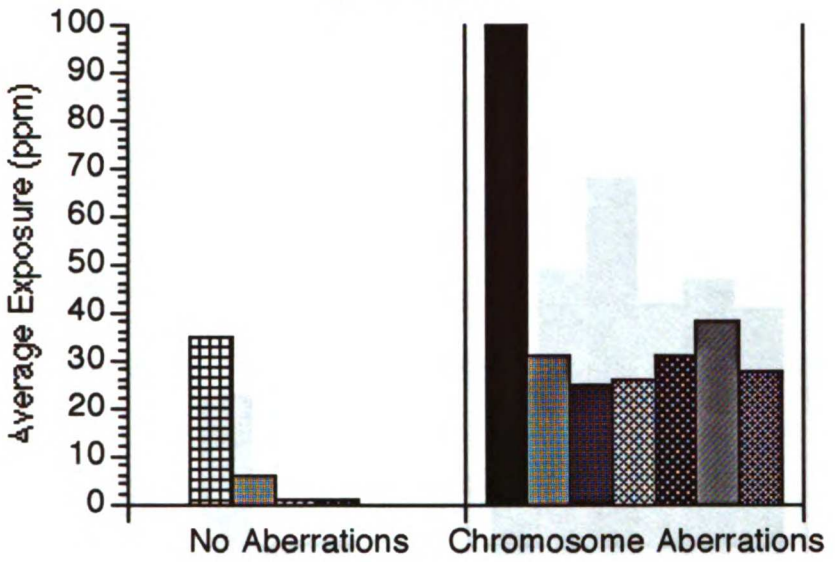
Discussion

Toxicodynamics.

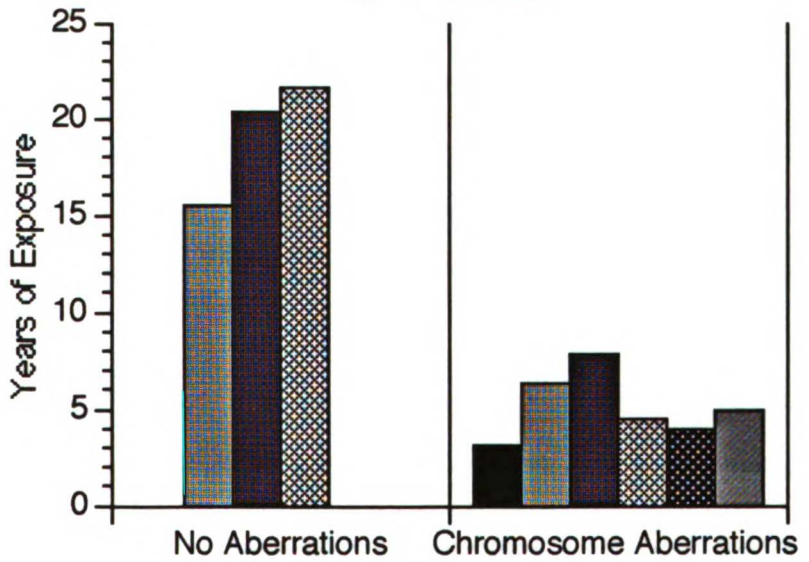
Three principal toxicodynamic parameters can be used to assess the effect of exposure to toxic substances. As examples, the peak level of an anesthetic agent in the brain is often associated with central nervous system changes; the duration of exposure for selected chemotherapeutic agents in treating cancer is an important treatment parameter[94, 95]; and the area under the concentration-time curve (AUC) may be the most important parameter for certain forms of chronic toxicity, including, for example, pack-years of smoking for lung cancer[96].

Figure 6-3: Correlation of chronic styrene exposure conditions with the presence of chromosome aberrations from 11 previous studies[27, 31, 90-93]. (Note that each bar represents one study, and that Fig. 6-3A presents two studies [farthest left bar of each of no aberrations and aberrations] that reported only average exposure.) Tau is the Kendall Rank Correlation Coefficient, and P is the probability that no association exists. A: Average Workplace Exposure (ppm) during working hours; B: Years of Exposure; C: Average Exposure (ppm)•Years.

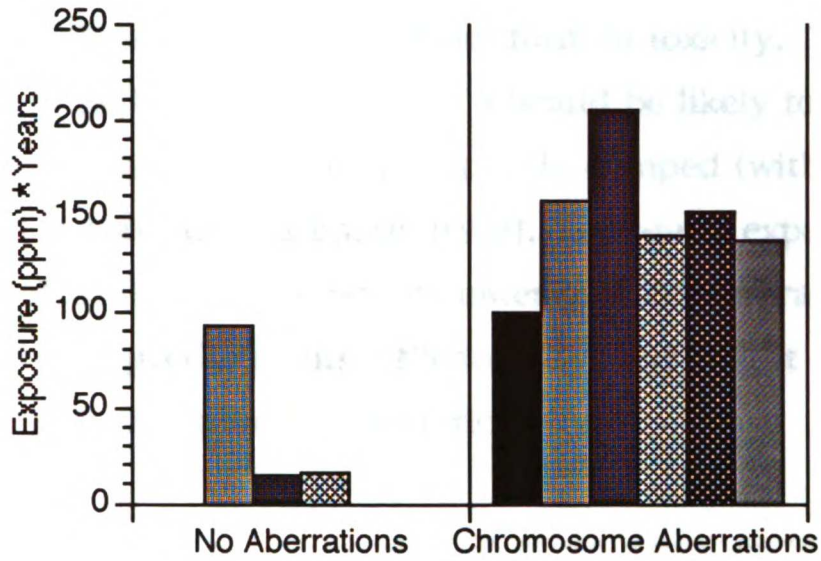
A. Average Workplace Exposure (ppm). Tau = 0.47, p = 0.045



B. Years of Exposure. Tau = 0.71, p = 0.008



C. Avg.•Years. Tau = -0.71, p = 0.008



Which of these parameters should be used depends on exposure frequency, target tissue damping, and the relevant toxicodynamic mechanism for a given form of toxicity. Thus, acute exposures to high concentrations of S would be likely to cause CNS impairment because brain tissue is poorly damped (with respect to blood, and in contrast to adipose tissue). Long-term exposure to the same total dose (and corresponding lower peak concentration) would be unlikely to produce this CNS toxicity, but might instead be associated with chromosomal aberrations.

Acute Toxicity From Occupational Exposure.

Simulated peak brain concentrations appear to be a predictor of acute toxicity (as discussed in chapter 3), suggesting that high levels of S in brain tissue correlate with CNS effects such as balance impairment, prolonged reaction times, listlessness and headache[19] [24]. These effects may simply be due to a structurally nonspecific induction of CNS impairment, as described by the Ferguson principle [75].

As seen in Figure 6-2, three principal factors contribute to the peak concentration of S in brain tissue: inhaled concentration, minute volume, and duration of exposure. Using a S brain concentration of 5 mg/L as a guideline, the combinations of these factors producing toxicity can be calculated (Table 6-2).

Table 6-2: Variation in exposure concentration (10 to 100 ppm), minute volume (rest-7.4, light work-30, moderate work-50 and hard work-70 L/min), and duration of exposure (15, 30, 60 and 120 min), and expected CNS toxicity. ϕ indicates no expected toxicity (brain level < 5 mg/L), and shaded areas indicate expected toxicity (brain level > 5 mg/L). STEL is the US. Short-Term Exposure Limit (100 ppm over a 15-min period). Existing US. and California state 8-hour average standards (50 and 100 ppm, respectively) are indicated at the bottom of each table. Simulations were carried out to 120 minutes, at which time steady state is nearly achieved.

Rest - Minute Volume of 7.4 L/min.

Duration (min)	Exposure (ppm)									
	10	20	30	40	50	60	70	80	90	100
15-STEL	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ
30	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ
60	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ
120	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ

US. Limit CA Limit

Light Work - Minute Volume of 30 L/min.

Duration (min)	Exposure (ppm)									
	10	20	30	40	50	60	70	80	90	100
15-STEL	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ
30	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ		
60	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ				
120	ϕ	ϕ	ϕ	ϕ	ϕ					

US. Limit CA Limit

Moderate Work - Minute Volume of 50 L/min.

Duration (min)	Exposure (ppm)									
	10	20	30	40	50	60	70	80	90	100
15-STEL	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	;	;
30	ϕ	ϕ	ϕ	ϕ	ϕ	;	;	;	;	;
60	ϕ	ϕ	ϕ	;	;	;	;	;	;	;
120	ϕ	ϕ	;	;	;	;	;	;	;	;

US. Limit CA Limit

Hard Work - Minute Volume of 70 L/min.

Duration (min)	Exposure (ppm)									
	10	20	30	40	50	60	70	80	90	100
15-STEL	ϕ	ϕ	ϕ	ϕ	ϕ	ϕ	;	;	;	;
30	ϕ	ϕ	ϕ	;	;	;	;	;	;	;
60	ϕ	ϕ	;	;	;	;	;	;	;	;
120	ϕ	ϕ	;	;	;	;	;	;	;	;

US. Limit CA Limit

Table 6-2 indicates that both the California standard (Cal-OSHA, 100 ppm time-weighted average [TWA]) and US federal standard (OSHA, 50 ppm) are protective against acute CNS toxicity at a resting minute volume. However, there are conditions, as shaded in Table 6-2, where acute CNS toxicity would be expected at higher minute volumes. Average minute volume for S-exposed workers manufacturing reinforced polyester boats has been estimated at 20 L/min[97].

Variations in frequency of exposure, at the same average exposure level, can also have a marked effect on peak brain level, as seen in Table 6-1 and Figure 6-1. At a light work breathing rate, the peak brain level was 25-50% higher in the lower vs. higher frequency exposure (240 vs. 15 min periods, respectively).

These results support the hypothesis that minute volume and exposure frequency can have an important effect on acute CNS toxicity from S exposure. This suggests that occupational standards should be reexamined with respect to working conditions, especially minute volume and frequency of exposure. Moreover, these conclusions are predicated on the response of a group of young, healthy volunteer subjects. The 300,000 people occupationally exposed to S could include individuals more sensitive to CNS toxicity.

In this investigation, the physiologic model has proved to be an insightful tool to measure target site toxicokinetics. In contrast to other models, the physiologic model has allowed for a correlation of target site-specific kinetics with dynamics.

Chronic Toxicity From Occupational Exposure.

The three most widely reported forms of toxicity reported for S exposure are mucous membrane irritation and CNS effects from acute exposure, and chromosomal abnormalities from chronic exposure (chapter 1). While a number of examinations of S-exposed workers have found CNS-related toxicity, such as increased reaction times, "disturbances of the nervous system"[54], and logical memory decrements[22], it is difficult to separate effects from acute and chronic exposure in this population. Long-term neurologic damage from S exposure has not as yet been clearly identified. Moreover, in a recent investigation of retired aircraft painters exposed to dichloromethane, it was found that nervous system function returned to normal after cessation of exposure[98].

Occupational exposure to S has also been found to depress δ-aminolevulinate dehydratase (ALA-D) levels in erythrocytes[35]. Authors of this work suggest that the obligate reactive intermediate, styrene-7,8-oxide, may react with ALA-D or a precursor in the bone marrow. The presence of decreased ALA-D concentrations in bone marrow and erythrocytes, but not in hepatic tissue, suggests that rapid sequential metabolism of S to SO and then to styrene glycol occurs more efficiently in hepatic vs. bone marrow tissue. Similarly, varied results of S mutagenicity in different in vitro and in vivo test systems[9] may be explained by the ratio of SO forming (P-450) and detoxifying (epoxide hydrolase, glutathione-S-transferase) activities in various tissues. This issue is more fully discussed in chapter 7.

The data in Figure 6-3 suggest that the presence of chromosome aberrations has a stronger association with total dose (Exposure•Years of exposure), than either of these variables independently. Figure 6-3B suggests an unexpected reciprocal relationship between duration of exposure and the presence of chromosomal damage. A possible explanation of these observations is the shifting of workers with seniority to lower-exposure jobs; this explanation is supported by an apparent reciprocal relationship between average exposure and duration of exposure, as seen in Figure 6-4.

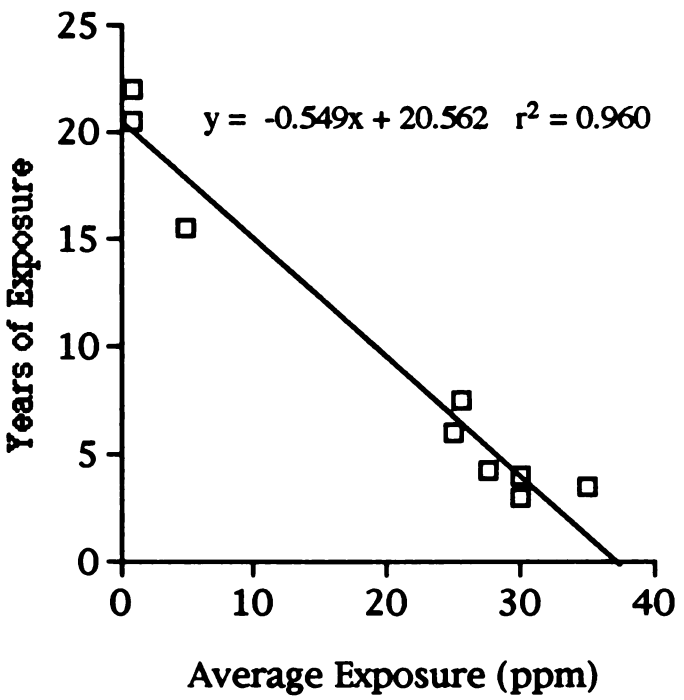


Figure 6-4: Relationship between average exposure level and duration of exposure among styrene workers. The correlation coefficient (R^2) of 0.960 indicates the strength of association.

An alternative explanation is the difference in aberration-detection methodologies. Norppa et al.[31] tended to examine workers with shorter durations of exposure (7.50 ± 6.41 years) and reported more aberrations. Thiess[90] and Thiess et al.[92] tended to examine workers with longer exposures (17.10 ± 2.77 years) and reported fewer aberrations. One study (the high-exposure group of Thiess[90]), criticized by other authors[31], was removed as a potential outlier due to possible under-reporting of aberrations.

On the basis of the relationship between total exposure and aberrations presented in Figure 6-3C, a value of 100 ppm•years appears to divide studies reporting the absence or presence of aberrations. While both the most recent exposure history and the half-life of chromosome aberrations play roles in the use of this toxicity marker, these results suggest that a threshold delimits chromosome damage in workers. Therefore, in addition to protection from acute CNS effects, as discussed above, regulations must protect workers from chronic toxicity, for example, by restricting total exposure to less than 100 ppm•years. This corresponds to an average lifetime exposure during work hours of 2.5 ppm (assuming 40 years of work).

Implementation of New Standards.

Much like the dual standards used to protect workers from radionuclide exposure, acute and chronic standards are needed to prevent toxicity from S exposure. Table 6-2 provides guidelines for acceptable exposure concentrations, durations, and working conditions (minute volume). Thus, a 50 ppm standard would be expected to be protective of CNS toxicity in a job requiring light work. Maximum exposures under moderate work conditions would be 80 ppm for 15 min, 50 ppm for 30 min, 30 ppm for 60 min, and 20 ppm for 120 min. Hard work conditions would be limited to 60 ppm for 15 min, 30 ppm for 30 min, and 20 ppm for 120 min.

The suggested chronic exposure standard is a total dose of 100 ppm•years. This could be monitored by yearly evaluations of exposure. For example, an individual working at a job with an average of 10 ppm for a year will have accumulated 10% of a total lifetime dose. Options for reassignment to jobs with higher or lower levels of exposure should then be evaluated.

Chapter 7

Sequential Metabolism of Styrene and Implications For Toxicity

Kinetic and metabolic observations from S and SO exposures can provide insight into the difference in toxicities from administration of each of these substances. While the acute CNS effects from S exposure (chapters 1 and 3) are likely due to the parent compound, irreversible plasma protein binding and chromosome aberrations appear to be related to the presence of the metabolite SO (chapters 1 and 6). The toxicokinetics of SO at potential target sites may thus provide substantial insight into S and SO toxicodynamics. The focus of this chapter is on SO kinetics following S and SO dosing.

It is of interest to consider why the principal obligate metabolite of S is considered a human carcinogen, while the parent compound shows a much weaker association with carcinogenesis (chapter 1). As observed previously, "*This ability of epoxides to alkylate or arylate nucleic acid, thereby presumably leading to germinal or somatic mutations, is the major concern in the question of intermediary metabolism of styrene.*"[2].

Styrene Clearance and Half-lives in Rats

The toxicokinetic properties of S are of primary interest in understanding its toxicity because they provide essential information regarding the intensity and duration of exposure. Blood clearance and apparent half-lives describe how the body distributes and eliminates S, providing information about peak tissue levels and duration of internal exposure.

More than any other kinetic parameters, clearance and half-life values can give an instant picture of the kinetics of an exogenous (or endogenous) substance. Blood clearance is defined as the volume of blood completely cleared of S per unit time. Clearance indicates the rate at which the body eliminates that substance relative to blood concentration, and can suggest which organ is primarily responsible for elimination. A value of blood clearance approaching hepatic blood flow for a metabolized drug suggests removal of most of the drug entering the liver; the value of renal clearance suggests how the drug is handled by the kidney. Half-lives give information about the rapidity of kinetic events, for example, how long it takes to reach a steady-state concentration during constant input, and how long it takes to eliminate a substance from the body.

Rodents have been used to assess S and SO toxicities. To be predictive of potential human effects, an animal model must demonstrate kinetic similarities. In this chapter, S and SO kinetics are evaluated in rats. Following administration of S or SO, S, SO, and styrene glycol (SG) were measured in venous blood. The physiologic model (chapter 3) was modified as described below to simulate S and

SO disposition in the rat, and the resulting clearance and half-life values were compared with the values obtained in vivo.

Materials and Methods.

Animal Protocol.

Male Sprague-Dawley rats, weighing between 250 and 400 g, were lightly anesthetized with ether until loss of sternal recumbancy and subsequently given 45 mg/kg intraperitoneal (i.p.) doses of sodium pentobarbital. After 10 minutes, a single 1-10 mg/kg dose (10-100 μl, determined by weighing injection syringe before and after administration) of S or SO in 70% ethanol (required to keep S in solution), was injected intravenously over 15 seconds into a rat penile vein. Blood samples of 100 μl were taken from a tail vein after immersion of the tail in warm water and superficial incision across the vein.

Upon response to hind leg or tail pinch tests, or after 75 minutes, animals were given an additional 9 mg/kg i.p. dose of pentobarbital. Maintaining anesthesia with repeated administrations, without seriously jeopardizing the survival of the rats, limited sampling time to 200 minutes. Five rats were used for two studies, for which injections were separated by at least four weeks. Following final blood collection, animals were euthanized with 200 mg/kg pentobarbital injections and bilateral thoracotomy. This protocol was approved by the UC San Francisco Committee on Animal Research.

Sample Handling and Analysis.

Venous blood samples (collected in capillary tubes), were flushed into 2 ml vials containing 100 μl of a solution of ethyl benzene (EB, 1 μg/ml) in 5% ethanol. After vortexing, 1 ml of headspace air was removed and injected into the gas chromatograph for S analysis, as described in chapter 3.

Because SO is unstable in blood (88-min degradation half-life at room temperature) it was extracted with pentane (1 ml) within 5 minutes of blood sampling. The pentane phase was transferred to a tube containing 1 ml of 0.5 M H_2SO_4 to hydrolyze the SO to SG[59]. Allyl benzene glycol, prepared using the method of Duverger-Van Bogaert et al.[99], was used as the internal standard. The sample was then hydrolyzed, extracted with ethyl acetate, evaporated to dryness and reconstituted in 0.5 ml toluene with 0.4% pyridine. Subsequent derivatization was accomplished with the addition of one μl of pentafluorobenzoyl chloride and incubation at 60°C for 20 minutes. The sample was then evaporated to dryness and reconstituted in 200 μl of 90% methanol and 400 μl of pentane.[99] Styrene glycol (SG), which remained in the aqueous phase, was extracted with 1 ml ethyl acetate, followed by evaporation and derivatization as described above.

One μl of the pentane phase was injected into the gas chromatograph (described in chapter 3) using a Supelco (Bellafonte, PA) SBP-5 column (30 m, 0.32 mm ID) and an electron capture detector at an oven temperature of 230 °C (injection port and

detector temperatures were 220 and 250 °C, respectively). Retention times were 7.4 and 9.2 min for the SG and allyl benzene glycol derivatives, respectively. Sample handling and analysis procedures are outlined in Figure 7-1.

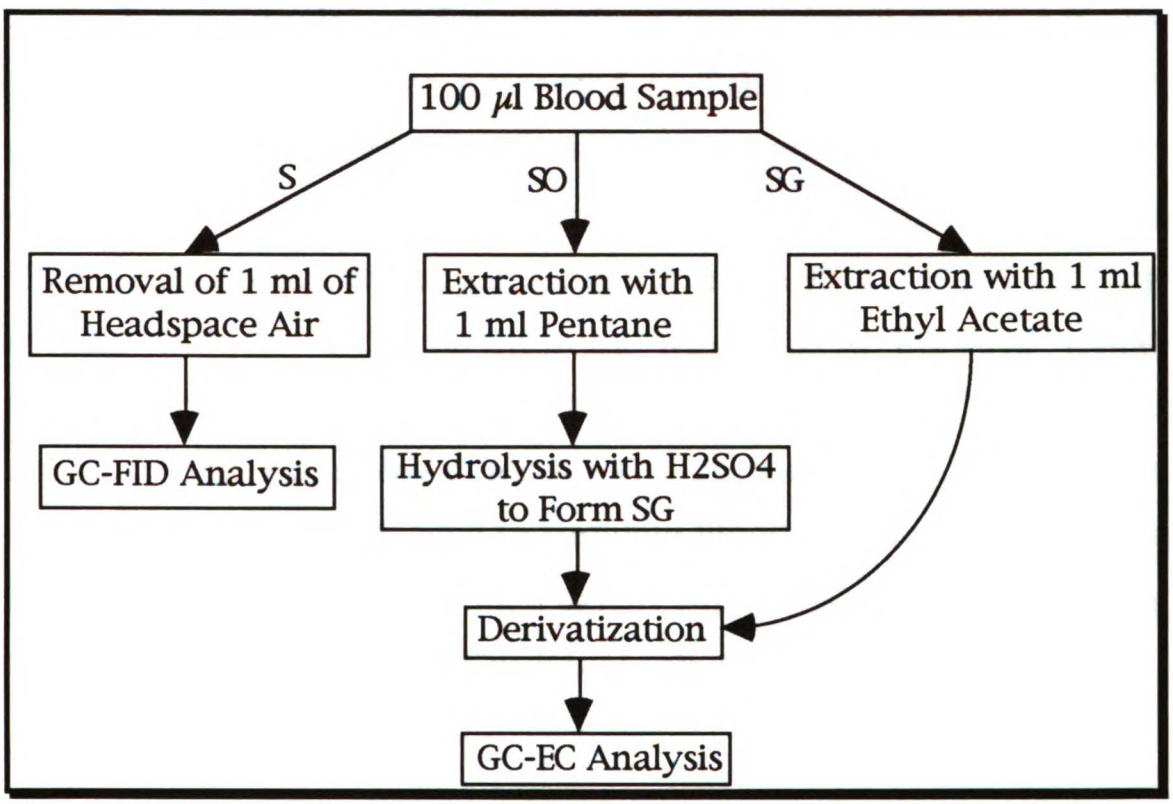


Figure 7-1: Sample handling and styrene oxide/glycol analysis scheme. GC-FID indicates gas chromatography with flame ionization detection, and EC indicates electron-capture detection.

Physiologic modeling.

The human S physiologic model (chapter 3) was modified for the rat by integrating the brain compartment into the well-perfused tissue compartment (as CNS effects were no longer studied), and replacing tissue volumes and blood flows with values for the rat. As in the human model, all S metabolism was assumed to occur in the liver. Model values are presented in Table 7-1. Arterial blood concentrations were simulated in the blood pool compartment. To simulate venous tail vein sampling in the rats, the model combined 1% of venous blood draining adipose tissue and 99% of blood draining muscle tissue. This partitioning was chosen because visual examination of the tail revealed very little adipose tissue. In contrast, the physiologic model for humans used a 15%/85% adipose/muscle partitioning to simulate blood samples taken from a forearm vein (chapter 3), which drains superficial tissues containing a larger proportion of fat. The actual ratios were arbitrarily chosen.

Styrene Oxide Model.

To obtain tissue/blood partition coefficients for SO, existing S partition coefficients were adjusted by the relative water solubilities of S and SO. Styrene oxide is 10 times more soluble than S in water (0.3 vs 0.03%). Accordingly, partition coefficients for SO were calculated by reducing the "lipid solubility component" of each tissue by 10. Using SO solubility in blood as a reference (coefficient of 1), the lipid component was estimated as the S partition coefficient

(Kp,s) minus one. Thus, SO tissue/blood partition coefficients were calculated from

$$K_{p,so} = \frac{K_{p,s} - 1}{10} + 1$$

In contrast to S (chapter 3), SO is metabolized in many tissues[100-102]. The disposition of SO was simulated using a model similar to that used for S. This model was linked to the S model through liver compartments so that SO created in the S model could be instantaneously input into the SO model (Fig. 7-2). This allowed for the study of SO disposition after both S and SO administration.

Styrene oxide is metabolized by two primary pathways in rats: hydrolysis to the glycol via epoxide hydrolase and glutathione addition mediated by glutathione-S-transferase (GST)[10]. The latter pathway may account for about 10% of S metabolism (when a single 250 mg/kg i.p. injection was given to 5 rats, mercapturic acids in urine accounted for 10.5% of the total dose[103]). To include the contribution of both of these pathways to metabolic activity, in vitro results were incorporated into the model. Cantoni et al.[100], Salmona et al.[101], and Pacifici et al.[102] measured enzyme activities in vitro using SO as substrate. In vitro measurements of epoxide hydrolase and glutathione-S-transferase activities in various tissues are presented in Table 7-2. The use of these values in the model assumes that a larger fraction of SO is metabolized by GST, in contrast to the in vivo observation cited above[103].

Table 7-1: Blood flows, partition coefficients, organ volumes, and metabolic activities used in the rat physiologic model for styrene. All values from Ramsey and Anderson[44] unless otherwise noted.

Tissue	Q_t (ml/min)	K_p	V_t (ml)
Adipose	39.6[a]	106[b]	27
Blood	-	1	21
Liver	35.2	2.7	12
Muscle	28.3[a]	1	219
Well-Perfused[c]	39.2	5.7	15
V_{max}: 0.06 mg/min		K_m: 0.36 mg/Liter[d]	

Table notes: [a]Fit to observed data . [b]Determined as described in text. [c]Includes brain, heart, kidneys, lungs, adrenal and thyroid glands, and skin (without subcutaneous fat); liver is considered separately. [d]Based on S concentrations in hepatic venous blood.

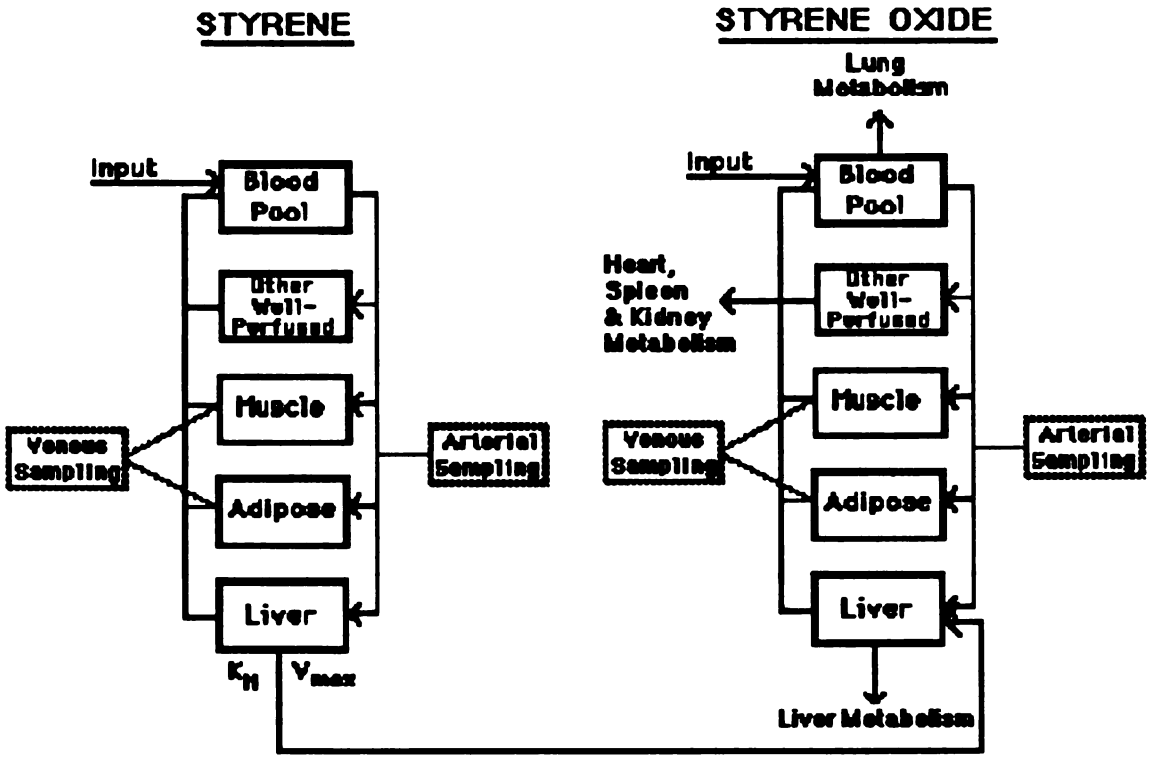


Figure 7-2: Linked physiologic models for styrene and styrene oxide in the rat. Styrene oxide generated by styrene metabolism in the liver (left model) is immediately input to the liver compartment of the styrene oxide model (right) for metabolism in liver, heart, spleen, kidneys, and lungs.

Table 7-2: In vitro measurements of epoxide hydrolase and glutathione-S-transferase activities in various male rat tissues using styrene oxide as substrate.

Heart	Spleen	Kidneys	Lungs	Liver	Reference
Epoxide Hydrolase Activity (nmol product/mg protein-min)					
2.42	1.39	3.11	1.63	8.66	[100]
<0.1	0.30	0.71	<0.1	6.10	[101]
-	-	1.3	0.6	5.2	[102]
Glutathione-S-transferase Activity (nmol product/mg protein-min)					
-	-	67	12.3	87	[102]

In order to use the SO in vitro enzyme activity measurements in the physiologic model, these measurements (Table 7-2) were scaled using the Michaelis constant K_m and maximum reaction rate V_{max} for S metabolism (S to SO) suggested by Ramsey and Anderson[44] (Table 7-1) to occur in rats. In the physiologic model used, the liver was conceptualized as a well-stirred compartment in which enzyme activity can be expressed as the intrinsic clearance, CL_{int}. Stated mathematically, $CL_{int} = (V_{max})/(K_m + C)$, where C is the concentration in the well-stirred compartment as reflected by the venous blood leaving that compartment. At low concentrations ($<< K_m$), the CL_{int} is approximately equal to V_{max}/K_m.

By multiplying the known V_{max}/K_m ratio in rats (167 ml/min, Table 7-1) for S metabolism by the epoxide hydrolase or GST activity (Table 7-2) for each tissue, and dividing by the P450 IIE1 activity (3.275 nmoL/min-mg protein[100, 101]) for S metabolism, a relative intrinsic clearance at low concentrations for the EH or GST activity in each tissue can be obtained. Values used in the rat SO model, including tissue metabolic activities, are presented in Table 7-3.

Table 7-3: Styrene oxide parameters, including blood flows, partition coefficients, organ volumes, and metabolic activities used in the rat physiologic model (300 g rat). All values from Ramsey and Andersen[44] unless otherwise noted.

Tissue	Q_t* (ml/min)	K_p[a]	V_t** (ml)	Epoxide Hydrolase activity (mg/min)	GST[b] activity (mg/min)
Adipose	39.6[c]	11.5	27	0	0
Blood	-	1	21[d]	0.04[e]	0.627[e]
Liver	35.2	1.17	12	0.34	4.44
Muscle[f]	28.3[c]	1	219	0	0
Well-Perfused[g]	39.2	1.47	15	0.088	3.42

Table notes: *Blood flow to tissue. **Volume of tissue. [a]Adjusted from Table 7-1 values as described in text. [b]Intrinsic clearance of SO by glutathione-S-transferase. [c]Fit to observed data. [d]Estimated as 7% of a 300 g rat. [e]Metabolism in lung. [f]Includes skin and other poorly-perfused tissues. [g]Includes brain, heart, kidneys, lungs, adrenal and thyroid glands; liver is considered separately.

Toxicokinetic Parameters.

Clearance.

Where applicable, monoexponential decline curves of the form $C = C_o * \exp(-kt)$ were fit to the concentration-time data in the rats by the least squares method. For these curves, area under the curve (AUC) was estimated from C_o/k. For biexponential declines, the area was estimated by dividing the overall curve into two regions in which monoexponential declines were evident. The AUC was calculated from $(C_1 - C_2)/k_1 + C_2/k_2$, where C_1 and C_2 are the concentrations at time zero (extrapolated value) and at the beginning of the second decline. The values of k_1 and k_2 are the slopes of the declines of the respective curves on a semilogarithmic scale.

The model-predicted clearance was derived in two ways: 1) by simulating an i.v. dose of 7.5 mg/kg, determining the AUC, and using the expression CL = Dose/AUC; and 2) by simulation of exposure to a nominal constant rate of infusion R_o, (0.004 mg/min-kg), determination of the resultant steady-state concentration C_{ss}, and use of the expression $CL = R_o/C_{ss}$.

Half-lives.

Half-lives ($t_{1/2}$) in individual rat experiments were determined by fitting mono- or bi-exponential decline curves of the form $C = \sum_i C_{oi} \cdot \exp(-k_i t)$ (where C_{oi} is the S blood concentration at the beginning of each phase and k_i is the corresponding elimination rate constant), and using the expression $t_{1/2} = \ln 2/k$. As in the human studies, the

simulated slopes were not constant with time. To evaluate the model behavior, instantaneous half-lives ($t_{1/2,instantaneous}$) were calculated from the fractional rate of decline ($k_{instantaneous} = (dC/dt)/C$) of the blood concentrations, using the relationship $t_{1/2,\ instantaneous} = \ln 2/k_{instantaneous}$.

Volume of Distribution.

The initial volume of distribution of S in rats was assessed by $V = Dose/C_o$, where C_o is the extrapolated zero time value from the equation $C = C_o exp(-kt)$, which was fit to the early time points of each animal.

Results.

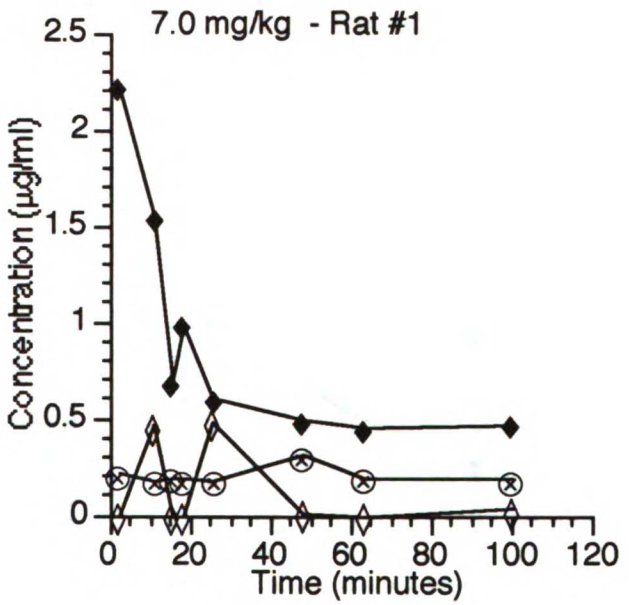
Styrene-Dosed Animals.

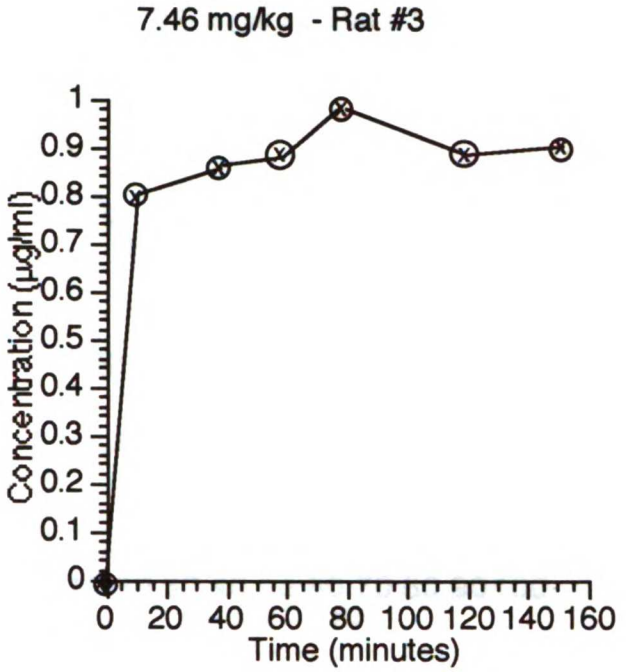
Concentration-time data.

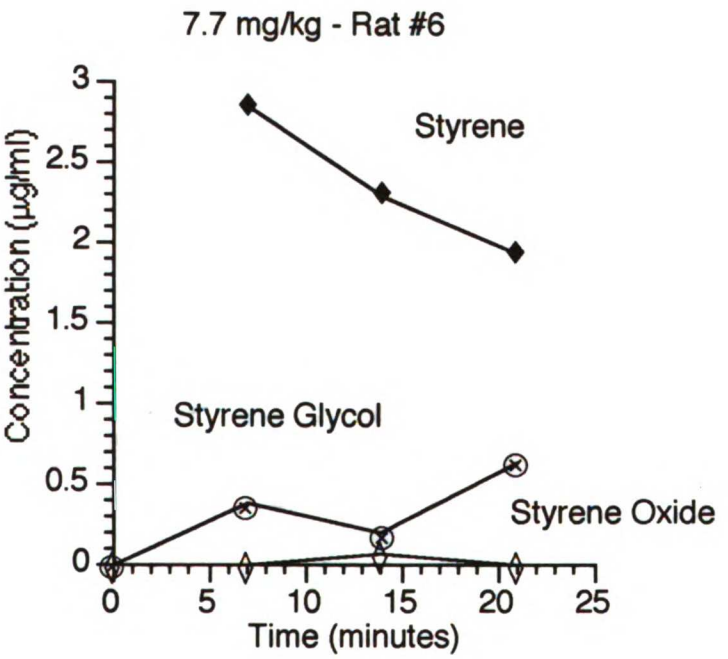
Styrene was given to rats #1, 3, 6, 7, 7B (where B indicates the reuse of an animal), 9, 11, 12, 13, and 14, at a nominal dose of 5-10 mg/kg. Concentration-time plots for S-dosed rats, including analysis for S, SO and SG, are presented in Figure 7-3. For three of the rats (#7B, 9, and 12), an increasing concentration of S with time suggested that doses were administered extravascularly to these animals. These apparent extravascular administrations reflect the difficulty of delivering S directly into the penile vein. Assuming minimal metabolism in the tissues surrounding the penile vein, the

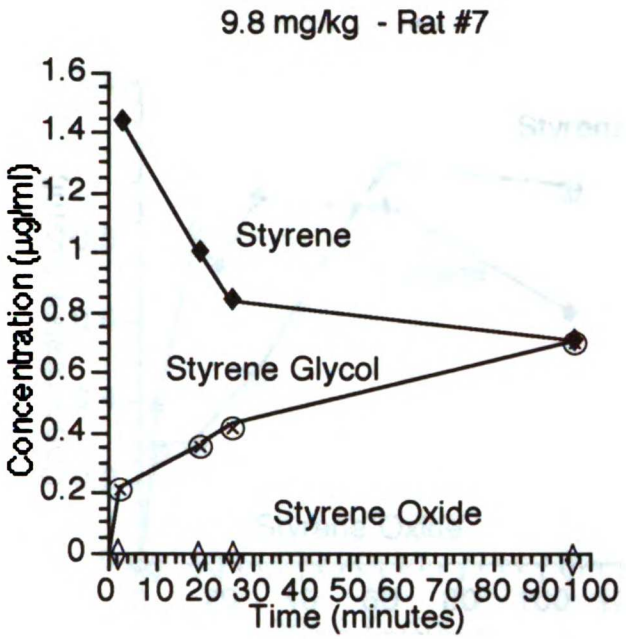
extravascular doses were assumed to completely enter the systemic circulation (F=1). Administration of S resulted in a corresponding immediate appearance of SG, while the intermediate metabolite SO was not detected.

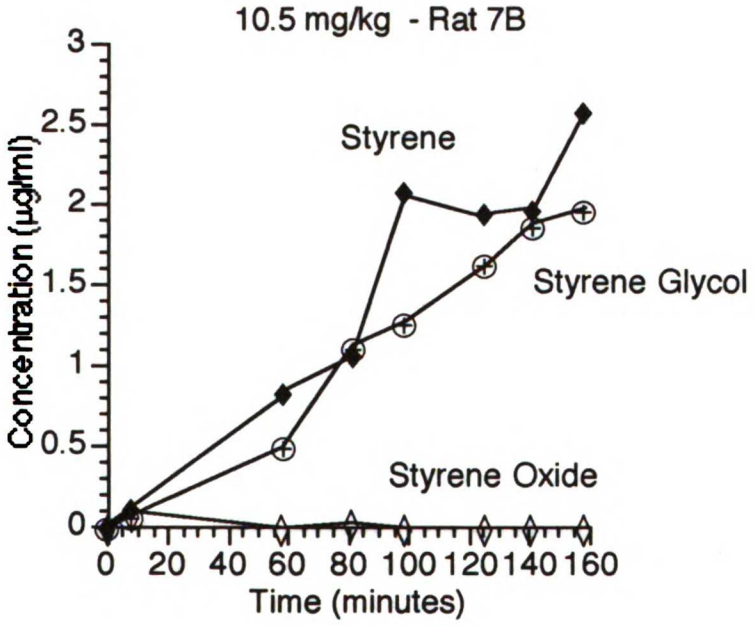
Figure 7-3: Styrene, styrene oxide, and styrene glycol in blood following nominal i.v. administration of styrene to male Sprague-Dawley rats ("Rat _B" indicates reuse of an animal). (Note that both x and y scales vary from graph to graph.) Styrene levels in Rat #3 were measured at ca 50x higher than those seen in other rats. This was assumed to be due to a source of contamination, and styrene data were not included for this animal. Key: Styrene, -♦-; Styrene Oxide, -◊-; Styrene Glycol, -⊗-.

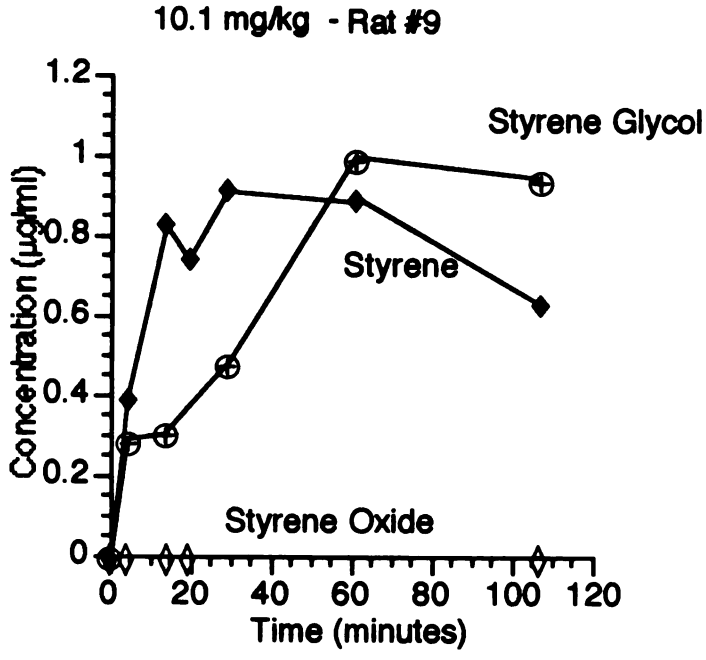


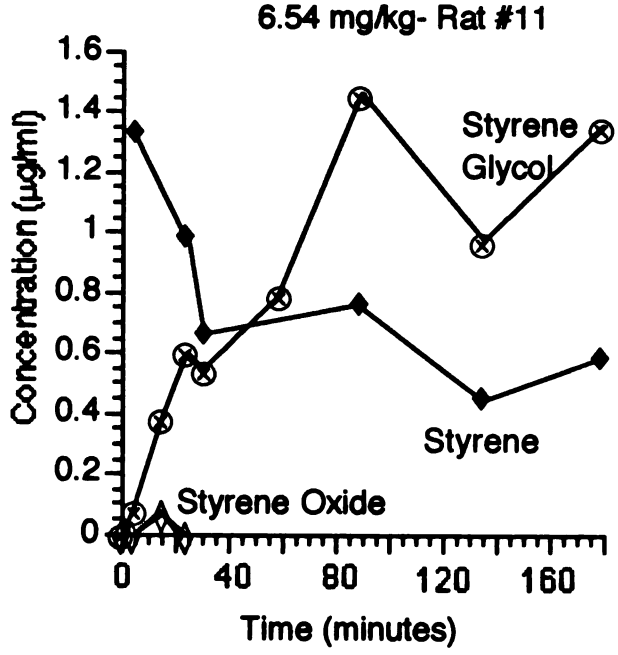

7.46 mg/kg - Rat #3
Concentration (µg/ml)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 20 40 60 80 100 120 140 160
Time (minutes)

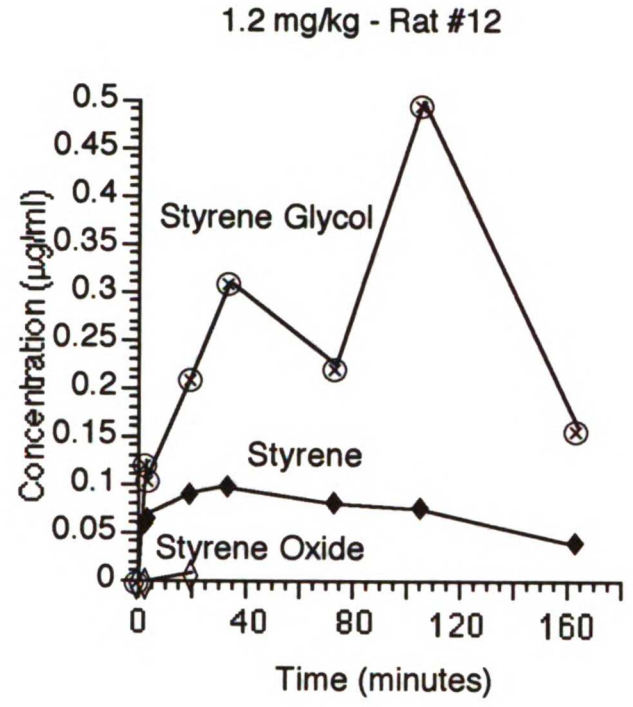

7.7 mg/kg - Rat #6
Concentration (µg/ml)
3
2.5
2
1.5
1
0.5
0
Styrene
Styrene Glycol
Styrene Oxide
0 5 10 15 20 25
Time (minutes)

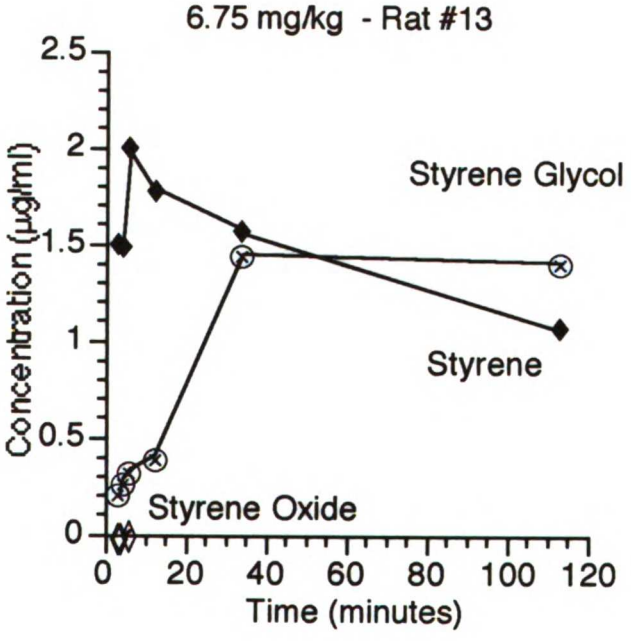

9.8 mg/kg - Rat #7
Concentration (µg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Styrene
Styrene Glycol
Styrene Oxide
0 10 20 30 40 50 60 70 80 90 100
Time (minutes)

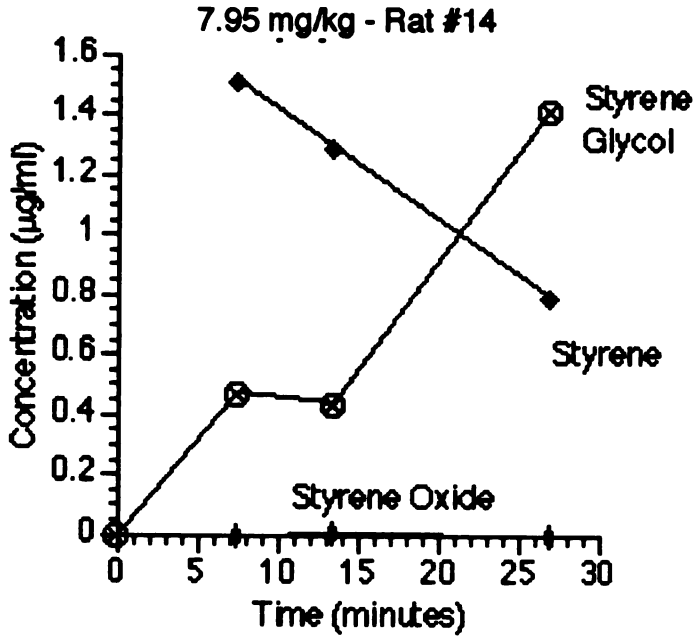

10.5 mg/kg - Rat 7B
Concentration (µg/ml)
3
2.5
2
1.5
1
0.5
0
Styrene
Styrene Glycol
Styrene Oxide
0 20 40 60 80 100 120 140 160
Time (minutes)


10.1 mg/kg - Rat #9
Concentration (µg/ml)
1.2
1
0.8
0.6
0.4
0.2
0
Styrene Glycol
Styrene
Styrene Oxide
0
20
40
60
80
100
120
Time (minutes)


6.54 mg/kg- Rat #11
Concentration (µg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Styrene Glycol
Styrene
Styrene Oxide
0
40
80
120
160
Time (minutes)


1.2 mg/kg - Rat #12
Concentration (µg/ml)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Styrene Glycol
Styrene
Styrene Oxide
0
40
80
120
160
Time (minutes)


6.75 mg/kg - Rat #13
Concentration (µg/ml)
2.5
2
1.5
1
0.5
0
Styrene Glycol
Styrene
Styrene Oxide
0
20
40
60
80
100
120
Time (minutes)


7.95 mg/kg - Rat #14
Concentration (µg/ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
Time (minutes)
Styrene Glycol
Styrene
Styrene Oxide

Predicted vs. observed levels of S in the rat.

Venous blood concentrations predicted by the physiologic model (parameters given in Table 7-1), and those observed in intravenous-dosed rats, normalized to a 7.5 mg/kg dose, are presented in Figure 7-4.

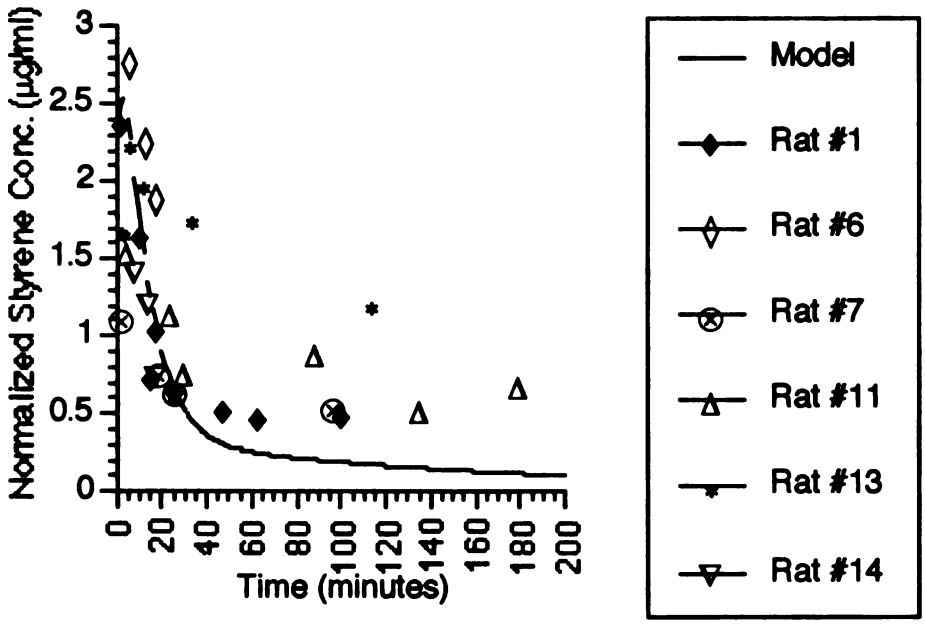


Figure 7-4: Physiologic model-predicted (solid line) and measured levels of styrene in male rats given styrene i.v. doses. The styrene concentrations are normalized to a 7.5 mg/kg dose.

Clearance of S in rats.

Clearance values (based upon measurement of S in venous blood) from the S-dosed rats and model simulations are presented in Table 7-4.

Half-lives of S in rats.

The S decline curves were fit by biexponential decline curves (with the exception of data from rats #6 & 14, which were fit monoexponentially). The apparent first half-lives of S observed in the rats and simulated in the model are presented in Table 7-5. A second S half-life found in the rats, while consistent with the model-predicted value of 115 min, is not presented because the short experimental period could not provide a precise estimate.

Volume of distribution.

The apparent volume of distribution calculated for each animal is presented in Table 7-6.

Table 7-4: Clearance values in styrene-dosed rats and from model simulations.

Animal ID	Dose (mg/kg)	Route	AUC (mg-min/L)	CL (ml/min-kg)
1	7.0	i.v.	137	51
7	9.8	i.v.	406	24
9	10.1	SQ[a]	213	47
11	6.54	i.v.	259	25
12	1.2	SQ	33.5	36
13	6.75	i.v.	346	20

Mean Clearance = 34 ± 13 ml/min-kg

Model-Predicted Clearance

1) CL = Dose/AUC = 7.5 mg/kg / 75 mg-min/L
= 100 ml/min-kg

2) CL = R_o/C_{ss} = 0.004 mg/min-kg / 0.03 mg/L
= 133 ml/min-kg

Table note: [a]Experiments in which the decline curve indicated that the dose was not given intravenously were considered to be subcutaneous administrations.

Table 7-5: Styrene half-lives observed in rats and simulated with the physiologic model.

Animal	First $t_{1/2}$ (min)
1	12.5
6	26
7	30.1
11	28.1
13	34.8
14	19
Mean (±S.D.)	25.1 ± 8.1
Model-Predicted	20

Table 7-6: Initial volumes of distribution for styrene-dosed animals.

Animal	Initial Volume of Distribution (l/kg)
1	2.7
6	2.2
7	6.3
11	4.8
13	3.1
14	4.1
Mean (±S.D.)	3.9 ± 1.5

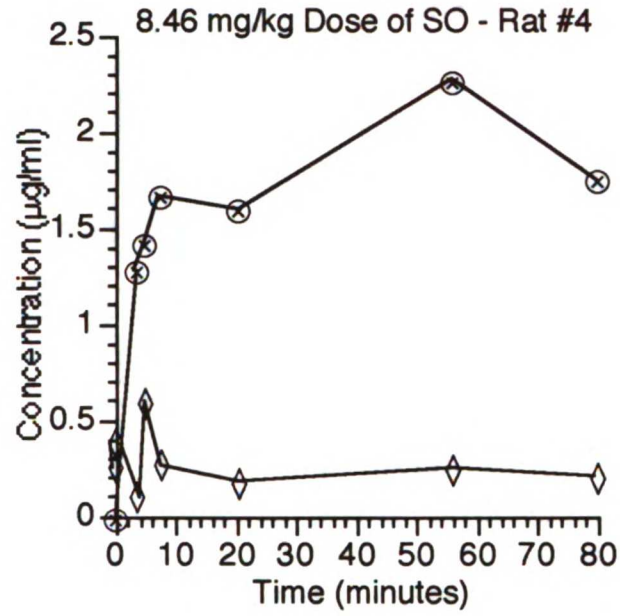
Styrene Oxide-Dosed Animals.

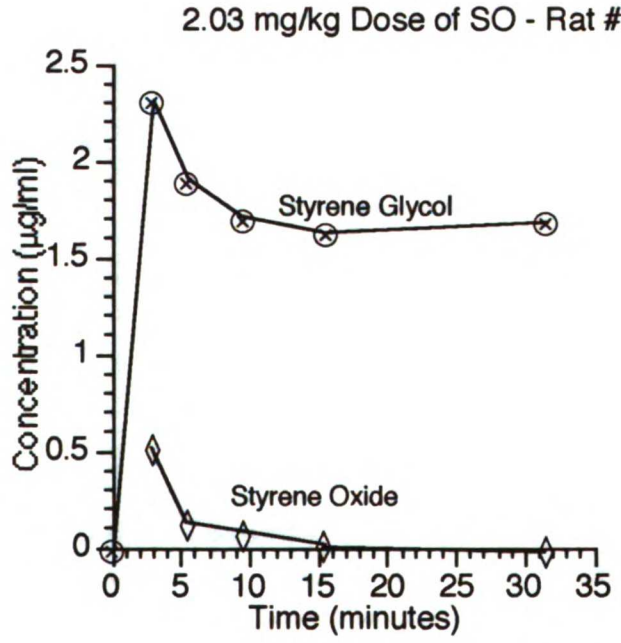
Concentration-time data.

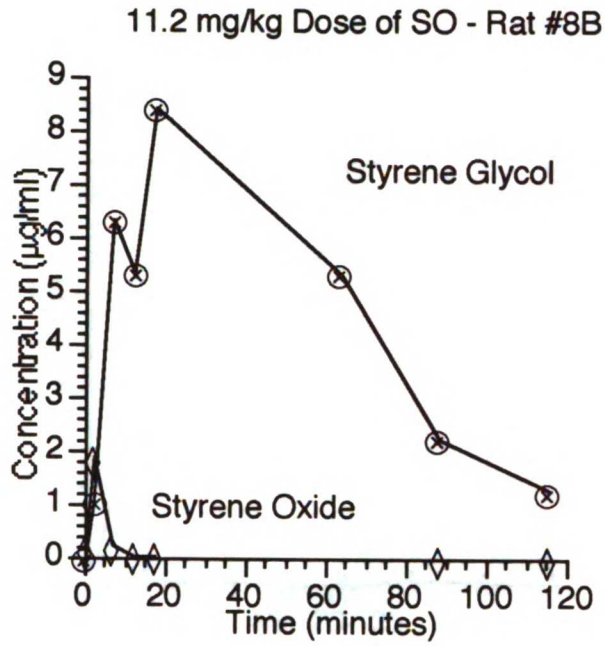
Rats #4, 8, 8B, 11B, 13B, and 14B were given 2-11 mg/kg i.v. doses of SO. Blood concentrations of SO and glycol following administration are presented in the graphs of Figure 7-5. (Rat #13B died following the first sample and only a single time point was available. A separate plot for this experiment is therefore not presented, although the point is included in the composite graph [Fig. 7-6].)

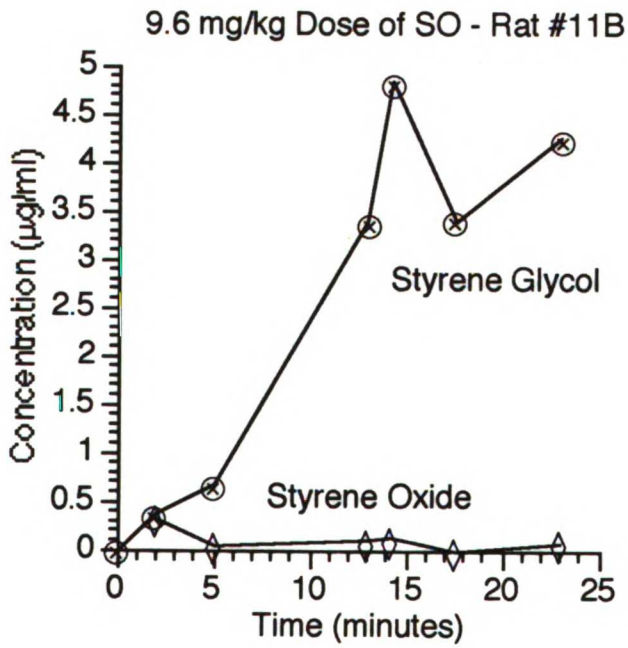
The constant SO level seen in rat #4 is likely due to a co-chromatographing contaminant present in all samples for this animal. Rat #13B died after only one sample was obtained (death was presumably due to the combined narcotic effects of pentobarbital, SO, and the 70% alcohol diluent). The measured level of SO with no corresponding SG present suggests that SO entered the systemic circulation but had not been metabolized (principally in the liver) at the time of death.

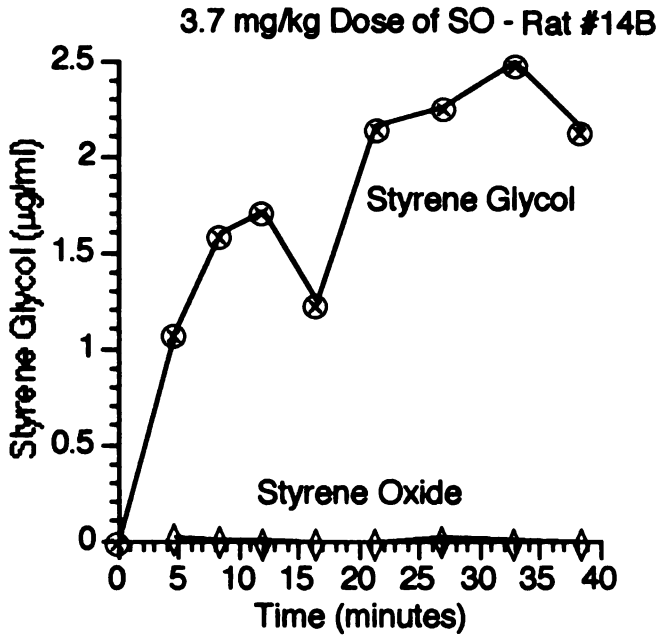
Figure 7-5: Styrene oxide (◊) and styrene glycol (⊗) in blood following i.v. administration of styrene oxide to rats. (Note differences in scale in both x and y axes.)






11.2 mg/kg Dose of SO - Rat #8B
Concentration (µg/ml)
Styrene Glycol
Styrene Oxide
Time (minutes)
0
1
2
3
4
5
6
7
8
9
0
20
40
60
80
100
120


9.6 mg/kg Dose of SO - Rat #11B
Concentration (µg/ml)
Styrene Glycol
Styrene Oxide
Time (minutes)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
5
10
15
20
25


3.7 mg/kg Dose of SO - Rat #14B
Styrene Glycol (µg/ml)
2.5
2
1.5
1
0.5
0
Styrene Glycol
Styrene Oxide
0 5 10 15 20 25 30 35 40
Time (minutes)

Predicted vs. measured levels of SO.

The combined model-predicted and measured concentration-time profiles of SO after SO administration are presented in Figure 7-6.

Clearance of SO in rats.

Measured and predicted values of SO clearance in the SO-dosed rats are presented in Table 7-7. There is a wide variation in calculated clearance values due to the difficulty in obtaining samples and measuring the resultant area under the curve from the rapid loss of SO from blood within the first 5 min.

Half-life of SO in rats.

Determination of SO half-life values was limited by the ability to obtain blood samples within the first 10 minutes of administration. Estimation of SO half-life from rats 4, 8, 8B, 11B, and 14B suggests a half-life not inconsistent with the model-predicted value of 6.4 minutes.

A

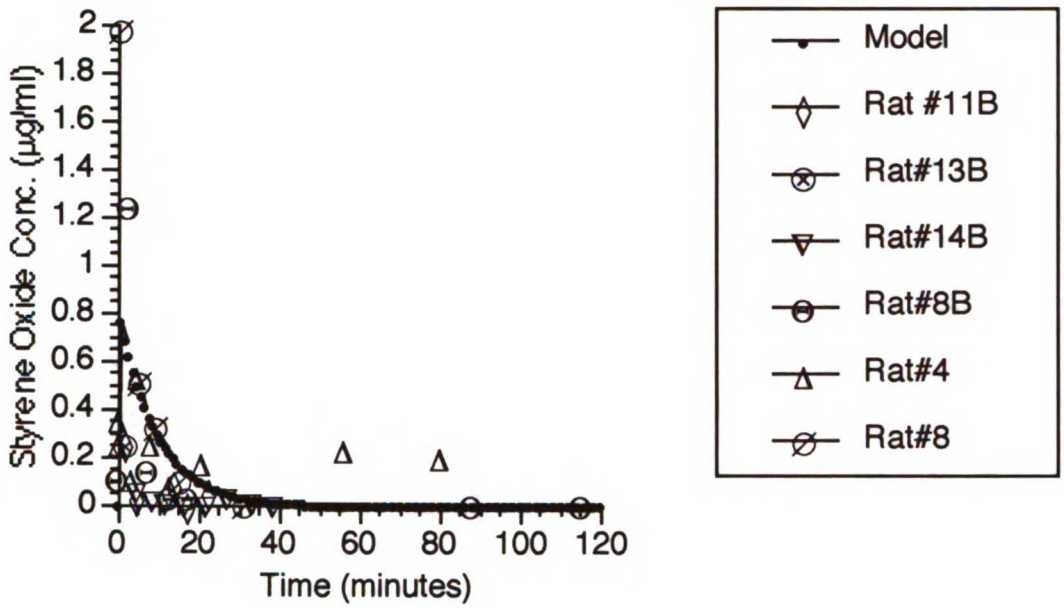


Figure 7-6: Model-predicted and measured levels of styrene oxide in rats following i.v. dosing of styrene oxide: A. Linear plot, B. Log-log plot (to more easily distinguish the predicted value and measured values for different rats) (on following page).

B

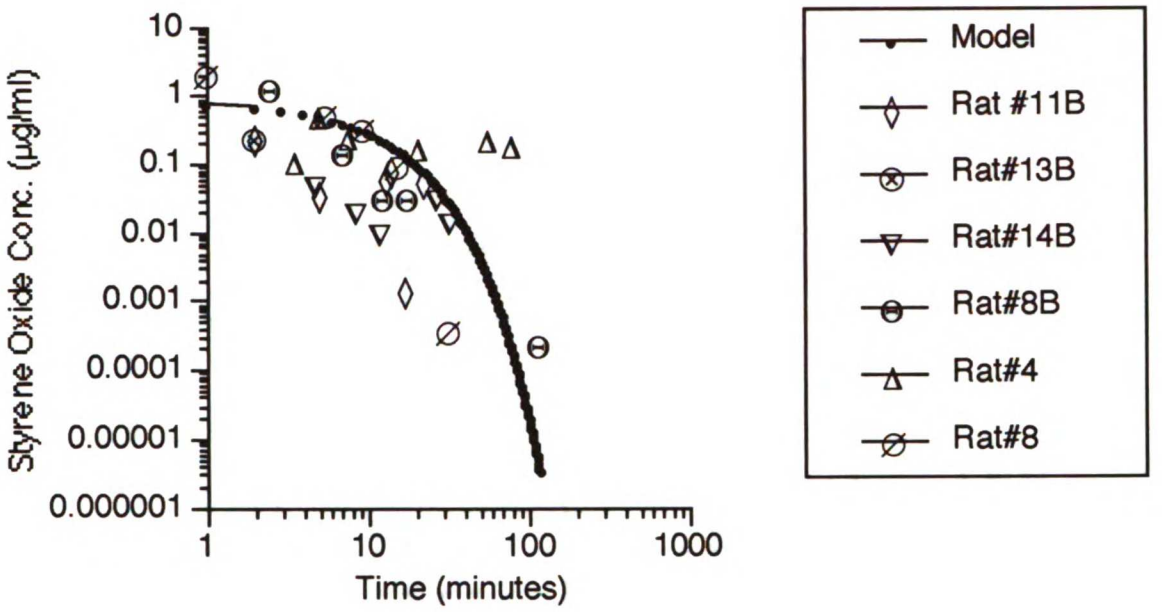

Styrene Oxide Conc. (µg/ml)
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
1
10
100
1000
Time (minutes)
Model
Rat #11B
Rat#13B
Rat#14B
Rat#8B
Rat#4
Rat#8

Table 7-7: Measured and predicted values of clearance in the styrene oxide-dosed rats.

Animal #	Dose (mg/kg)	AUC (μg-min/ml)	CL (ml/min-kg)
4	8.46	8.72[a]	970
8	2.03	4.20	480
8B	11.2	7.87	1,400
11B[b]	9.6	1.87	5,100
14B	3.7	0.25	15,000
Model-predicted	7.5	8.13	922

Table notes: [a]Obtained by fitting 5 and 7.75 min time points only to exponential decay curve and calculating area under this curve. [b]Previously dosed with styrene.

Discussion.

Styrene-Dosed Animals.

While there was substantial scatter within the concentration-time data, a number of trends were evident. Analysis of blood from S-dosed rats indicates metabolism through SO, with rapid further metabolism to SG. Rats #7B, 9, and 12 received subcutaneous (or other nonvascular site) doses of S, as determined by the respective concentration-time profiles. The persistence of SG in blood suggests that this metabolite has a longer half-life than S.

The decline of S levels following i.v. administration appeared to be biphasic. Model parameters used by Ramsey and Anderson[44] did not provide a good fit to the observations. To better match the data, both muscle and adipose tissue blood flow were adjusted. The observed rapid decline in the first phase was modeled by increasing the blood flow to muscle by a factor of 2.5. This adjustment suggests a more rapid equilibrium between S in blood and the large mass of muscle tissue, allowing a faster initial decline in venous levels. The size of this adjustment is not particularly alarming, when it is considered that normal blood flow is known to vary by a factor of at least ten. In addition, samples were taken from the tail, which has a much lower portion of fat than other muscular sampling sites (e.g., leg), and as such may have higher blood flow.

A higher than expected level of S in the second phase (notably rats #1, 6, 7, 13) (Fig. 7-4) suggested a more rapid uptake into and release from well-perfused tissues containing some adipose tissue than was seen in the human model. To include this consideration, and better fit the observations in the rat, adipose tissue blood flow was increased by a factor of 4.75 over a value used by other authors[44]. This suggests that not all adipose tissue is poorly perfused but that the fat in "marbled" tissue such as vascular endothelial lining, kidney, and muscle may have a higher apparent blood flow.

As seen in Figure 7-4, the physiologic model provided a good fit to early observed S concentrations, but a poor fit to later concentrations. Clearance values determined in the animals and from model simulations also differ by a factor of three (Table 7-4). The two model-derived values of clearance, 100 and 133 ml/min-kg, suggest that S is highly cleared by the liver (hepatic blood flow = 117 ml/min-kg[44]). In addition, Ramsey and Young[61] determined clearances of 68 and 58.5 ml/min (in rats of unspecified size) following exposures to 80 ppm for 6 or 24 hours. Styrene levels measured in these studies (Fig. 7-4) suggest that S is more slowly cleared from blood. These differences may be a result of the use of model metabolic parameters K_m and V_{max} that overestimated the rate of S metabolism, and therefore underestimate S concentration. This possibility highlights a central problem in the use of physiologic models - that of metabolic parameter estimation.

Accurate estimation of metabolic parameters K_m and V_{max} requires a series of animal experiments in which a range of low to

saturating doses are given, and measurement of metabolites specific to the enzymatic pathway of interest. These data are often difficult to acquire, and as such, model metabolic parameters are frequently estimated or fit to observed data. As such, the physiologic model is best used as a flexible tool, providing toxicokinetic information while continually being improved with experimental observations.

A second possible explanation for the disparity between predicted and measured S blood concentrations is noise in the measurement of lower S levels at later time points, which may have led to an overestimation of observed levels.

The physiologic model provided a good prediction of the measured "first" half-life observed in rats (20 vs. 25.1 min, Table 7-5). The second half-life seen in the rats, while difficult to accurately estimate (given the limitation of sampling times to < 200 min), was not inconsistent with the model-predicted value of 115 min.

Two other investigations[61, 104] used two-compartment models to follow S disposition in the rat. Ramsey and Young[61] found two half-lives, of 15.6 ± 2.4 min, and 216 ± 166 min. In contrast, Withey[104] found an initial half-life of 2.6 - 7.1 min following an i.v. dose of 1.3-9.4 mg/kg S, and a value of 6.3 min following inhalation exposure to 44.8 ppm S over 5 hours. This half-life was only observed at early time points (< 20 min) and may represent distribution of S from blood to well-perfused tissues.

The longer half-life (modeled value of 115 min) is likely a function of S loss from adipose tissue, a speculation supported by Ramsey and Young[61]. The second half-life found by Withey, 13.5-63.6 min and 49.5 min, for the i.v. and inhalation studies,

respectively, may also represent this process. A potential explanation for this variability among studies is differences in animal species, strain, and age. Such differences could be the basis for differing "well-perfused" and "poorly-perfused" adipose tissue stores (chapter 5), leading to varying estimates of the terminal half-life.

The volume of distribution of S found in the rats was 3.86 ± 1.5 L/kg. This large initial dilution space (calculated as V_D = Dose/C_o) is consistent with the presence of adipose tissue in well-perfused tissues such as the endothelial lining of the vascular system, the kidney, and muscle. Withey[104] found a much smaller volume of distribution, 0.60 ± 0.06 L/kg in four rats given S intravenously. Ramsey and Young[61] found a central compartment volume of distribution of 5.3 L/kg. These differences suggest a rapidly-changing volume of distribution that may indeed be affected by S adsorption to adipose tissue in well-perfused tissues.

Styrene Oxide-Dosed Animals.

SO was measured in all rats dosed with SO (albeit at low levels in the animals pretreated with S - rats #11B, 13B, and 14B). The physiologic model for SO provided a good fit to the SO data (Fig. 7-6).

Styrene oxide was much more rapidly metabolized to the glycol than S, as evidenced by the appearance of SG levels in the range of 1.5-5 μg/ml by 20 min post-dosing, in contrast to levels of 0.2-1 μg/ml following S administration. This observation suggests that the rate-limiting step in S metabolism is the initial oxidation to SO.

A decline of glycol levels was evident after 20-100 minutes (rats #1, 4, 8B, 9, 11, and 12). Observation of the S-dosed and SO-dosed animal data (Figs. 7-3 and 7-5) indicates the relative order of apparent half-lives: SO $t_{1/2}$ < S $t_{1/2}$ < SG $t_{1/2}$.

Although the number of determinations is small, the model-predicted and measured rates of SO clearance in animals not previously dosed with S (#4, 8, 8B) are similar (922 and 959 ml/min-kg, Table 7-7). The observed half-life is consistent with the predicted value of 6.4 minutes. This value is similar to a 3.4 min half-life found in the mouse[10].

An interesting observation was that there was some indication of induction of SO metabolism by S pretreatment (with concomitant pretreatment with pentobarbital as the anesthetic agent), although the data are limited in this regard. This can be seen by comparing SO levels in SO-dosed animals that were previously used in S dosing experiments (rats #11B, 13B and 14B), to those of the other rats (4, 8 and 8B), as presented in Figure 7-8. Although based on sparse data, clearance values in these two groups also appear to be different (9,970 ± 6,800 vs. 960 ± 470 ml/min-kg). Induction of SO metabolism is supported by previous observations in which pretreatment of rats with phenobarbital or 3-methylcholanthrene stimulated SO biotransformation[37]. Another investigation found that a single 2,000 mg/kg i.p. injection of S to Wistar rats increased epoxide hydrolase activity, while lower doses (100 and 500 mg/kg) injected from 3-6 times daily did not show this effect[37]. Pretreatment of rats with phenobarbital given i.p. increased the rate of appearance of S metabolites in urine following S (455 mg/kg), but

not SO (527 mg/kg) administration[37]. Given the very high rate of SO clearance, systemic absorption of an intraperitoneal dose of SO would be the rate-limiting step in SO decline. Therefore, induction of metabolic activity should not be apparent following i.p. administration of SO. The possible induction of SO metabolism observed in these studies could be explained by an increase in extrahepatic enzyme activity following the initial S dosing. Increased levels of enzymes in the liver would not significantly increase hepatic clearance because the hepatic extraction ratio is high and metabolism in this organ is limited by blood flow.

Styrene Oxide Following S vs. SO Administration.

Evidence from S and SO administration to rats suggests that SO is present at much higher levels for longer periods (greater AUC) after SO vs. S dosing, and that S is sequentially metabolized, S → SO → SG. This finding is supported by a study done by Sbrana et al.[18], who gave a 150 mg/kg SO gavage dose to male mice. Examination of these data indicates that the AUC following a 150 mg/kg dose of SO is approximately 150 μg-min/ml. (Under these conditions, metabolic saturation may have disproportionately *increased* the AUC, relative to a non-saturating dose.) The expected AUC of SO after a 7.5 mg/kg dose of S or SO (given that virtually all the S is metabolized through SO) is 7.5 μg-min/ml. This expectation is in stark contrast to the nearly zero SO AUC observed in our studies following S administration. Sbrana et al. also found no measurable presence of

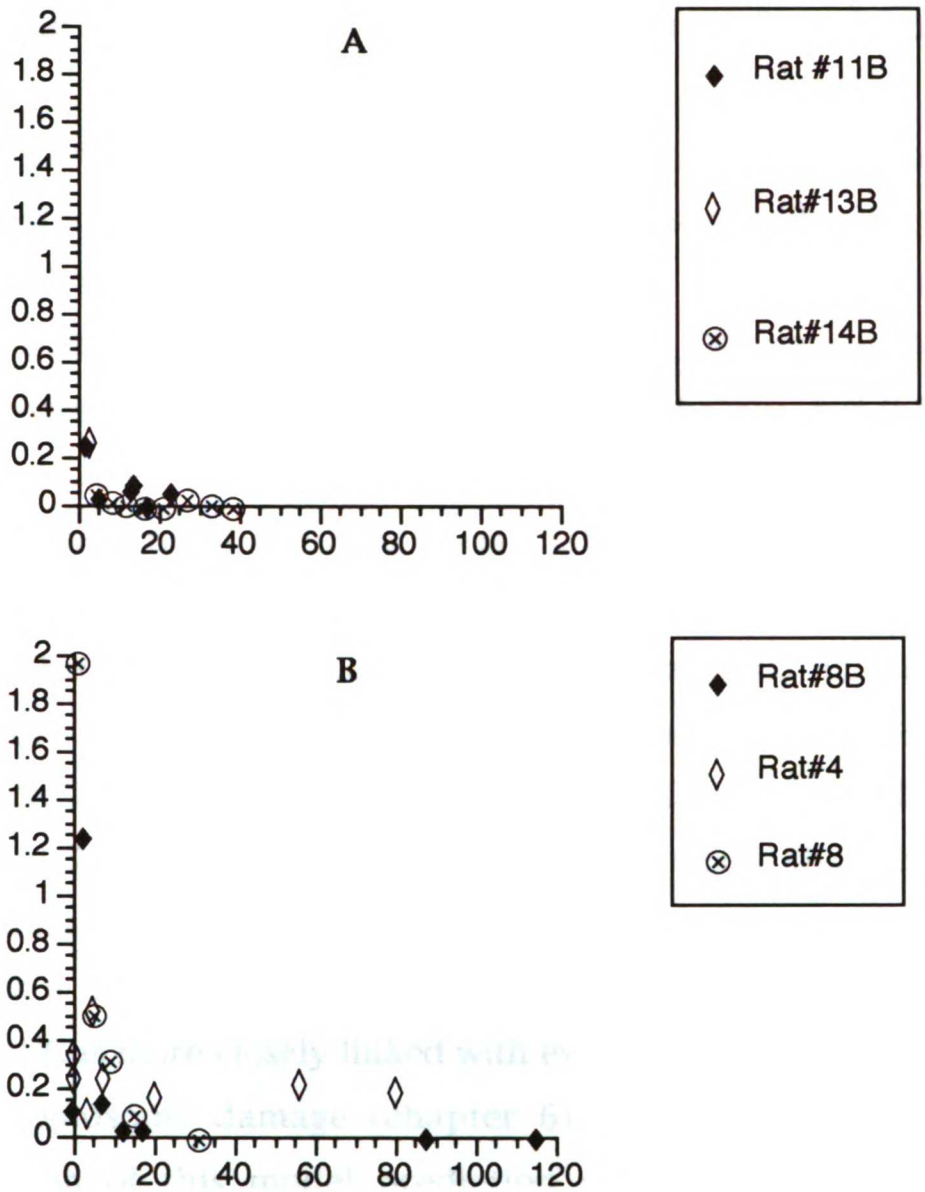


Figure 7-8: Styrene oxide concentration-time profiles in styrene pre-treated (A) and the other rats (B) following SO administration.

SO in male mice given daily oral doses of 200 mg/kg S for 1 or 70 days.

In further support of this idea are the physiologic model-simulated levels of SO following S or SO administration (Fig. 7-9), which indicate that very low systemic levels of SO result from S dosing. Indeed, as found by other investigators, "*Styrene oxide ... has been proposed as an intermediate in the metabolism, but it has not been found in vivo.*"[2].

A more surprising result was that the model-predicted SO persisted longer, albeit at low levels, following S vs. SO exposure (Fig. 7-9B). This was due to the relatively long terminal half-life of S, with subsequent rapid conversion through the obligate metabolite SO, a condition in which the decline of SO is rate-limited by the decline of S. Although a single exposure to S would thus provide a longer duration of exposure to SO (at very low levels), exposure to S provides a much lower SO total dose (AUC) than from SO dosing, where AUC is more closely linked with events such as carcinogenesis and chromosome damage (chapter 6). However, experimental verification of this model prediction was not obtained due to detection limitations.

A comparison of the relative AUCs of SO following simulated S or SO administration is presented in Table 7-9. These data are consistent with the apparent strong and weak carcinogenic effects of SO and S, respectively, in animal models (chapter 1), based on the idea that the circulating levels of SO are related to carcinogenicity.

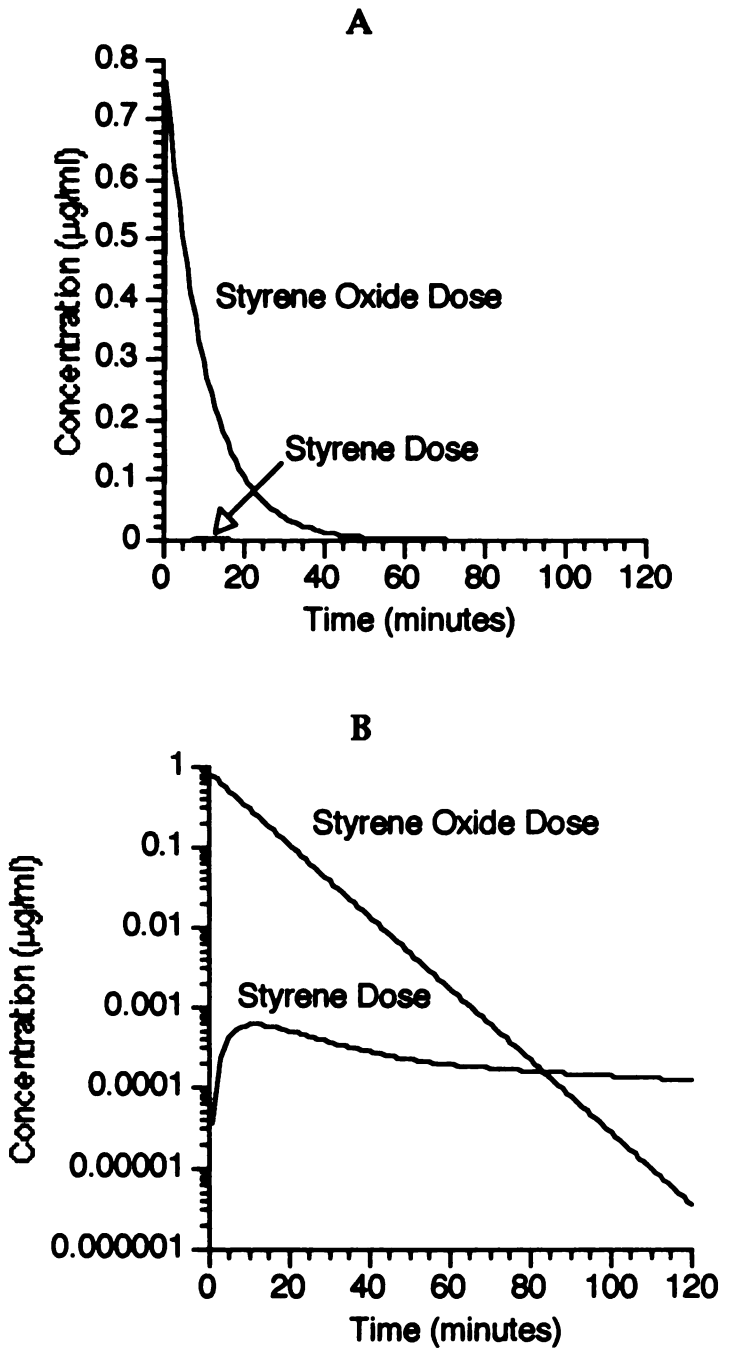


Figure 7-9: Physiologic model-simulated levels of styrene oxide following 7.5 mg/kg i.v. doses of styrene or styrene oxide in blood. A. Linear plot. B. Semilogarithmic plot.

Table 7-9: Relative areas under the concentration-time curve (AUC) of styrene oxide following simulated i.v. administration of styrene or styrene oxide.

Dose	Styrene Oxide AUC (μg-min/ml)	AUC/ Dose[a]
7.5 mg/kg Styrene	0.05	0.0067
7.5 mg/kg Styrene Oxide	8.13	1.08

Table note: [a]Dose of SO assumes all S is metabolized through SO.

Tissue and Species Sensitivity.

Both S and SO produce plasma protein, hemoglobin, and DNA adducts when given at doses of 1.1 mmol/kg i.p. to mice[10]. The DNA adducts from both administrations were identified as N^7-alkylguanine. This finding, along with other macromolecular binding evidence (chapter 1), suggests that both tissue-specific formation of SO from S, and administration of SO can result in toxicity. Thus, there is very strong evidence that SO, whether administered directly or formed from S, is responsible for macromolecular binding and carcinogenesis.

The greater systemic exposure to SO when SO is administered compared to S (Table 7-9) is associated with a higher incidence of macromolecular binding and the greater carcinogenicity of SO. While virtually all S is metabolized through SO, the sequential nature of S metabolism greatly diminishes the systemic exposure to SO.

Following S exposure, both the toxicokinetics of S and the relative rates of bioactivation to SO and detoxification to SG (chapter 1) can affect the toxicodynamic outcome. Regarding the former, there is evidence that both rats and humans (this chapter and chapter 3) show high hepatic extraction of S. This result is consistent with the use of the same Michaelis constant K_m in both models, and with the scaling of both hepatic blood flow and maximal reaction rate V_{max}, determined in rats, to humans, using the relative body surface area (body weight$^{0.7}$) of each[44].

A second issue is tissue and species-specific activating vs. detoxifying metabolic activity. Cantoni et al.[100] measured SO-forming (P450) and degrading (epoxide hydrolase) activities in vitro in rats, mice, guinea pigs and rabbits. The lowest SO degrading/forming activities were found in the male and female mouse lung (0.27 and 0.80), male and female rabbit lung (0.24 and 0.72) and female rat spleen (0.58). These authors speculated that these tissues might be sensitive to SO-induced toxicity, a point of view echoed elsewhere[2]. Assuming that in these species S is sequentially metabolized with a high tissue extraction of SO, as seen in the rat, these tissues would indeed be expected to be more sensitive to SO-related toxic events following exposure to S.

As presented in chapter 1, there is evidence for carcinogenicity at sites thought to be sensitive to SO toxicity. Male B6C3F1 mice exposed to 78 weeks of daily gavage dosing (150 mg/kg) showed an increase in alveolar/bronchiolar adenomas and carcinomas[14]. Increased appearance of lung tumors in male and female mouse offspring was observed after S dosing of dams[14].

In addition to greater SO forming versus degrading activity, a rapid elimination of SO is necessary for sequential metabolism to play a role in mediating toxicity. Thus, if SO were slowly metabolized, circulating levels of SO would be evident following S dosing, and the contribution of site-specific formation of SO to toxicity would be minor.

While the principal organ of S elimination is the liver, P-450 activity is distributed throughout the body, and as such many tissues may be able to form SO. In order to qualitatively assess the potential

risk to human lung tissue, the relative activity of the SO forming and degrading enzymes can be calculated. However, because different authors use varied in vitro preparation techniques, it would be best to use activities determined under the same conditions. The goal of this calculation was to determine the ratio of degrading/forming enzyme activities in the lung of humans vs. that in an apparently sensitive species, the mouse. For the mouse and human, the P450 activity ($P450_m$ and $P450_h$, respectively) represents metabolic activation. The mouse uses both epoxide hydrolase (EH_m) and glutathione-S-transferase (GST_m) activities to degrade SO[10], whereas humans use virtually only EH (EH_h) (chapter 1).

Therefore, using the in vitro enzyme activities presented in Table 7-10 in the lung, $EH_m/P450_m = (0.27 + 0.80)/2 = 0.54$ (average of values from males and females[100]). The ratio of GST to EH activity in the mouse is given by: $GST_m/EH_m = 14.7/0.6 = 24.5$[102]. The total mouse SO degrading/forming ratio is then $EH_m/P450_m + (EH_m/P450_m * GST_m/EH_m) = 0.54 + (0.54*24.5) = 13.8$.

Human EH and P450 activities have been measured in comparison to the rat (EH_r and $P450_r$), such that $EH_h/EH_r = 2.0/0.6 = 3.3$[102]. Knowing that $EH_r/P450_r = (4.79 + 3.23)/2 = 4.0$ (average of male and female rats[100]), then $EH_h/P450_r = (EH_h/EH_r)*(EH_r/P450_r) = 3.3*4.0 = 13.2$. To get the human lung activity ratio, the P450 activity in human vs. rat can be used: $P450_r/P450_h = 10$ (average of 0-20 range[16]). Combination with the previous ratio gives $(EH_h/P450_r)*(P450_r/P450_h) = EH_h/P450_h = 13.2*10 = 132$. Finally, to compare degrading/forming rates in human vs. mouse lung, the following equation can be solved:

$(EH_h/P450_h) / ((EH_m + GST_m)/P450_m) = 132/13.8 = 9.6$. These calculations, based on measured in vitro metabolic rates of SO-forming (P450) and SO-degrading (EH and GST) enzymes, suggest that the human lung may be 10 times more resistant than the mouse lung to carcinogenesis from SO produced in lung tissue following S administration.

Table 7-10: Selected mouse, rat, and human epoxide hydrolase, P450, and GST activities in vitro in the lung

Species	Enzyme	Activity (nmoles/min-mg protein)	Reference
Mouse	Epoxide Hydrolase	0.83 (male)	[100]
		2.51 (female)	[102]
		0.6	
	P450	2.97 (male)	[100]
		3.11 (female)	
	GST	14.7	[102]
Human	Epoxide Hydrolase	2.0	[102]
Rat	Epoxide Hydrolase	0.6	[102]
		1.63 (male)	[100]
		0.97 (female)	
	P450	0.34 (male)	[100]
		0.30 (female)	

Conclusions.

The model simulations and data from other investigators suggest that S is rapidly metabolized in the liver, with blood clearance near hepatic blood flow. However, in these studies a much lower clearance was calculated from the experimental data. Further experiments are needed to resolve this difference. Very low to undetectable levels of SO were seen in rat blood following S administration. Styrene appeared to be lost from rat blood in two phases, with half-lives of about 20 and 115 min, possibly representing hepatic elimination from well-perfused tissues and desorption from adipose tissue, respectively.

Styrene oxide was rapidly metabolized, and the apparent clearance indicated extensive extrahepatic metabolism. Extrahepatic metabolism of SO may be induced following pretreatment with S. The measured presence of SO in rat blood after SO, but not S, administration suggested that S is rapidly metabolized through SO to SG, in a sequential metabolic process.

There is strong evidence for a lower SO AUC following S administration than would be expected, given the principal metabolic pathway S → SO → SG. These findings are consistent with the observed in vitro and in vivo toxicity studies performed in test animals (chapter 1), particularly the much greater carcinogenic effect of SO. In vitro SO-forming and SO-degrading enzyme activities in lung tissue suggest that following S exposure, humans may be more

resistant to toxicity than the mouse, a species in which tumors have been observed (chapter 1).

Dissertation Summary.

Styrene is an aromatic hydrocarbon with widespread occupational and environmental exposure. Occupational exposure to S causes CNS disturbances and chromosome aberrations. The obligate reactive intermediate in the metabolism of S, SO, is considered to be carcinogenic in animals and humans. A physiologically-based model proved to be a valuable tool to investigate SO toxicokinetics and S toxicokinetics and toxicodynamics.

Simulation of blood concentrations in a forearm vein was accomplished by assuming that 85% of venous blood drained muscle tissue and 15% of venous blood drained adipose tissue. Blood S concentrations from volunteers experimentally exposed to S were adequately predicted with the model, and simulation-derived half-lives of 0.5, 45, and 3,220 min help to explain the wide range of previously-reported values. Styrene clearance was found to approach hepatic blood flow, indicating high hepatic extraction. Although no CNS disturbances were found in this study, analysis of data from the literature suggest that the appearance of CNS disturbances is associated with simulated S brain concentrations above 5 μg/ml. Further model simulations indicated that arteriovenous differences can be an order of magnitude at the onset of inhalation exposure, and that arterial concentrations are approximately 30% lower than venous concentrations in the terminal phase following cessation of exposure.

Styrene was measured at about 1 μg/g in the adipose tissue of non-occupationally exposed persons. Use of this value, along with the derivation of an apparent clearance of S from adipose tissue, and previously measured levels of S in ambient air, suggests that there may be a number of undiscovered sources of environmental S exposure, or that elimination of S from adipose tissue may be slower than previously thought.

Examination of predicted S brain concentrations indicated that physiologic damping plays an important role in mediating CNS toxicity from different exposure frequencies (with total dose held constant). Minute volume (corresponding to work load) was found to be a pivotal consideration in a suggested redefinition of occupational exposure standards. A chronic exposure standard of 100 ppm work hour-years was suggested to protect workers from chromosome aberrations.

Examination of previous findings, and administration of S and SO intravenously to rats showed that S is sequentially metabolized through SO to SG. The difference in carcinogenic risk from S and SO may be explained by the degree of systemic exposure to SO, which is much less following S administration because of rapid sequential metabolism. The ratio of tissue-specific enzyme activities for forming and degrading SO are also determinants of carcinogenic risk from S exposure.

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