SUMMARY REPORT

OF THE EXTERNAL PEER REVIEW OF THE DRAFT

TOXICOLOGICAL PROFILE FOR

STYRENE

Submitted to:

The Agency for Toxic Substances and Disease Registry
Division of Toxicology
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SECTION I

PEER REVIEWERS' SUMMARY COMMENTS

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Comments on Styrene Draft Review

From George Cruzan, PhD, DABT

July 20, 2007

In general this is a good review. I have made some comments on text. There are a few issues I would like to raise. I do not have access to high speed photocopying, so I have not provided copies of some of the articles cited, but have provided full citations.

1. In several cases, differences from controls are listed in tables or text as "impaired", e.g., "impaired color vision". It should be noted that none of these studies reported "impaired color vision". I.e., in no cases are the responses of the styrene exposed workers outside the normal range for color discrimination. I have enclosed a copy of a review article by Drs. Gregory Good and Jason Nichols from Ohio State U., which was published in the SIRC Review. CCI values for the styrene exposed workers range from 1.10 to 1.30 in various studies. Congenitally color blind people score 2.5 or higher, while those with an anomalous pigment score greater than 1.5. A better term would be "decreased color discrimination". (Copy of The SIRC Review included)

2. The meta analysis by Benignus et al. (2005) is accepted as a valuable contribution to understanding the neuropsychological effects of styrene and is used as the basis for an MRL (p. 9 and 16). It needs to be noted that 2 of the 4 studies included by Benignus for reaction time are not studies generally considered as reaction time studies. They also were quite selective in the studies included for color discrimination. Further, they found a time-response only because of they assumed there was and estimated effects for different amounts of exposure. Only one of the studies had data for people exposed for different amounts of time; that study concluded that duration of exposure had no impact.. I have included an analysis of the Benignus paper performed by 4 independent consultants (for SIRC). – (Commentary article- final.doc)

I do not think this should be the basis for MRL.

3. Comments are included that styrene increases prolactin in humans. The authors of the papers listed make those claims. In all the studies cited, nearly all individuals (exposed or not) have prolactin values within the normal range <20 ng/ml for females and <15 ng/ml in males. The Arfini etal. study used a supraphysiologic dose of TRH to elicit a response,

so the significance of the observations are unclear; in fact the editor of the journal included a note to be cautious in interpreting the results.

4. p. 22 Discusses human studies of respiratory system. You should add: Odkvist et al. (1985) found no cellular changes in the nose of a small group of workers exposed to at least 50 ppm in reinforced plastics work. Dalton et al. (2001)[in your reference list] reported that styrene exposure in reinforced plastics workers did not result in a change in the ability to detect standard odors or in the odor detection threshold of phenylethanol.

p. 29 cites a number of acute studies; the study by Ska et al. 2003 should be included. Volunteers were exposed for 8 hours at either 25 or 50 ppm TWA styrene.

5. PBPK models – p. 61. The Filser et al. (1994, 2003) model is useful in understanding that the mode of action for styrene induction of mouse lung tumors is NOT related to styrene oxide. It is NOT useful for understanding carcinogenic risk from styrene because it does not explain the differences between rats and mice and does not explain the MOA for styrene. The 2003 version of the model treats lung as a uniform organ, assumes metabolism of styrene is uniform throughout the 40 cell types in the lung, and that styrene metabolite concentration is uniform across cell types. Studies from Carlson's lab deomstrated the styrene metabolism occurs only in Clara cells. The model predicts relatively small differences in average lung styrene oxide between rats and mice. A recent study by Dr. Filser's lab using isolated perfused lungs verified that there was little difference in styrene oxide in rat and mouse lung and concluded that styrene oxide was not the carcinogenic agent. (Hofmann et al., 2006). The Sarangapani et al. 2002 model should be included because it attempts to relate pharmacokinetics to mode of action. Because toxicity (and metabolism) occurs only in terminal bronchioles, the Sarangapani model estimates the concentration of styrene oxide in terminal bronchioles, as well as liver.

6. It has often been presumed that styrene-7,8-oxide is the causative agent (via genotoxicity or non-genotoxicity) for any tumorigenic properties of styrene. This is doubtful. 1. Blood level of styrene oxide is more than 100 times higher in rats at non-tumorigenic doses (1000 ppm) than in mice at tumorigenic doses (40 ppm). 2. Oral administration of styrene oxide did not result in lung tumors in rats or mice. 3. Blood levels of styrene oxide from the oral doses in the mouse carcinogenicity study are about 100 fold higher than from inhalation of styrene at 40 ppm.

The mode of action data are summarized below:

Postulation of a Mode of Action for Tumors Induced by Styrene in the Mouse Lung

1. Description of Postulated Mode of Action

The characteristics of the styrene tumor data (increased lung tumors in mice after 2 years, but not 18 months, of exposure by inhalation; by oral exposure lung tumors equivocally increased in mice; no other organs in mice had increased tumor incidence and none were

increased in rats exposed by oral or inhalation routes) suggest a non-genotoxic mode of action. The metabolism of styrene in mouse lung Clara cells produces high levels of styrene metabolites which cause Clara cell toxicity. The cellular damage results in reparative responses involving increased Clara cell proliferation and hyperplasia of Clara cells; an increase in lung tumors occurred after 24 months. Rats and humans have a lower metabolism of styrene in lung and more rapid removal of metabolites, thus they do not develop toxicity, increased cell proliferation, hyperplasia, or lung tumors.

2. Key Events

In mouse or rat lung, styrene is metabolized nearly exclusively in Clara cells, not in type II cells. Styrene is metabolized by CYP2F2 to cytotoxic metabolites. CYP2E1 appears to play a less important role. Using enriched cell fractions, rat and mouse Clara cells produce about the same amount of the S enantiomer of SO per cell, but mice produce five-fold more per cell of R-SO than do rat Clara cells. The production of SO, especially the R enantiomer, is inhibited by 5-phenyl-1-pentyne, an inhibitor of CYP2F2. Using human lung tissue, very little, if any SO is produced and the S enantiomer predominates. Inhalation of 80 ppm or greater styrene also results in glutathione depletion in mouse, but not rat, lung tissue. In rats, no depletion is seen at levels below 300 ppm and only marginal depletion occurs at higher levels.

Compared to rats, a large proportion of the cells in the terminal bronchioles in mice are Clara cells, the cell with the highest metabolism of styrene. Humans have even fewer Clara cells than rats. In mice, exposure to 40 ppm or greater results in Clara cell cytotoxicity after a single exposure. Continued exposure results in increased Clara cell proliferation, measured by increased BrdU labeling for at least the first two weeks. By thirteen weeks, cellular crowding is seen histologically in the terminal bronchioles. By 12 months, hyperplasia is seen histologically in the terminal bronchioles. With continued exposure, the extent of the hyperplasia increases and it is seen at lower styrene concentrations and a greater percentage of the mice are affected. No toxicity, cell replication or hyperplasia is seen in mouse type II cells. Similarly, no toxicity, increased cell replication, or hyperplasia are seen in rat lung, either for type II or Clara cells. Furthermore, inhibition of styrene metabolism by 5-phenyl-1-pentyne eliminated the cytotoxicity and increased cell replication in mouse Clara cells.

In summary, the metabolism of styrene by CYP2F2, primarily to R-SO, in mouse lung Clara cells results in cytotoxicity, increased cell proliferation, cell crowding, hyperplasia and eventually lung tumors. Rat lung tissue, with fewer Clara cells and much less CYP2F4 in those Clara cells, does not produce toxic quantities of styrene metabolites, as evidenced by lack of increased cell proliferation, absence of histopathologic effects in lungs and no increase in lung tumors. In humans, with barely detectable levels of CYP2F1 (the human equivalent of CYP2F2) and no measurable production of SO by CYP2F1, even less SO is produced than in rats and that which is produced or diffuses into lung from the blood stream is removed more efficiently by epoxide hydrolase. Thus humans have even less likelihood of developing styrene-induced lung cytotoxicity, increased cell proliferation or lung tumors than rats.

3. Strength, Consistency, Specificity

Mice have a much greater number of Clara cells than do rats in the lung, which have a much greater number than humans. In addition, mouse Clara cells have much more CYP2F and produce much more R-SO than do rat Clara cells; human lung cells have barely detectable levels of CYP2F and no detectable production of SO. Thus mice have the greatest number of target cells for styrene toxicity and those target cells have the greatest capacity to produce toxic metabolites of styrene. Increased production of SO, especially R-SO, in mouse lung has been shown by the use of microsomes and isolated cell fractions. Using i.p. injection, R-SO was shown to be more pneumotoxic (increased GGT and LDH in BALF) than S-SO, consistent with the increased production of R-SO in mouse lung. Thus, styrene-induced toxicity in mice occurs in the organ where increased tumors are seen.

Styrene toxicity in mice occurs in 3 organs: liver (acute necrosis, but cells replaced with resistant hepatocytes); nasal olfactory mucosa (chronic cytotoxicity, limited cellular replacement, cells replaced with respiratory-like cells), and lung (chronic cytotoxicity, rapid cellular replacement in kind, hyperplasia). These are the organs highest in CYP2F2. In both lung Clara cells and nasal olfactory mucosa, metabolism of styrene per cell is markedly greater than that in mouse hepatocytes and the formation of R-SO is preferred even more than in mouse hepatocytes. Toxicity in both olfactory mucosa and Clara cells is prevented if CYP2F2 is inhibited by 5-phenyl-1-pentyne. In rat lung and liver, with very little CYP2F2, styrene is metabolized via CYP2E1. Rat nasal olfactory tissue contains a moderate amount of CYP2F2, in addition to CYP2E1. In rat lung and liver the ratio of R-SO to S-SO is less than 1, but in rat nasal olfactory tissue, it is about 3. Thus the only rat tissue that contains measurable amounts of CYP2F2 (rat olfactory epithelium) is the only tissue that produces a high proportion of R-SO and is the only rat tissue to display styrene induced cytotoxicity.

The induction of lung tumors in mice does not appear to be related to the formation of styrene-DNA adducts because:

- The formation of DNA adducts following styrene exposure is low in rats, mice and humans (<1 per 107 nucleotides).
- No unique styrene-DNA adducts have been found in mouse lung.
- The level of DNA adducts in mouse lung is less than in mouse liver.
- The level of DNA adducts in mice is not greater than in rats.

The induction of lung tumors in mice appears to involve mouse lung-specific metabolism of styrene, and is independent of the blood level of SO. In the chronic inhalation studies, the blood levels of SO in rats exposed to 1000 or 200 ppm styrene were 170 and 47 μg/l (male and female averaged), respectively, while mice exposed to 160 ppm had blood SO levels of 27 μg/l. It should be noted that in the chronic study 11 of 20 mice exposed to 40 ppm for 73 weeks had no detectable blood SO, and none of those exposed to 20 ppm had detectable blood SO. Yet Clara cell toxicity was seen in mice exposed to 20 ppm and greater. Effects seen in mouse lung are not likely the result of the influx of SO from the bloodstream because: 1) exposure concentrations in rats (up to 1000 ppm) that did not

cause increased lung tumors resulted in higher blood levels of SO than exposure levels in mice that did result in increased lung tumors, and 2) effects were seen in mouse lung following exposure to 20 ppm where SO was not detected in the blood.

4. Dose/Response

The non-neoplastic key events of styrene in mouse lung occurred at the same exposure concentrations that resulted in increased lung tumors. Decreased staining of the Clara cells (an indicator of cytotoxicity) was reported in 50-70% of the mice exposed to 20 ppm for 12, 18 or 24 months and in more than 80% of those exposed to 40, 80, or 160 ppm. Increased cell proliferation has been reported at concentrations of 40 ppm or greater (20 ppm has not been examined). Bronchiolar hyperplasia was seen in a few mice exposed to 40 ppm for 12 months and in most mice exposed to 80 or 160 ppm; by 24 months bronchiolar hyperplasia was seen in up to 40% of the mice exposed to 20 ppm and in more than 75% of those exposed to 40, 80 or 160 ppm. Because the non-neoplastic effects of styrene are seen in nearly all mice at exposure levels of 40 ppm and above, it is not surprising that there is not a clear dose-response in tumor formation.

5. Temporality

In mice, increased lung tumors were seen only at the end of the study. Short-term studies and interim assessments during the chronic study indicate that all the described precursor lesions preceded the formation of lung tumors. Single exposures to styrene result in the production of large amounts of metabolites in mouse lung and produce cytotoxicity following a single exposure. After a few exposures, increased repair is measurable as increased labeling of Clara cells. Continued exposures of a few weeks result in Clara cells that stain less well with eosin and crowding of Clara cells, which could be described as an increased number of cells but not extra layers of cells. Sometime between 13 and 52 weeks, this increase in cells become great enough that extra layers of cells are detectable; this is described as hyperplasia of the terminal bronchioles. With continued exposure, the hyperplasia extends into the alveolar ducts. Lastly between 18 and 24 months increased lung tumors appear.

6. Plausibility and Coherence

The lung tumors found in styrene exposed mice fit with a non-genotoxic mode (single species, single organ, late occurring). In addition, there is no support for genotoxicity from styrene in mouse lung (no increase in CAs in mouse lungs, very low levels of non-specific DNA adducts in mouse lungs, negative in A/J mouse screening assay), as well as no increase in HGPRT mutations in hamster lung V79 cells. Lack of lung tumors as a factor contributory to death, except in a few high exposure females, provides additional support for a non-genotoxic mode of action.

Styrene causes mouse-specific lung cytotoxicity due to mouse-specific metabolism of styrene. Cytotoxicity, with compensatory cell replication, often results in increased tumor incidence; this is believed to be due to the increased probability of fixing spontaneous

mutations in dividing cells because of the increased cell replication and the resulting decreased time for repair of mutations.

Depletion of GSH occurs in mouse lungs at levels that resulted in increased tumor formation. Depletion of GSH results in an inability to detoxify normal oxidative compounds inhaled or generated during normal metabolism. These oxidative compounds can cause increased DNA adducts, such as 8-dehydroxy-2-deoxyguanosine, which lead to DNA strand breaks and potentially to increased tumor formation.

The data are also consistent with CYP2F catalyzed metabolism of styrene to largely R-SO. In mice, lung Clara cells and nasal tissue have large amounts of CYP2F, produce much more R-SO than S-SO, and develop cytotoxicity. Inhibition of CYP2F by 5-phenyl-1-pentyne in mice for three days eliminated the nasal and Clara cell toxicity from exposure to 160 ppm styrene. In rats, CYP2F has been detected only in nasal tissue; correspondingly, cytotoxicity is only seen in the olfactory region of rats, as well as in mice. Rat Clara cells have very limited amounts of CYP2F , produce more S-SO than R-SO, and do not develop cytotoxicity.

Naphthalene is another aromatic hydrocarbon that is metabolized to a much greater extent in mouse lung than in any other tissue, also by CYP2F2 to produce a high ratio of one enantiomer. Similar to styrene, naphthalene is largely negative in genotoxicity assays and results in increased lung tumors in mice (NTP, 1992; Abdo, et al., 1992). Like styrene, no other tumor type was increased in mice. No lung tumors were reported in rats exposed by inhalation to naphthalene for two years (NTP, 2000; Abdo, et al., 2001). A considerable body of evidence has been developed on the effects of naphthalene exposure on mouse lung Clara cell cytotoxicity, cellular repair, and the role of CYP2F2. These show a similar pattern of effects as seen with styrene.

The current data indicate a greater production of R-SO in tissues where cytotoxicity is seen; however, very low levels of bound metabolites, and specifically low levels of DNA adducts, suggest that the cytotoxicity may be caused by further metabolism of R-SO.

In summary, the styrene data fit with a non-genotoxic mode of action. Lack of genotoxic reactions in mouse lung tissue and lack of tumor response in tissues other than mouse lung support this conclusion. Mouse lung specific metabolism and cytotoxicity of styrene and elimination of the cytotoxicity by inhibition of the metabolism of styrene provide further support for the non-genotoxic mode of action.

7. Other Modes

The default assumption of tumor formation involves a genotoxic mode of action. Some positive genotoxic findings have been reported for styrene. Further, styrene is metabolized to SO, which consistently has demonstrated genotoxic effects in in vitro studies. SO reacts with DNA to form adducts; DNA adducts have been demonstrated in rats, mice and humans exposed to styrene and/or SO. Thus there is evidence of a genotoxic material reaching the target organ and reacting with DNA in the target organ,

which could be interpreted to mean that the default assumption is correct. The data also demonstrate the presence of that genotoxic agent in human blood and reaction of that agent with human (lymphocyte) DNA. However, the styrene database presents important inconsistencies with the default assumption.

In general, genotoxic agents cause increased tumors in more than one species and/or at more than one site (EPA, 1996). Frequently there is good evidence of increased malignant tumors at multiple sites. None of these attributes fit the styrene database. Increased malignant tumors (lung) were found only in high exposure female mice and only in one of 5 studies. In three of the five studies increased benign (and combined benign and malignant) lung tumors were identified as the only site, but the increase was not consistent as to the sex affected. No tumor sites were consistently increased in the 8 rat studies. The overall body burden of SO in rats and mice in the chronic studies does not support an SO-mediated genotoxic mode of action. SO levels, as measured by blood SO, were higher in at least some of the rat studies (up to 1000 ppm or 1000 mg/kg/day styrene) than in the mouse studies where increased lung tumors were observed. This suggests that target organ metabolism, not circulating blood levels of SO, is important for tumor formation. The metabolism of styrene to SO occurs to a greater extent in mouse lungs than in rat lungs and to a greater extent in mouse lung than in mouse liver, suggesting that mouse lungs are more likely to sustain genotoxic reactions from SO than are rat lungs or mouse liver. However, the limited data available indicate that styrene is not genotoxic in mouse lung tissue; i.e., no increase in CAs in mouse lung tissue and negative in A/J mouse screening assay. In addition, styrene exposure results in very low levels of DNA adducts (<1 per 107 nucleotides) in mouse lungs, which are not unique to lung tissue and do not occur at a greater frequency than in mouse liver or rat lung.

Thus while styrene and SO have some genotoxic properties, the overall evidence does not support a genotoxic mode of action for the induction of mouse lung tumors.

8. Mode of Action Conclusions

The mode of action data for styrene fit with a non-genotoxic action as a result of the high metabolism of styrene by CYP2F2 in mouse lung Clara cells. Data have been presented which show the specificity of the metabolism in the target cell, cytotoxicity in the target cell, increased cell replication in the target cell, leading to hyperplasia and increased tumors at the end of the study. Inhibition of the styrene metabolism eliminated the cytotoxic effects of styrene exposure in mouse lung (and nasal tissue). Styrene has been shown to have a weak genotoxic potential, and measures of genotoxicity in mouse lung are negative.

Although the cell of origin for the styrene-induced mouse lung tumors has not been established, no difference in tumors between the styrene-exposed and control mice have been seen, further suggesting a promotional-type increase in spontaneous tumors.

Based on the tumor data for styrene, evidence of cytotoxicity and cell proliferation dependent on the mouse lung specific metabolism of styrene, and the lack of evidence of

a genotoxic mode of action, it is concluded that styrene induces tumors in mouse lung tissue by a non-genotoxic mode of action.

9. Human Relevance

The carcinogenic potential of styrene has been studied in a number of cohorts of workers exposed to styrene in the workplace. No consistent evidence of an increased risk of cancer from styrene exposure is found in these studies. In the reinforced plastics industry, past exposures may have consistently exceeded 100 ppm. Environmental exposures are not greater than the low ppb range, and most are below 1 ppb.

The sharp contrast between the positive mouse tumor data and the negative rat and human data is not surprising in light of the mode of action for the styrene-induced mouse lung tumors. Exposure of mice to styrene results in metabolism by a number of cytochrome P450 isoforms. In lung and nasal tissue, CYP2F appears to be the most important, leading to a high production of R-SO and to subsequent metabolites. Cytotoxicity is seen in both tissues. In nasal tissue, damaged olfactory cells are replaced with respiratory-like cells which appear to be resistant to effects from styrene exposure. In lungs, Clara cells are damaged and rapidly replaced by more Clara cells which in turn are damaged and replaced, eventually leading to an increase in lung tumors.

Rat lung Clara cells have much lower levels of CYP2F, which leads to a significantly lower production of R-SO in rat lung; this explains why exposure of rats to much higher levels of styrene does not result in cytotoxicity or increased lung tumors. Humans have even fewer Clara cells than rats and those Clara cells have much less CYP2F (human equivalent of CYP2F) than rats. Limited human studies show little if any production of SO from styrene by CYP2F and little if any SO production by microsomes prepared from human lung tissue. Thus, neither cytotoxicity nor lung tumors are expected in humans exposed to environmental or occupational exposures to styrene. This is supported by a lack of styrene-related increased lung tumors in the cohort mortality studies of workers exposed to styrene.

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The Toxicological Profile on Styrene compiles an extensive amount of literature data on the health effects of styrene. The document has captured the relevant health effects of styrene in both humans and animals and relevant pharmacokinetic and exposure information to put the potential for human exposure into context. The identified data gaps are also helpful for guiding future research to evaluate risk from exposure to styrene. Overall the profile addressed the majority of goals that ATSDR outlined for each of the chapters in the document. However there were some areas in the text that needed clarification or additional references, or that should be moved to other sections or deleted because of redundancy. Those specific comments are annotated in the hard copy of the profile. Below is a summary of my comments in response to specific issues raised by the guidelines for reviewing the profile. If a specific chapter or section is not listed below, only minor comments were marked in the text, but there were no major issues.

Children's Health Issues

The document discusses the general issues relevant to child health and identifies the lack of data in several areas. However one area that needed to be discussed more thoroughly or at least put into context was information included in Section 6.6, Exposure of Children. The text only mentioned exposure of breastfed babies without comparison to formula-fed babies and their potential exposure. In addition the sample size of 12 is small. Nursing has many benefits for infants, and this should be added along with the limitation and lack of information on infants' other sources of exposure, which may be greater than breastmilk.

Chapter 1. Public Health Statement

The majority of the chapter was written as factual and in a style suitable for the average citizen, had answers relevant to the public, and had statements that were consistent and supported by the discussion in the remainder of the profile. There were a few areas that may need further clarification including:

1) Does the average citizen understand the units ppb for an air concentration? Should an explanation or example be included on page 3? Also the air concentrations don't seem to match those given on page 8 of Chapter 2.
2) On page 3, consider adding showering or bathing in water containing styrene as a potential exposure to styrene.
3) All the exposure routes are given, but then only health effects from inhalation are listed on page 4. A brief statement about the other exposure routes and possible effects should

be included or either a statement that the most likely health effects are from inhalation exposure.

4) On page 5, the answer to whether children are likely to have similar effects as adults doesn't seem to have any supporting documentation in the rest of the profile. The answer should just mention the lack of studies in children, and that we don't know whether their susceptibility is different. In addition, what is known about formula-fed babies? If their water is contaminated, formula-fed babies may also be exposed. Are there studies on the concentration of styrene in the powder or liquid formulas? This information needs to be listed in addition to stating that nursing infants are exposed through breastmilk. As pointed out above, mothers should not be warned about possible chemical exposures that are not put into context with possible benefits from nursing.
5) Breaking children out as a specific group in answers to the questions on the bottom of page 5 and on page 6 is slightly confusing, since the answer to whether styrene can affect children was that children would have the same effects as adults. These answers would make more sense if the chapter only stated the effects on children are not known. For example, stating that the doctor may inquire is children were exposed, limiting smoking to limit children's exposure, and a separate drinking water standard aren't consistent with children reacting the same as adults.
6) Page 6, lines 14 to 18 should be broken down into separate statements, so that the sentence isn't so long and complex.

Chapter 2. Relevance to Public Health

As a general note, the introductory paragraph of this section could be eliminated if the chapters on the production, use, and exposure were presented first. The chapter does provide a good overview of effects known to occur in humans and effects in animals likely to be relevant to humans. As noted in the profile, switching in the text between human and animals in the same paragraphs was slightly confusing. For example, on page 10, lines 17 to 20, the statements switch from rats to humans and presumably back to rats, although the sentence on lines 19 to 20 does not specify a species. The information would be more coherent if the human data was presented separately from the animal data.

Overall the exposure conditions were adequately described. There were a few examples where more information was needed. On page 10, lines 19 to 20 for the liver effect and then lines 28 on fetal death need more description. The species showing eye irritation on page 13, line 18 needs to

be given. For the vestibular effects on page 14, lines 10–11, more information needs to be given as how 87 ppm was selected as LOAEL, when on lines 19–20 vestibular effects were noted in workers at 18–36 ppm. On page 17, line 11, there appears to be an error from the Benignus (2005) paper. The text cites the 1.7 additional years of age given in Benignus for the loss in color perception, but I think the number should have been 2.9 years (see text annotation).

Chapter 3. Health Effects

Sections 3.1 to 3.3

There are minor annotations in the text concerning the human studies and animal studies. Overall the information provided seemed adequate and met goals stated in guidelines.

As for the Level of Significant Exposure (LSE) tables and figures, I assume the user's guide is given in close proximity to the tables and figures, otherwise they will be difficult to impossible to interpret. The reader should be directed to the user's guide before being directed to the tables and figures. As for categorization of effects as serious or less serious, the neurological effects in humans such as impaired color vision, slowed reaction time, and impaired vestibular function might be serious as they could affect job performance and safety. Part of the problem is only having 2 categories for effects.

The minimal risk levels (MRL)s are given in Chapter 2 and not 3, however adequate justification was given for them in that chapter. As noted in the text, subtitles should be given for each MRL for clarity. In Chapter 3 on page 75, line 34, the text states an inhalation acute-duration MRL could not be established, although one is given on page 13 in Chapter 2. In addition page 76 the text states an oral acute-duration MRL could not be established, but one is listed on page 17. These discrepancies should be corrected.

Section 3.4 Toxicokinetics

This section provided inadequate information to evaluate the role of kinetics in the toxicity of styrene. The title chapter should be changed to Pharmacokinetics since later physiologically based pharmacokinetic (PBPK) models are discussed, or the modeling should be changed to PBTK for consistency. Overall the section provides very limited discussion of the metabolism of styrene. Information lacking includes isoforms of cytochrome P450 involved in metabolism, comparison of species in vitro rates of metabolism, species differences in hydrolysis of styrene

oxide, and stereochemistry. Some of this information is given but in other sections (for example bottom of page 62).

The discussion on the actual styrene PBPK modeling was limited given the general details included on PBPK models. The text did not discuss the adequacy of the models' ability to simulate styrene pharmacokinetic data, whether the models had been validated, or the utility of the models for predicting species differences in styrene pharmacokinetics. The information would be important for determining whether the models could help extrapolate animal toxicity data to humans. There are also suggested changes to Figure 3-4, the conceptual representation of a PBPK model.

Section 3.5.1 Mechanisms of Action

The information given for the pharmacokinetic mechanisms does not seem relevant. This section should address the species differences in epoxide concentrations, activation versus detoxication of styrene oxide, and tissue-specific metabolism in target tissues. For example the information on lines 29 to 34 on page 62 belong in this section. Other specific examples are annotated in the text.

Section 3.9 Interactions with Other Chemicals

Although the text repeatedly mentions that the effects in humans are confounded by exposure to butadiene or acrylonitrile, references to studies in animals looking at interactions were not included. Relevant references that show metabolic interactions between styrene and these compounds are listed below:

Laib, R. J., Tucholski, M., Filser, J. G., and Csanady, G. A. (1992). Pharmacokinetic interaction between 1,3-butadiene and styrene in sprague-dawley rats. Arch Toxicol **66,** 310-314.

Leavens, T. L., Farris, G. M., James, R. A., Shah, R., Wong, V. A., Marshall, M. W., and Bond, J. A. (1997). Genotoxicity and cytotoxicity in male b6c3f1 mice following exposure to mixtures of 1,3-butadiene and styrene. Environ Mol Mutagen **29,** 335-345.

Leavens, T. L., Moss, O. R., Turner, M. J., Janszen, D. B., and Bond, J. A. (1996). Metabolic interactions of 1,3-butadiene and styrene in male b6c3f1 mice. Toxicol Appl Pharmacol **141,** 628-636.

Normandeau, J., Chakrabarti, S., and Brodeur, J. (1984). Influence of simultaneous exposure to acrylonitrile and styrene on the toxicity and metabolism of styrene in rats. Toxicol Appl Pharmacol **75**, 346-349.

Section 3.10 Populations that are Unusually Susceptible

This section should consistently reference the supporting text as with the reproductive effects for styrene-exposed workers.

Section 3.11 Methods for Reducing Toxic Effects

The one issue that deserves attention in this section is the suggestion of ipecac as treatment to reduce absorption, since this is controversial. The information given on p 82, lines 17 to 19, should be substituted for the current text.

Section 3.12 Adequacy of the Database

The current form of this section reads more like a brief synopsis of the chapter. The section needs to be rewritten to just identify the data needs for effects similar to the chapter on exposure. In addition as pointed out previously, the section also mistakenly states that acute-duration inhalation and oral MRLs were not established.

Chapter 6. Potential for Human Exposure

The one issue that should be addressed in this chapter is the exposure from nursing that has been raised previously. The text needs to include possible exposure of infants via formula and baby food for comparison. Breastfeeding has been shown to be beneficial to infants compared to formula, and statements concerning contaminants in breastmilk that are not put into context could persuade mothers to not breastfeed their children.

SUMMARY COMMENTS RECEIVED FROM

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Jean Rabovsky, Ph.D.

1 August, 2007

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Lexington, MA 02421-3136

Re: Review of ATSDR Profile on Styrene

Dear Ms. Waite:

Thank you for the opportunity to review the Agency for Toxic Substances and Disease Registry (ATSDR) *Toxicological Profile for Styrene, Draft 2* (referred to hereafter as *the Profile*). The document was prepared under contract by Syracuse Research Corporation (referred to hereafter as the authors) and dated 12 July 2007. As we discussed during previous phone calls, my review is focused on those areas for which I believe I can make the best contribution. Also, given the short review time (20 hours) I believe it would be prudent to carefully consider fewer sections rather than superficially scan the total document. Accordingly, the reviewed sections are Inhalation Minimal Risk Levels, Inhalation Neurotoxicity, Cancer Bioassay and Genotoxicity. At the beginning of the review a comment is included on Children's Health, as directed by the *Guidelines for Peer Review of ATSDR's Toxicological Profiles* (referred to hereafter as the *Guidelines*). At the end of the review are short sections on Other Comments and Typographical errors. A Reference list is also provided. The citations in this list were not found in the Reference section of the *Profile*.

The lack of commentary on the remaining sections does not imply concurrence with or disagreement with the contents of those sections.

My approach to reviewing the *Profile* is to keep in mind protection of public health to the maximum extent possible. I believe this approach is consistent with the *Guidelines* that were provided by ATSDR. I understand the goal of the *Profile* is to provide a document that is comprehensible to the general public and therefore detailed technical information is avoided. However, in those cases where I believe lack of such information prevents an understanding of the context in which a study is discussed, such details are included in my review. I believe inclusion of these details is in the best interest of public health protection. Recommendations follow the discussions on specific issues. I hope the comments and recommendations are helpful

to ATSDR as the final document is prepared. If you have questions or need additional information, please do not hesitate to contact me.

Sincerely yours,

Jean Rabovsky, Ph.D.

REVIEW OF ATSDR PROFILE ON STYENE

CHILDREN'S HEALTH

The *Guidelines* request feedback on the completeness of the *Profile* regarding children's health, i.e., child heath and development as well as other issues. Regarding developmental effects, this area is outside of my area of expertise and I suggest a review by someone who is knowledgeable about this area of research. The completeness and accuracy of this section is extremely important and may impact on the final minimal risk level (MRL) if this toxicity endpoint is more sensitive than neurotoxicity, the endpoint currently used in the development of the chronic MRL. The authors of the *Profile* recognize the need for additional studies on developmental toxicity (p.79). Regarding child health, in general, I am concerned from my review of the *Profile,* the calculations of acute and chronic MRLs for styrene do not take into account potentially increased adverse effects for children. In the absence of conclusive data on developmental neurotoxicity, children may represent a population that is more sensitive to the neurotoxic effects of styrene than the worker population used in the MRL calculations. Also, as discussed in the *Profile*, children's physiology is different from adults. Klaus et al (2005) also noted that exposures to the same levels may result in higher internal doses to children.

Section 6.6, p. 101, lines 18-22. Another study on exposures to volatile organic chemicals (VOCs) in children found an exposure ranking of personal > indoor > outdoor for many VOCs including styrene (Adgate, 2004).

MINIMAL RISK LEVELS (MRLs)

Sect. 2.3, pg. 12-13. The second and third paragraphs are identical, with the exception of a USEPA date (1990 or 1989d). (See also p. 7 of this letter).

Inhalation MRLs

Sect. 2.3, p. 14, lines 11-15, Acute MRL. The nasal irritation data reported by Cruzan et al (2001) is included with a discussion on nervous system function. Apparently, the *Profile*

authors want to discount the mouse nasal irritation results, although the styrene lowest observed adverse effect levels (LOAELs) for both endpoints were similar. The basis for omitting the mouse nasal irritation data, is the enhanced cytochrome P450 (CYP)-dependent formation of styrene-7,8-oxide and its decreased degradation rate in mice compared to humans. The use of toxicokinetic differences requires more discussion. From a public health perspective, the presence of similar metabolic systems (see Sect. 3.4.3, p. 56) and the presence of similar toxicity endpoints (see Stewart et al 1968, described on p. 13 of the *Profile*) suggest nasal irritation is a reasonable basis for the development of an acute MRL, provided issues of exposure levels are taken into account. A criterion for the use of a study for an MRL calculation is the choice of the most sensitive target. If the authors believe the neurotoxicity endpoint is the most appropriate, the decision should be based on criteria that go beyond one metabolic pathway that exists in mice and in humans. Regarding the respiratory tract endpoint, Moscato et al (1987, listed in the reference section but not cited in the text, see legend to references, p. 113) report two subjects with occupational asthma who exhibited serious upper respiratory symptoms when exposed to styrene).

> ***Recommendation.*** *Discuss in greater detail the upper respiratory effects of styrene in mice and in humans. Include information on the presence of a known human population (occupationally derived asthmatics) that is more sensitive to the respiratory effects of styrene. Include dose-response data and compare to the neurotoxic effects.*

Sect. 2.3, pg. 16-17, Chronic MRL. The discussion does not allow the reader to understand the neurotoxic effects are serious effects, not "less serious" as indicated in Table 3-1 (see discussion on p. 4 of this letter). A disturbance to any of the pathways that contribute to central nervous system (CNS) function may have implications throughout the lifetime of an individual. The implications of a single neurobehavioral decrement in the context of lifelong experiences can be found in Benginus et al (2005) and are discussed further in the next section of this letter.

> ***Recommendation.*** *Expand the discussion on chronic MRL based on neurotoxicity to include the serious nature of these toxic effects.*

Other agencies (Federal and state, e.g., California) have developed recommended air styrene levels for chronic environmental exposures. Examination of these exposure levels reveals differences that result from the choice of study and/or the use of different methodologies. Such information informs the reader about the nuances of such calculations and will add to their understanding of the assessment process.

> ***Recommendation.*** *Include in the section on chronic inhalation MRL development a comparison with other agencies (Federal and state) and justify the approach taken by the authors of the Profile.*

NEUROTOXICITY – INHALATION

Section 3.2.1.4

P. 30, lines 10-14. The authors conclude mental disorders and suicide were probably related to factors other than direct exposure to styrene, because the metric decreased with increasing exposure duration. A common confounder in human occupational studies is the healthy worker effect. Workers who are resistant to the effects of a toxic chemical will be more likely to remain at work, while more susceptible workers will be more likely to leave the workforce and hence the group under study. The issue here is not the basis for the specific endpoints of mental disorders and suicide but the omission of discussion about the healthy worker effect in human studies.

> ***Recommendation.*** *Discuss the concept of healthy worker effect in the context of human studies.*

P. 30, lines 31-34 and P. 31, lines 1-24. The authors present styrene doses in terms of urinary mandelic acid and phenylglyoxylic acid without explanation The explanation may be found in the section on metabolism (Sect. 3.4.3, p. 56 and Fig. 3.3). However, this information should also be in this section in order for the reader to understand the relationship between urinary mandelic and phenylglyoxylic acids and air styrene exposure. This relationship is important because it is the basis for many calculations on absorbed styrene doses, e.g., see the

analysis of Guillemin et al (1982). The following recommendation applies to the whole section on Neurotoxicity.

> ***Recommendation.*** *Explain the significance of urinary mandelic acid and phenylglyoxylic acid for styrene exposure.*

P. 32, lines 4-23, Neurobehavioral tests. The *Profile* authors describe decrements in responses to a variety of neurobehavioral tests, e.g., reaction time, digit span, memory. Among the studies, Mutti et al (1984) is included with data from specific test results. However, Mutti et al (1984) extended their analysis to the number of abnormal tests among the respondents as a function of styrene dose (i.e., the urinary styrene metabolites, mandelic and phenylglyoxylic acids). Their results exhibited a dose-response relationship over the dose range and an effect of exposure duration. Using the quantal data of Mutti et al (1984), Rabovsky et al (2001, cited in the reference section but not in the text) determined the lowest dose group was a LOAEL. As discussed in Rabovsky et al (2001) and OEHHA (2000), CNS dysfunction as a total domain provides a more sensitive analysis because the number of affected individuals at low doses may be too low to be observed with only one test. The analysis of Benginus et al (2005) also utilizes this approach by analyzing data on two neurotoxicity endpoints (choice reaction time and color-confusion index). Such an approach is consistent with biochemical studies that suggest styrene exposure may be associated with one or more neurotransmitters that control more than one CNS function.

> ***Recommendation*** *. On the topic of neurobehavioral testing, expand on the Mutti et al (1984) study to include consideration of CNS function as a total domain. Explain how such consideration may affect the development of a chronic inhalation MRL.*

Table 3-1, Levels of Significant Exposure to Styrene-inhalation, Chronic. All neurologic effects are listed as "less serious", but no objective criteria are given to justify the decision. The implication is decrements in these specific functions may not lead to serious or life-threatening conditions to the affected individuals. Proper cognitive function, however, depends on many pathways and a decrement in one function may impact on the expression of another one. Also, impaired cognitive function may affect an individual's ability to function on a daily basis, e.g., work duties, self-care or social interactions. The negative implications of decreased reaction time

and deficiencies in color-vision discrimination are discussed by Benignus et al (2005) and the *Profile* authors. Reduced reaction times have implications for economic level and personal performance, and the inability to accurately discriminate between colors has implications for work and school performances.

> ***Recommendation.*** *Consider the adverse effects of chronic styrene exposure as serious effects unless substantiated by objective criteria. (This recommendation is applied to the concept of "less serious" effects, which appear throughout the Profile.)*

The Draft 2-Summary Table (Deliverable No. 34) appears to be a combination of Tables 3-1 and 3-2 found in Deliverable No. 28. However, a "legend" page that includes footnote (a,b,c) explanations was not found. The legend page at the beginning of the table in Deliverable No. 34 only defines abbreviations. Also, an entry for the analysis of Benginus et al (2005) was not found in the Summary Table in Deliverable No. 34. This analysis is the basis for the chronic MRL (see Table 3-1 of Deliverable No. 28). Why was this study dropped from the Summary Table in Deliverable No. 34?

> *If the Draft 2-Summary Table replaces the original Tables 3-1 and 3-2, include footnote definitions. Enter the Benginus et al (2005) analysis in this table.*

<u>Table 8-1. Regulations and Guidelines Applicable to Styrene.</u> Regulatory and recommended exposure levels are listed for international and United States Federal agencies. No state standards are listed. At least one state, California, has adopted a styrene inhalation reference exposure level (OEHHA 2000).

> ***Recommendation.*** *Where state exposure levels for styrene have been adopted, list the values in Table 8-1.*

For the medium of air, different units are used, i.e., mg/m^3 and ppm. A reader who is unfamiliar with the calculations used to obtain these numbers, will be unable to directly compare them.

> ***Recommendation.*** *Convert all air levels to one set of units. Alternatively, provide two columns, one for ppm and one for mg/m^3. A legend that gives the formula for the conversion (see Table 4-2 of the Profile) will be helpful.*

CANCER

Animal studies

Sect. 3.2.1.7, pg. 43-44. The *Profile* authors discuss laboratory animal studies carried out under the inhalation route of exposure. In keeping with the ATSDR approach the authors provide summaries of findings and conclusions rather than detailed descriptions of study results and analyses. Some omitted details, however, detract from the overall picture of laboratory animal styrene-related carcinogenicity, and they are discussed below.

P. 43, lines 21-23. The authors of the *Profile* conclude the increased mammary tumor incidence was not dose-dependent because two out of six responses did not smoothly follow the dose-response curve. Perfect fits of experimental animal bioassay data with model-derived curves are difficult to obtain and it is unfair to discount the total study because two points do not meet expected results. IARC (1994) reported a positive trend (Cochran-Armitage) for the malignant mammary tumor data reported by Conti et al (1988).

P. 43, lines. 26-28. The *Profile* authors note the inconsistency between the rat mammary tumor data of Conti et al (1988) and that reported by Cruzan et al (1998). Specifically, the former study described a dose dependent increase in incidence, whereas the latter study described a decrease in incidence although the doses in the latter study were higher and the exposure duration was longer. As discussed by the authors of the *Profile*, the studies may not be directly comparable. Styrene is associated with non-cancer toxicities (see previous sections of the *Profile*) that may compete with the carcinogenic responses at the higher exposure levels. Hence the rats described in the Conti et al (1988) study, which were exposed to lower styrene levels, may not have been as susceptible to the non-cancer toxicities as the rat exposed to the higher levels in the Cruzan et al (1998) study.

> ***Recommendation.*** *Expand the discussion on rat mammary tumors. Include in the discussion the presence of an overall dose-response in the data reported by Conti et al (1988). Discuss the apparent inconsistency between the data of Conti et al (1988) and Cruzan et al (1998) in terms of the effect of higher styrene doses on different toxicity endpoints.*

Pg. 43-44, lines 33 to end. The *Profile* authors minimize the relevance of the mouse lung tumors reported by Cruzan et al (2001) by referring to the reviews of Cruzan et al (2002) and IARC (2002). The issues are toxicokinetic/metabolism data that show differences between humans and mice in Clara cell styrene metabolism, differences in styrene-7,8-oxide levels in terminal bronchioles and the effect of styrene-7,8-oxide (the putative carcinogenic metabolite) chirality on the carcinogenic response. In terms of the relationship between styrene metabolism and a specific toxicity endpoint – in this case, cancer - the differences between mouse and human data as described on pg. 43 and 44 require more discussion. According to Cruzan et al (2001) immunohistochemical staining data identified a preponderance of alveolar Type II cells compared to bronchiolar Clara cells in the mouse lung tumors. The authors of the *Profile* discuss only Clara cell metabolism and omit the fact that alveolar Type II cells also possess metabolic activity, including CYP-dependent enzymes. The CYP-dependent pathway is considered the primary metabolic mechanism for styrene carcinogenicity, although other metabolic pathways may be involved. While the levels of CYP-dependent monooxygenases in Type II cells may be less than in Clara cells, the predominance of Type II cells in the mouse lung tumors (Cruzan et al, 2001) suggests that greater attention be paid to the role of the alveolar region in styrene-related lung tumorigenicity. Perhaps the CYP-dependent pathway is only one mechanism for the metabolic formation of a lung tumorigen.

> *In the discussion of the mouse lung tumor data, discuss the potential role of alveolar Type II cells, which were reported by Cruzan et al (2001) to be a major cell type in these tumors.*

The enantiomeric specificity of the tumorigenic response is more complex than the R/S ratios of styrene-7,8-oxide levels in the lungs of mice (2 to 3) and rats (< 1) (see p. 44, lines 7 to 9). The *Profile* authors state the R- enantiomer possesses higher pneumotoxicity than the S-enantiomer and conclude mice are more sensitive than rats to styrene by a mechanism not relevant to humans. The pneumotoxic endpoint is not defined. Other studies looked at endpoints associated with mutagenicity and the results of the relation between R- and S forms of styrene-7,8-oxide were not straightforward. Sinsheimer et al (1993) reported an R/S ratio less than one for chromosomal aberrations and sister chromatid exchanges in the bone

marrow of mice receiving styrene-7,8-oxide by intraperitoneal (ip) injection. Bacterial mutagenicity studies, however, revealed R/S ratios for the response to styrene-7,8-oxide as greater than one (Pagano et al, 1982; Seiler, 1990; Sinsheimer et al., 1993).

Discuss in more detail the varying responses of different toxicity endpoints to different styrene-7,8-oxide enantiomers.

From a public health perspective, the presence of chemical related tumors in experimental animals is cause for concern about carcinogenicity in another animal, e.g., humans.

Figure 3-1.

Levels of Significant Exposure to Styrene-Inhalation-Chronic (≥365 days). Under the endpoint, cancer, the authors show 160 ppm styrene for mouse. According to the legend, this dose represents the lowest tested dose that produced a tumorigenic response. The data reported by Cruzan et al (2001), however, show tumorigenic responses at styrene levels as low as 40 ppm (male mice) and 16 ppm (female mice). These tumorigenic responses were adenomas, which are benign tumors. Lung adenomas are considered precursors to lung adenocarcinomas and their presence among the styrene treated mice is not trivial.

Recommendation. *Lower the level of significant exposure for cancer due to chronic exposure to styrene.*

GENOTOXICITY

Sect. 3.3, pg. 51-52, lines 27 to end. Results of genotoxicity studies are important for understanding carcinogenicity data because of the basic assumption that damage to genetic material will ultimately be expressed as neoplastic events in all species. The *Profile* describes, in summary form, numerous genotoxicity studies in humans, laboratory animals and microorganisms. Significant details, however, are missing. While the *Guidelines* are not meant to "present extensive details for users to weigh all the evidence themselves..." (ATSDR, 2004), the ability of a reader to understand the relationship between genotoxicity studies and carcinogenicity requires additional information in this section.

Recommendation. *Genotoxicity tests. Explain the principles underlying the genotoxicity tests that are discussed in this section. Discuss the advantages and disadvantages that are associated with in vivo and in vitro tests.*

P. 51, lines 27-36 and p. 52, lines 1-16. Human studies. Among the human studies, differentiate between those performed on cells isolated from the blood of styrene exposed workers and those performed on cells obtained from unexposed humans and then treated with styrene.

P. 52, lines 25-30. Putative mutagen. Specifiy which metabolic system is under discussion. The major mechanism by which styrene is metabolized to styrene-7,8-oxide (considered to be the major mutagen) is the CYP-dependent monooxygenase system. However, oxidative metabolism through non-CYP-dependent enzymes has been described (Belvedere and Tursi 1981, Norppa and Tursi, 1984, Oritz de Montellano and Catalano 1985, Guengerich 1990, Tuynman et al 2000). Although these non-CYP systems may operate at rates lower than the CYP enzymes, in organs with low CYP-dependent monooxygenase levels, they may play an important role. Other evidence suggests another styrene metabolite, i.e., styrene-3,4-oxide, may also play role in the mutagenicity of styrene (Pantarotto et al 1978, Bakke and Scheline, 1970, Pfaffli et al 1981, Watabe et al 1984, Carlson et al 2001). While the section on Metabolism (Sect. 3.4.3, pg. 56-57), contains a couple of sentences on the presence of other styrene metabolites, there is no discussion on the relationship between these metabolites and styrene mutagenicity. Such discussion is necessary in the section on Genotoxicity to better understand the role of genotoxic metabolites in styrene mutagenicity.

OTHER COMMENTS

P. 126-130, References. Documents from the United States Environmental Protection Agency (USEPA) are listed under EPA. The term, EPA implies there in only one such agency in the

country. In at least one state, California, there is also an agency named Environmental Protection Agency, abbreviated CalEPA.

Recommendation. *Specify the Federal Environmental Protection Agency as USEPA.*

CD References. The references for Rabovsky et al (2001) and Moscato et al (1987) were not found not on the CD reference list.. These references are in Section 9 of the *Profile*, although they are not cited in the text. They were, however cited in this letter.

> ***Recommendation.*** *Enter the Rabovsky et al (2001) and Moscato et al (1987) citations in the CD list of references. When the papers are discussed in the next draft of the Profile, use the "*" to indicate their citations in the text.*

On the CD (A-K), the second pdf file is damaged and cannot be opened. A rapid scan of other pdf files did not reveal any problems, but a file by file search was not attempted.

> ***Recommendation.*** *Check and repair damaged pdf files.*

Typographical errors that need correction.

Sect. 2.3, pg. 12-13. The second and third paragraphs are identical.

Sect. 3.2, p. 21, lines 6, 9, 14. The start parentheses are missing.

Sect. 3.4.3, p. 56, lines 20-35, Metabolism. The abbreviations, MA and PGA are introduced without definition. Include the full chemical names the first time they are used. The full names, mandelic acid and phenylglyoxylic acid, appear in earlier sections but without abbreviations.

Figure 3-3. Metabolic Pathways of Styrene. According to the structure for phenylglyoxylic acid, carbon has five bonds and is incorrect. The correct structure can be found in IARC (2002, p.481).

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SECTION II

ADDITIONAL REFERENCES AND DATA SUBMITTED BY THE PEER REVIEWERS

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Introduction

The Styrene Information and Research Center (SIRC) is pleased to present this 10th volume of its journal, *The SIRC Review*. Since producing our first issue in 1990, SIRC has periodically published *The SIRC Review* to serve as a compendium of recent research on styrene, bringing together the best science on the chemical. The readership of *The SIRC Review* includes persons with interests in styrene from many sectors – regulators, scientists, industry, academia, and communications. It is SIRC's hope that this journal continues to aid these people in assessing the potential health and environmental effects of styrene.

Since our last issue, SIRC has continued its active research program on the health effects of styrene, and this new volume reflects SIRC's ongoing mission to expand styrene's already substantial scientific database. Following our past efforts to assemble commonly-themed reports, Volume 10 focuses on two key areas of research – reproductive and developmental toxicity in section one, and neurobehavioral effects in section two.

Of particular note in the first section is the executive summary of an important monograph from the National Toxicology Program's Center for the Evaluation of Risks to Human Reproduction (CERHR). The CERHR recently issued its final report on an expert panel review it sponsored on the potential reproductive and developmental toxicity effects of styrene. The CERHR review, which SIRC followed with great interest, provided a thorough assessment of the available data on styrene, and offers a sound, independent conclusion on its potential to cause human reproductive effects. The report's summary conclusion, with which SIRC concurs, noted that "NTP judges the total scientific evidence sufficient to conclude that it is unlikely that styrene exposures to the general population or to styrene-exposed workers in the United States will adversely affect human development or reproduction."

We have included the NTP report summary as a preface to two articles, both originally published in *Birth Defects Research*, on which much of the CERHR's deliberations were based: "Developmental Neurotoxicity Study of Styrene by Inhalation in Crl-CD Rats" and "Two Generation Reproduction Study of Styrene by Inhalation in Crl-CD Rats," both by Cruzan *et al.* These two studies, conducted by WIL Laboratories of Ashland, Ohio, represent the most sophisticated data to date on styrene reproductive and developmental toxicity effects. The developmental neurotoxicity study concluded that there is no specific effect on nervous system development observable at styrene exposures up to 500 ppm. The reproductive study confirmed previous observations of slight body weight effects of styrene exposure at 500 ppm or greater in rats and further demonstrated a lack of styrene effects on gonadal function, reproductive performance, and offspring survival. This enhanced the conclusions of an earlier 3-generation reproduction study of styrene in drinking water. SIRC provided funding for these studies, along with the Styrenics Steering Committee in Europe and the Japan Styrene Industry Association.

In the second section are two articles reprinted from the *American Journal of Industrial Medicine*: "Olfactory Function in Workers Exposed to Styrene in the Reinforced-Plastics Industry" by Dalton *et al.*, and "Exposure Assessment for Study of Olfactory Function in Workers Exposed to Styrene in the Reinforced-Plastics Industry" by Lees *et al.* These studies addressed previous data that suggested a potential for styrene exposure to affect the sense of smell. Working with a cohort in the comparatively highly-exposed reinforced plastics industry, Dalton *et al* concluded that there was no evidence to suggest that styrene is an olfactory toxicant in humans.

In addition to the reprinted articles, the second section also includes three original articles that are being published for the first time in *The SIRC Review*. These articles reflect SIRC's efforts to better understand the potential neurotoxicolgical effects of styrene, in particular on workers exposed to styrene. Bagirzadeh *et al.'s* review of the neuropsychological effects data updates earlier reviews, including those published previously in this journal. Drs. Good and Nichols of Ohio State University have provided two articles on potential color vision

cate that exposure to styrene can cause some types of DNA damage, it is uncertain whether genetic mutations are induced. In a recent review of the effects of styrene on DNA, Henderson and Speit (2005) concluded that there is no clear evidence that gene mutations result from worker exposures to styrene. Therefore, it is unlikely that occupational styrene exposures would lead to infertility or genetic disorders in subsequent generations.

ARE CURRENT STYRENE EXPOSURES IN THE U.S. GENERAL POPULATION HIGH ENOUGH TO CAUSE CONCERN?

Probably Not. It is estimated that styrene exposure in nonsmokers is less than 0.3 μg/kg body weight/day. Smokers are estimated to be exposed to 3 μg/kg body weight/day. These exposures are approximately 1 million-fold and 100,000-fold less, respectively, than doses that showed no adverse reproductive or developmental effects in laboratory animals.

ARE CURRENT U.S. OCCUPATIONAL EXPOSURES TO STYRENE HIGH ENOUGH TO CAUSE CONCERN?

Probably Not. Occupational exposures to styrene can be considerably higher than exposures to the general population. However, in animal studies, doses of 600 ppm by inhalation or 300 mg/kg body weight/day by oral gavage did not result in developmental toxicity. In reproductive studies, doses of 500 ppm by inhalation or 250 ppm in drinking water did not result in reproductive toxicity. These styrene doses are approximately 6-fold to 200-fold greater than the averages for occupational exposures in the United States.

The NTP reached the following conclusions based on estimates of general population exposure, information on occupational exposures, and studies in laboratory animals (Figure 3).

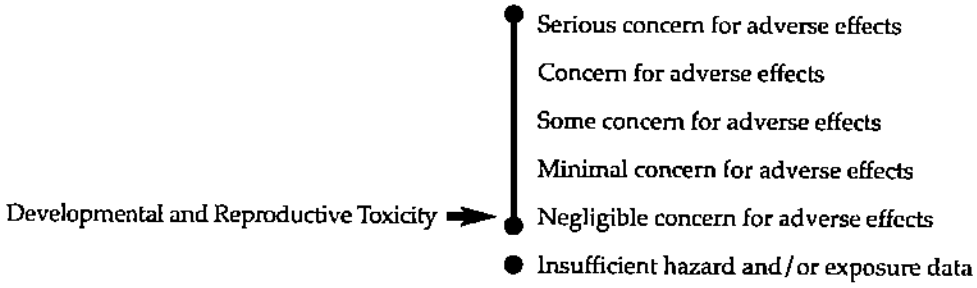
The NTP concurs with the CERHR Styrene Expert Panel that there is negligible concern for adverse developmental and reproductive effects in humans exposed to styrene, including exposures to the general population and exposures in the workplace.

This conclusion is based on the low levels of estimated human exposures to styrene in the general population and in the workplace. No clear evidence of developmental or reproductive toxicity effects in experimental animals has been reported, even at comparatively high doses of styrene. Further, there have been no reports of an association between styrene exposures and developmental or reproductive toxicity in humans.

These conclusions are based on the information available at the time this brief was prepared. As new information on toxicity and exposure accumulate, it may form the basis for either lowering or raising the levels of concern expressed in the conclusions.

FIGURE 3

NTP conclusions regarding the possibilities that human development or reproduction might be adversely affected by exposure to styrene



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APPENDIX I

NTP-CERHR STYRENE EXPERT PANEL

A 10-member panel of scientists covering disciplines such as toxicology, occupational exposure, and epidemiology, was recommended by the Core Committee and approved by the Associate Director of the National Toxicology Program. Prior to the expert panel meeting, the panelists critically reviewed articles from the scientific literature, as well as a variety of other relevant documents. Based on this material they identified key studies and issues for panel discussions. At a public meeting held June 1-3, 2005, the expert panel discussed these studies, the adequacy of available data, and identified data needed to improve future assessments. The expert panel reached conclusions on whether estimated exposures to styrene might result in adverse effects on human reproduction or development. Panel conclusions were based on the scientific evidence available at the time of the meeting. The expert panel report was made available for public comment on July 18, 2005, and the deadline for public comments was September 1, 2005 (*Federal Register* Vol. 70, Number 139, pp 42064-42065). The Expert Panel Report on Styrene is provided in Appendix II and the public comments received on the report are in Appendix III. Input from the public and interested groups throughout the panel's deliberations was invaluable in helping to ensure completeness and accuracy of the report. **The Expert Panel Report on Styrene is available on the CERHR website <http://cerhr.niehs.nih.gov>.**

CERHR STYRENE EXPERT PANEL:

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George P. Daston, PhD	The Procter & Gamble Company
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Two Generation Reproduction Study of Styrene by Inhalation in Crl-CD rats

*George Cruzan[1] *, Willem D. Faber[2], Keith A. Johnson[3], Linda S. Roberts[4], Juergen Hellwig[5], Ed Carney[1], John T. Yarrington[6], Donald G. Stump[6]*

This study was conducted to evaluate the potential adverse effects of styrene on reproductive capability from whole-body inhalation exposure of F_0 and F_1 parental animals. Assessments included gonadal function, estrous cyclicity, mating behavior, conception rate, gestation, parturition, lactation, and weaning in the F_0 and F_1 generations, and F_1 generation offspring growth and development. Four groups of male and female Crl:CD(SD)IGS BR rats (25/sex/group) were exposed to 0, 50, 150, and 500 ppm styrene for 6 hr daily for at least 70 consecutive days prior to mating for the F_0 and F_1 generations. Inhalation exposure for the F_0 and F_1 females continued throughout mating and gestation through gestation day 20. Inhalation exposure of the F_0 and F_1 females was suspended from gestation day 21 through lactation day 4. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117, and 300 mg/kg/day (divided into three equal doses, approximately 2 hr apart). These oral dosages were calculated to provide similar maternal blood peak concentrations as provided by the inhalation exposures. Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5. Styrene exposure did not affect survival or clinical observations. Rats in the 150- and 500-ppm groups in both parental generations gained weight more slowly than the controls. There were no indications of adverse effects on reproductive performance in either the F_0 or F_1 generation. Male and female mating and fertility indices, pre-coital intervals, spermatogenic endpoints, reproductive organ weights, lengths of estrous cycle and gestation, live litter size and postnatal survival were similar in all exposure groups. Additionally, ovarian follicle counts and corpora lutea counts for the F_1 females in the high-exposure group were similar to the control values. No adverse exposure-related macroscopic pathology was noted at any exposure level in the F_0 and F_1 generations. A previously characterized pattern

Reproductive performance and offspring postnatal survival prior to weaning were not adversely affected by styrene exposure. Pre-weaning pup weights were unaffected by styrene exposure for the F_1 generation.

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Funded by: Styrene Information and Research Center (SIRC), Arlington, VA

of degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates occurred in the F_0 and F_1 generation animals from the 500-ppm group. In the 500-ppm group, F_2 birth-weights were reduced compared to the control and F_2 offspring from both the 150- and 500-ppm exposure groups gained weight more slowly than the controls. Based on the results of this study, an exposure level of 50 ppm was considered to be the NOAEL for F_0 and F_1 parental systemic toxicity; the NOAEL for F_0 and F_1 reproductive toxicity was 500 ppm or greater. *Birth Defects Res* B 74:211-220, 2005 ©2005 Wiley-Liss, Inc.

INTRODUCTION

Styrene (CAS no. 100-42-5) is a commercially important monomer, which is used in the manufacture of polystyrene products (packaging, insulation, etc.), acrylonitrile-butadiene-styrene (ABS) products (appliance cases, automotive parts, etc.), synthetic rubber, and reinforced plastics. Exposure to the general population occurs at levels of micrograms per day from ambient air and intake of food (IARC, 2002).

The reproductive and developmental effects of styrene have been extensively reviewed by Brown et al. (2000). Reports of styrene-related effects on human reproduction are limited and conflicting. A large study of US women concluded that styrene exposure did not affect menstrual cycle (Lemasters et al., 1985); however, Cho et al. (2001) concluded that exposure to styrene increased the risk of menstrual cycles longer than 35 days. One study (Jelnes, 1988) suggested increased sperm abnormalities in workers exposed to high levels of styrene in the reinforced plastics industry. A later study of 23 workers (Kolstad et al., 1999) found no effect on sperm abnormalities, but reported a decrease in sperm density during the first 6 months of exposure to styrene in the reinforced plastics industry. In a study of 220 male reinforced plastics workers exposed to high levels of styrene, there was no relationship between exposure and time to pregnancy of their partners (Kolstad et al., 2000). A study of female reinforced plastics workers reported a possible decrease (4%) in birth weight of offspring of mothers exposed to styrene above 80 ppm and other solvents (Lemasters, 1989). Birth weights were taken from mothers' memory, not birth records, and the difference was not statistically significant.

No effects on fertility or reproduction were found in three generations of male and female Sprague-Dawley rats exposed to 125 or 250 ppm styrene in their drinking water (Beliles et al., 1985). The concentration was limited by the solubility of styrene in water (approximately 300 ppm). Water consumption was significantly reduced in both groups compared to controls, indicating taste aversion. While this study demonstrated no effects on fertility, gestation, or reproduction, its value for risk assessment is limited due to the low doses achieved (<25 mg/kg/day).

No effects on ovarian or testicular pathology have been reported in several of the subchronic or chronic toxicity studies in rats (500 to 2,000 mg/kg/day gavage, 50-1,500 ppm inhalation) and mice (150-300 mg/kg/day gavage, 20 to 200 ppm inhalation) (NCI, 1979; Cruzan et al., 1997, 1998, 2001; Roycroft et al., 1995). On the other hand, testicular pathology and decreases in sperm count were reported in rats treated with 400 mg/kg/day styrene by gavage for 60 days (Srivastava et al., 1989). Decreased free testosterone in plasma was reported in pre-pubertal male C57BL/6 mice exposed to 50 mg/l styrene in drinking water for 4 weeks (12 mg/kg/day). There were no effects on body weight, testis weight, plasma cortisone, or plasma luteinizing hormone (Takao et al., 2000).

Because data on the reproductive effects of styrene were limited, a two-generation reproduction study was conducted via whole-body inhalation according to current regulatory guidelines. Developmental neurotoxicity evaluation of selected offspring from the second generation are reported in an accompanying article (Cruzan et al., 2005, this issue). In most reproduction studies conducted by inhalation, exposure is stopped on day 20 of gestation and reinstated on lactation day 5 to minimize stress on the offspring from the more than 6-hr separation that would occur during inhalation exposure of the dam. Because high concentrations of styrene may cause central nervous system (CNS) depression and significant development of the CNS occurs during the first few days after birth in rats, F_0 and F_1 dams were treated orally during lactation days 1-4 at doses estimated by physiologically based pharmacokinetic (PBPK) modeling to mimic a 6-hr inhalation exposure.

MATERIALS AND METHODS

Study Design

Four groups of male and female Crl:CD rats (25/sex/group) were exposed to vapor atmospheres of styrene at 0, 50, 150, or 500 ppm for 6 hr daily for at least 70 consecutive days prior to mating. Daily vaginal smears were performed for assessment of estrous cyclicity, beginning 21 days prior to pairing. Females were paired with males on a 1:1 basis for 14 days or until evidence of mating was observed. The F_0 and F_1 females continued inhalation exposure throughout mating and gesta-

tion through gestation day 20. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117, and 300 mg/kg/day (divided into three equal doses, approximately 2 hr apart) at a dose volume of 1 ml/kg/dose. The doses were calculated to mimic the peak maternal blood level of styrene during a 6-hr inhalation exposure at the target concentration based on the PBPK model of Sarangapani et al. (2002). Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5 and continued through the day prior to euthanasia. Offspring were weaned on lactation day 21; exposure of F_1 pups began on postnatal day (PND) 22. Inhalation exposure of the F_0 and F_1 males continued throughout mating, and through the day prior to euthanasia. Spermatogenic endpoints were recorded for all F_0 and F_1 males. Ovarian primordial follicle counts and the corpora lutea counts were recorded for all F_1 females in both the control and high-exposure groups and for F_1 females in the other exposure groups that did not mate or produce offspring.

Test Material

Styrene monomer (inhibited from self-reaction by 10 ppm t-butylcatechol), CAS No. 100-42-5, was provided by Chevron Phillips Chemical Company LLP, St. James, LA. The purity and stability of the styrene were verified by gas chromotography with flame ionization detection. When present in the chromatograms, the percentage of benzene, ethylbenzene, styrene oxide, and styrene dimers was also determined. Results obtained indicated the styrene was at least 99.9% pure.

Animals and Animal Husbandry

One hundred fifteen male and 116 female Crl:CD®(SD)IGS BR rats from different colonies (to avoid sibling matings) were received from Charles River Laboratories, Inc. (Raleigh, NC) on July 24, 2001. The animals were 37 and 38 days old upon receipt, respectively. At the conclusion of the acclimation period, all available F_0 animals were weighed and examined in detail for physical abnormalities. Animals judged to be in good health and meeting acceptable body weight requirements were randomized into treatment groups by a computerized program to ensure homogeneity of treatment groups.

Until pairing, all F_0 and F_1 parental test animals were individually housed in clean, wire-mesh cages suspended above cage-board. During cohabitation, the animals were paired for mating in the home cage of the male. Following positive evidence of mating, the males were housed in suspended wire-mesh cages until the scheduled necropsy of the parental generations, and the females were transferred to plastic maternity cages with nesting material (Bed-O'Cobs®; The Andersons, Industrial Products Division, Maumee, Ohio). The dams were housed in these cages until weaning on lactation day 21. Animals were housed in accordance with the Guide for the Care and Use of Laboratory Animals. The animal care program including animal facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International).

Animals were fed PMI Nutrition International, Inc., Certified Rodent LabDiet® 5002 ad libitum. Municipal water was reverse-osmosis-treated (on-site) and delivered by an automatic watering system to individual cages ad libitum, except by water bottles when water consumption was measured during gestation and lactation. No water was available during inhalation exposure. Animals were housed throughout the acclimation period and during the study in an environmentally controlled room.

Parental Observations

All animals were observed twice daily (at least 7 hr apart) for moribundity and mortality, appearance, behavior, and pharmacotoxic signs (prior to exposure/gavage dosing for the F_0 and F_1 animals). During inhalation exposures, approximately 50% of the F_0 and F_1 animals in each group were visible through the chamber windows; the visible animals were observed for appearance and behavior at the mid-point of exposure. The F_0 and F_1 animals were also observed within 1 hr following exposure/gavage dosing. Detailed physical examinations were recorded weekly for all F_0 and F_1 parental animals throughout the study period. F_0 and F_1 females expected to deliver were also observed twice daily during the period of expected parturition and at parturition for dystocia or other difficulties.

Individual F_0 and F_1 male body weights were recorded weekly throughout the study and prior to the scheduled necropsy. Individual F_0 and F_1 female body weights were recorded weekly until evidence of copulation was observed. Once evidence of mating was observed, female body weights were recorded on gestation days 0, 4, 7, 11, 14, and 20 and on lactation days 1, 4, 5 (pre-exposure), 7, 14, and 21.

Individual F_0 and F_1 male food consumption was measured on a weekly basis (except during the mating period) until the scheduled necropsy. Individual F_0 and F_1 female food consumption was measured on a weekly basis until the start of the mating period. Female food consumption was recorded on gestation days 0, 4, 7, 11, 14, and 20 and lactation days 1, 4, 5, 7, 14, and 21. Water

consumption was recorded daily during gestation and lactation for the F_0 and F_1 females.

Assessment of Reproductive Performance

Vaginal smears were prepared daily to determine the stage of estrus for each female, beginning 21 days prior to pairing and continuing until evidence of mating was observed. For females with no evidence of mating, smearing was continued until termination of the mating period. The average cycle length was calculated for complete estrous cycles.

After a minimum of 70 days of exposure, each female was housed overnight in the home cage of a randomly chosen male from the same exposure group, avoiding sibling matings. Each mating pair was examined daily for the presence of a copulatory plug or the presence of sperm in a vaginal smear. The day when evidence of mating was identified was termed day 0 of gestation. The animals were then separated and the female was housed in an individual plastic cage with nesting material. When evidence of mating was not apparent after 14 days, the female was placed for an additional 7 days with another male of the same exposure group that had successfully mated. If no evidence of copulation was obtained after 21 days, the animals were separated without further opportunity for mating, and the female was placed in a plastic cage containing nesting material. Following the second mating period, the females were euthanized on gestation day 15 (females that mated with the second male) or post-cohabitation day 15 (females that did not mate with the second male). Pre-coital intervals were calculated according to the number of 12-hr dark cycles prior to evidence of mating.

All females were allowed to deliver naturally and rear their young to weaning (PND 21). During the period of expected parturition, the females were observed twice daily for initiation and completion of parturition and for signs of dystocia. On the day parturition was judged complete (PND 0), the sex of each pup was determined and each was examined for gross malformations; the numbers of stillborn and live pups were recorded. Individual gestation lengths were calculated using the date delivery started.

Offspring Evaluations

All pups were individually identified by application of tattoo markings on the digits on PND 0. Each litter was examined twice daily for survival and signs of toxicity. Intact offspring dying from PND 0 to 4 were necropsied using a fresh dissection technique (Stuckhardt and Poppe, 1984). A detailed gross necropsy was performed on any pup dying after postnatal day 4 and prior to weaning and for all F_1 pups dying between PND 22 and 28.

To reduce variability among litter size, 10 F_1 and F_2 pups from each litter were randomly selected of equal sex distribution, if possible, on PND 4. The remaining F_1 and F_2 offspring were weighed, euthanized by intraperitoneal injection of sodium pentobarbital, and discarded on PND 4.

Pups were individually sexed on PND 0, 4, 7, 14, and 21. F_1 pups were individually weighed on PND 1, 4, 7, 14, and 21; F_2 pups were individually weighed on PND 1, 4, 7, 11, 13, 17, and 21. The following investigations were used to assess the maturation of the selected F_1 and F_2 pups: pinna detachment, surface righting response, hair growth, incisor eruption, eye opening, balanopreputial separation, and vaginal patency. Individual pups were weighed on the day of sexual maturation. These data are presented in the companion developmental neurotoxicity study (Cruzan et al., 2005, this issue).

Weaning and Selection for F_1

Each F_0 dam and its litter remained housed together (except during inhalation exposures of the dams 6 hr/day on lactation days 5-20) until weaning on lactation day 21. On PND 21, a computerized randomization procedure was used to select two F_1 male and two F_1 female weanlings per litter. These pups were exposed to the test article for 6 hr per day beginning on PND 22. Between PND 22 and 28, 6 male and 6 female control pups died; 0 from the 50-ppm group; 1 male and 2 females from the 150-ppm group, and 1 male and 2 females from the 500-ppm group died. One male and one female from each litter, when available, were randomly selected on PND 28 to comprise the F_1 generation (16, 23, 23, and 22 males and females for 0, 50, 150, and 500 ppm, respectively). Additional rats (9, 2, 2, and 3 males and females for 0, 50, 150, and 500 ppm) were randomly chosen from the remaining male and female from each litter to make 25 male and 25 female in each F_1 exposure group (0, 50, 150, and 500 ppm). The remaining pups were euthanized on PND 28.

Ovarian and Spermatogenic Endpoint Evaluations

A bilateral evaluation of one section of each ovary was performed for F_0 females. A quantitative histologic evaluation of five sections (at least 100 μm apart) from the inner third of each ovary was conducted on all F_1 females in the control and high-dose group. This examination included enumeration of the total number of primordial follicles and corpora lutea according to the methods of Bolon et al. (1997) and Bucci et al. (1997). The primordial follicles were defined as small oocyte with a nucleus sur-

rounded by a partial or unbroken single layer of flattened to cuboidal follicular/granulosa cells. Due to the size of corpora lutea (much larger than primordial follicles), each corpus luteum was possibly sectioned and counted multiple times, resulting in a value that was larger than would be expected.

Spermatogenic endpoints were evaluated using the methods described by Nemec et al. (2004). Immediately upon euthanasia, the right epididymis of each F_0 and F_1 male was exposed, excised, and weighed. Sperm motility was assessed using the Hamilton-Thorne HTM-IVOS Version 10 (Beverly, MA) computer-assisted sperm analysis (CASA) system. Sperm morphology was evaluated by light microscopy via a modification of the wet-mount evaluation technique (Linder et al., 1992).

The left testis and cauda epididymis from all F_0 and F_1 males from all exposure groups were weighed and stored frozen (approximately -20°C). These tissues from the control and 500-ppm groups were then thawed, homogenized, and a sample was evaluated for determination of homogenization-resistant spermatid count and sperm production rate (Blazak et al., 1985).

Pathology

All F_0 and F_1 adult animals were euthanized by isoflurane inhalation and exsanguination. Vaginal smears were performed on all females on the day of euthanasia to determine the stage of estrous cycle. All surviving males were euthanized approximately 3 weeks following completion of the parturition period. All surviving females that delivered were euthanized between 6 and 10 days after weaning of their litters. Females that mated but did not give birth were euthanized on presumed gestation day 25. Females that experienced total litter loss were euthanized within 24 hr. All surviving F_1 weanlings not selected for styrene exposure were euthanized on PND 21. All F_1 weanlings exposed to styrene PND 22-27 but not chosen to become F_1 parents, were euthanized on PND 28. All F_2 weanlings not selected for behavioral evaluation were euthanized on PND 21. A complete necropsy was conducted, selected organs were weighed, and selective histopathologic examination was performed.

Statistical Methods

Analyses were conducted using two-tailed tests (except as noted otherwise) for a minimum significance level of 5%, comparing each test article-treated group to the differences. Parental mating and fertility indices were analyzed using the Chi-square test with Yates' correction factor (Hollander and Wolfe, 1999). Mean parental (weekly, gestation and lactation) and F_2 offspring body weight data, food consumption and food efficiency data, organ weight data, maternal estrous cycle data, pre-coital intervals, gestation lengths, implantation sites, unaccounted sites, ovarian primordial follicle counts, mean number of pups born, live litter size, epididymal and testicular sperm numbers, and sperm production rates were analyzed for heterogeneity of variance (Levene, 1960) and normality (Royston, 1982). If the data were homogeneous and normal, a parametric one-way analysis of variance (ANOVA) was used to determine intergroup differences (Snedecor and Cochran, 1980). If the results of the ANOVA were significant ($p<0.05$), Dunnett's (1964) test was applied to compare the control group versus all treatment groups. If the data were not homogeneous and normal, the data were analyzed by the Kruskal-Wallis (1952) nonparametric ANOVA test to determine the intergroup differences. If the ANOVA revealed statistical significance ($p<0.05$), the Mann-Whitney U-test (Kruskal and Wallis, 1952) was used to compare the test article-treated groups to the control group. Pup weights through weaning were analyzed separately by sex by a nested analysis of covariance (ANCOVA). The number of pups born was used as the covariate. The following assumptions were made regarding the ANCOVA: homogeneity of regression slopes, linear relationship between the pup weights and number of pups born, and additive group and regression effects. Histopathologic findings in the test article-treated groups were compared to the control group using a two-tailed Fisher's Exact test (Steel and Torrie, 1980).

RESULTS

Exposures

Gas chromatographic analyses of chamber atmospheres demonstrated mean daily styrene exposure concentrations of 0, 50, 151, and 499 ppm for F_0 generation and 0, 50, 153, and 501 ppm for F_1 generation. Standard deviation/mean concentration never exceeded 3.5%. Low levels of styrene oxide were detected in exposure chambers on nearly 50% of analyses. Except for 5 occasions, the styrene oxide level was less than 1 ppm, was never greater than 2.3 ppm, and was not proportional to the chamber styrene concentration. Styrene dimer was detected in chamber atmospheres of ~20% of the samples tested; levels were less than 2 ppm.

Analyses of the oral dosing formulations used during lactation days 1-4 showed homogeneity and stability; analyzed concentrations were within 12% of target.

TABLE 1

Mean Body Weights (g) of F_0 and F_1 Rats During Premating, Gestation, and Lactation

	Exposure conc. (ppm)							
	Males				Females			
	0	50	150	500	0	50	150	500
F_0								
Week								
0	266	263	264	263	190	190	190	189
1	305	301	295	287	215	210	208	203
3	378	347	359	353*	251	246	243	231*
5	437	419	413	404*	273	269	262	252*
7	483	455	454*	443*	290	288	279	269*
10	530	503	503	494	306	303	296	282*
F_0 gestation								
Day								
0					302	300	298	285
7					330	323	325	312
14					362	354	357	346
20					427	424	428	418
F_1 males								
Week								
0	110	112	103	97	97	104	93	90
1	173	173	162	151*	141	147	135	133
3	292	285	272*	262*	196	199	187	187
5	375	369	349*	336*	240	242	227	224*
7	429	421	399*	393*	267	268	253	249*
10	481	468	448	443*	293	293	278	269*
F_1 gestation								
Day								
0					296	295	279	274*
7					325	324	308	307
4					356	354	340	334*
20					428	431	410	402

* Statistically significantly different from control, $p<0.05$.

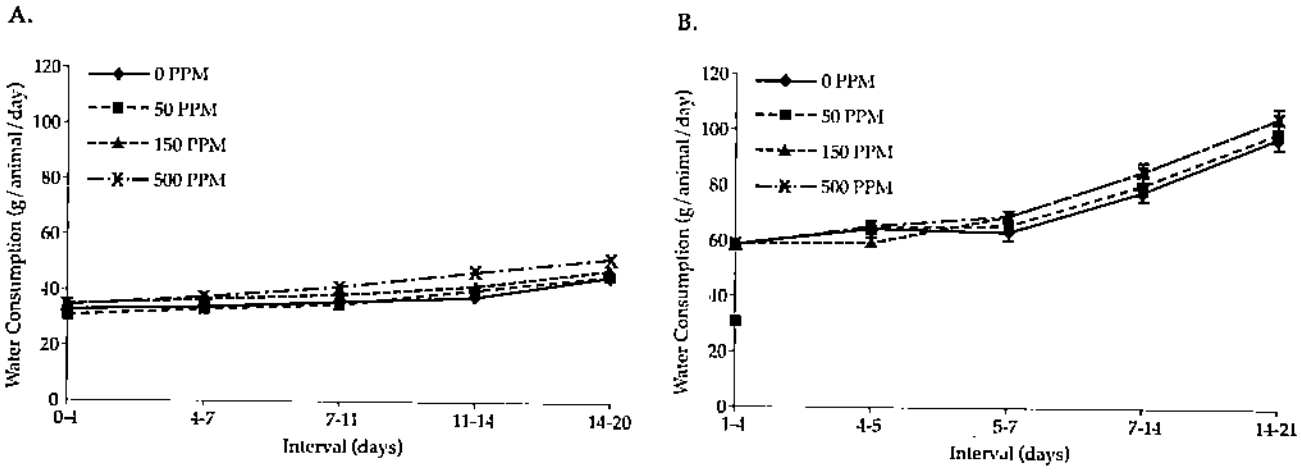
Parental Evaluations

No exposure-related clinical findings were noted at the weekly detailed physical examinations or at the observations made prior to, at the midpoint, or 1 hr following exposure in either F_0 or F_1 animals. Findings noted in the treated groups occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not exposure-related.

Body weight gain was slightly reduced in F_0 males and females at 500 ppm as evidenced by decreased mean body weights during premating weeks 3-10 (Table 1). In F_0 males exposed at 150 ppm, mean weight gain was reduced during the first week (31 vs. 39 g in the control group); body weight was significantly reduced at week 7. Body weights of F_1 males and females at 150 and 500 ppm were reduced compared to controls after exposure on PND 22-27 and remained reduced through the F_1 exposure period (Table 1). There was no effect on bodyweight

FIGURE 1

Mean water consumption of F_0 (A) and F_1 (B) females during gestation.



or bodyweight gain at 50 ppm in either the F_0 or F_1 exposure periods.

Only very minor differences in food consumption between exposed and control groups were reported (data not shown).

Gestation

Styrene exposure had no effect on body weight gain or food consumption during gestation in either the F_0 or F_1 dams (Table 1). Mean body weight reductions observed in the 500-ppm group F_1 females during gestation were attributed to the reduced body weight gain observed during the pre-mating period. At 500 ppm, water consumption during gestation was increased slightly, but statistically significantly, in both F_0 and F_1 dams (Fig. 1). There was no effect at 150 or 50 ppm.

The mean lengths of gestation were unaffected by test article exposure in the F_0 (Table 2) and F_1 (Table 3) treated rats. No signs of dystocia were noted at any exposure level.

Lactation

There was no effect of styrene exposure on body weight gain or food consumption during lactation in either the F_0 or F_1 dams (data not shown). Water consumption increased during lactation days 5-14 in F_0 dams exposed to 150 or 500 ppm, but not in F_1 dams. No effect was noted in F_0 or F_1 dams at 50 ppm.

Reproductive Performance

Exposure of F_0 females had no effect on mean estrous cycle length (Table 2). The means for all treated groups (4.2 to 5.1 days) were within the historical range (4.1 to 5.1 days), while the mean for the control females (5.8 days) was abnormally high due to 3 control females with abnormally long estrous cycles. Exposure of F_1 females had no effect on mean estrous cycle length (Table 3). No effects from exposure of F_0 or F_1 rats were observed on the mean numbers of days between pairing and coitus. Styrene exposure had no effects on F_0 or F_1 spermatogenic endpoints (Table 4). Exposure to styrene did not affect F_0 or F_1 male or female mating index, male or female fertility index, mean number of pups born, the number of former implantation sites, or the number of unaccounted sites (Tables 2, 3).

Pathology

At the scheduled F_0 and F_1 male and female necropsies, no macroscopic findings attributed to styrene were observed. Increases in relative (to final body weight) liver weights were observed in the 150- and 500-ppm group F_0 and F_1 males (data not shown). Other organ weights (absolute and relative to final body weight) were unaffected by the test article at all exposure levels.

Microscopic evaluations were performed only on tissues from all F_0 and F_1 parental animals in the 0- and 500-ppm groups and for those adult animals in the 50- and

TABLE 2

Reproductive and Offspring Parameters for F_0 Generation Rats Exposed to Styrene

	Parental exposure level (ppm)			
	0	50	150	500
Reproduction parameters (mean±SD)				
No. F_0 males/females assigned	25/25	25/25	25/25	25/25
Males/females died	0/0	0/0	0/0	0/0
Males/females euthanized[a]	0/7	0/2	0/2	0/3
Males/females completing study	25/18	25/23	25/23	25/22
Estrous cycle length (days)	5.8 ± 3.1	5.1 ± 2.0	5.1 ± 2.2	4.2 ± 0.5*
Male mating index (%)[b]	23/25 (92)	25/25 (100)	25/25 (100)	23/25 (92)
Female mating index (%)[b]	25/25 (100)	25/25 (100)	25/25 (100)	25/25 (100)
Pre-coital interval (days)	3.8 ± 3.9	2.0 ± 1.0	2.5±1.3	3.6 ± 3.5
Male fertility index (%)[c]	21/25 (84)	24/25 (96)	24/25 (96)	23/25 (92)
Female fertility index (%)[c]	22/25 (88)	24/25 (96)	24/25 (96)	25/25 (100)
Fertility determined via 2nd male	2	0	0	2
Females delivering litters	20	23	24	23
Gestation length (days)	21.9 ± 0.3	21.9 ± 0.5	21.8 ± 0.6	22.0 ± 0.3
Implantation sites	15.2 ± 1.8	14.8 ± 3.6	15.8 ± 1.8	15.3 ± 1.8
Number born/litter	14.5 ± 1.9	13.8 ± 3.2	14.9 ± 1.8	14.3 ± 1.6
Live litter size	14.3 ± 1.8	13.5 ± 3.3	14.3 ± 2.0	14.1 ± 1.6
Live birth index (%)[d]	99.0 ± 2.5	97.6 ± 4.5	96.1 ± 6.2	98.2 ± 3.5
Females with surviving pups at weaning (PND 21)	18	23	23	22
F_1 offspring parameters (mean±SD)				
Sex distribution at birth (% Males)	46.6 ± 14.9	51.0 ± 11.6	46.8 ± 12.1	48.9 ± 11.8
Survival (%)				
Birth to PND 4 (pre-culling)	98.7 ± 2.8	96.1 ± 5.9	94.5 ± 8.2	92.7 ± 17.1
PND 4-21 (post-culling)	91.1 ± 23.6	98.6 ± 4.7	98.3 ± 5.8	93.5 ± 20.8
Body weight gain (g)				
Males PND 1-4 (pre-culling)	2.6 ± 0.6	3.0 ± 1.1	2.9 ± 0.6	2.7 ± 1.1
Females PND 1-4 (pre-culling)	2.5 ± 0.6	3.0 ± 1.3	2.7 ± 0.8	2.5 ± 1.0
PND 21 body weight (g)				
Males	38.4 ± 6.3	41.4 ± 5.5	39.1 ± 5.2	38.5 ± 3.8
Females	37.6 ± 5.8	40.7 ± 7.1	37.1 ± 5.4	37.3 ± 3.9

[a]One female each from the control and 150-ppm groups were euthanized in extremis after the mating trial; one female each from the control and 500-ppm groups were euthanized during lactation due to total litter loss; all others were euthanized for pregnancy determination after no evidence of mating.

[b]Mating Index: Proportion of males/females showing evidence of mating relative to the number cohabited x 100; positive evidence of mating included vaginal sperm, copulatory plug, and or/pregnancy.

[c]Fertility Index: Proportion of pregnancies relative to the number showing evidence of mating x 100; males were considered to have sired a litter if the paired female was gravid, regardless of delivery status. Females that did not show evidence of mating during cohabitation with the first male were paired with a second male; these outcome data were used only for calculating fertility indices.

[d]Live Birth Index: Number born live relative to the total number born x 100.

*Dunnett's Test, significantly different from control, $p \leq 0.05$. No other significantly different effects.

TABLE 3

Reproductive and Offspring Parameters for F_1 Generation Rats Exposed to Styrene

	Parental exposure level (ppm)			
	0	50	150	500
Reproduction parameters (mean±SD)				
F_1 Males/females assigned (PND28)	25/25	25/25	25/25	25/25
Males/females died	0/1	0/0	0/0	0/0
Males/females euthanized[a]	0/3	0/1	0/4	0/3
Males/females completing study	25/21	25/24	25/21	25/22
Estrous cycle length (days)	4.9 ± 1.1	4.9 ± 1.4	4.8 ± 1.4	4.5 ± 0.8
Male mating index (%)[b]	23/25 (92)	25/25 (100)	22/25 (88)	24/25 (96)
Female mating index (%)[b]	24/25 (96)	25/25 (100)	24/25 (96)	25/25 (100)
Pre-coital interval (days)	4.7 ± 3.3	3.4 ± 1.7	4.6 ± 4.2	3.3 ± 3.5
Male fertility index (%)[c]	23/25 (92)	24/25 (96)	21/25 (84)	24/25 (96)
Female fertility index (%)[c]	23/25 (92)	24/25 (96)	23/25 (92)	25/25 (100)
Fertility determined via 2nd male	2	0	3	1
Females delivering litters	23	24	21	24
Gestation length (days)	22.0 ± 0.6	22.0 ± 0.4	22.1 ± 0.2	21.9 ± 0.5
Implantation sites/litter	14.3 ± 2.6	14.6 ± 2.2	14.8 ± 2.3	14.3 ± 4.4
Number born/litter	13.6 ± 2.5	14.1 ± 2.4	13.8 ± 2.2	13.4 ± 4.7
Live litter size	12.7 ± 3.6	13.7 ± 2.5	13.8 ± 2.2	13.1 ± 5.0
Live birth index (%)[d]	93.3 ± 21.3	97.1 ± 5.9	99.7 ± 1.4	95.0 ± 20.4
Females with surviving pups pups at weaning (PND 21)	21	24	21	22
F_1 Offspring parameters (mean±SD)				
Sex distribution at birth (% males)	47.1 ± 14.5	51.2 ± 12.2	47.9 ± 15.6	55.2 ± 13.8
Survival (%)				
Birth to PND 4 (pre-culling)	92.7 ± 21.5	91.2 ± 16.0	96.9 ± 7.7	89.2 ± 28.0
PND 4-21 (post-culling)	99.5 ± 2.2	96.9 ± 6.2	99.5 ± 2.2	97.7 ± 5.3
Body weight gain (g)				
Males PND 1-4 (pre-culling)	3.3 ± 0.9	3.0 ± 1.0	3.3 ± 0.7	2.9 ± 1.0
Females PND 1-4 (pre-culling)	3.0 ± 0.9	2.9 ± 0.8	3.1 ± 0.7	2.6 ± 0.7
PND 21 body weight (g)				
Males	42.6 ± 5.3	40.3 ± 5.2	38.2 ± 5.1*	38.0 ± 6.2*
Females	40.5 ± 4.7	39.1 ± 5.0	37.4 ± 4.8	35.4 ± 5.7*

[a]One female from the control group and two females from the 500-ppm group were euthanized during the lactation period due to total litter loss; all others were euthanized for pregnancy determination after no evidence of mating.
[b]Mating Index: Proportion of males/females showing evidence of mating relative to the number cohabited x 100; positive evidence of mating included vaginal sperm, copulatory plug and or/pregnancy.
[c]Fertility Index: Proportion of pregnancies relative to the number showing evidence of mating x 100; males were considered to have sired a litter if the paired female was gravid, regardless of delivery status. Females that did not show evidence of mating during cohabitation with the first male were paired with a second male; these outcome data were used only for calculating fertility indices.
[d]Live Birth Index: Number born live relative to the total number born x 100.
*Dunnett's Test: significantly different from control; $p \leq 0.05$. No other significantly different effects.

TABLE 4

Spermatogenic Endpoints of F_0 and F_1 Males[a]

	Group (ppm)			
	0	50	150	500
F_0 males				
Sperm number (millions/g tissue)				
Left testis	79.1 ± 17.0	ND	ND	78.6 ± 11.2
Left cauda epididymis	739 ± 148	ND	ND	727 ± 157
Motile sperm (%)	88.6 ± 7.5	87.6 ± 8.1	87.5 ± 9.0	91.5 ± 4.1
Progressive motile sperm (%)	75.6 ± 9.9	72.4 ± 11.3	74.8 ± 12.1	78.4 ± 5.7
Sperm morphology (%)				
Normal	99.5	99.1	98.5	99.6
Normal head separated from flagellum	0.3	0.6	1.0	0.3
Head absent, normal flagellum	0.2	0.3	0.5	0.1
F_1 males				
Sperm number (millions/g tissue)				
Left testis	96.6 ± 42.9	ND	ND	106.9 ± 39.9
Left cauda epididymis	619 ± 148	ND	ND	639 ± 171
Motile sperm (%)	84.3 ± 12.7	80.6 ± 19.9	76.7 ± 25.8	86.4 ± 8.7
Progressive motile sperm (%)	73.0 ± 15.9	69.4 ± 20.5	65.9 ± 23.2	75.0 ± 10.7
Sperm morphology (%)				
Normal	99.3	98.3	98.0	98.7
Normal head separated from flagellum	0.4	1.1	1.4	0.6
Head absent, normal flagellum	0.2	0.6	0.6	0.6

[a]ND=Not determined. No values were statistically significantly different from control.

150-ppm exposure groups that were found dead or were euthanized in extremis. In the 500-ppm males and females, exposure-related microscopic findings were confined to the nasal cavity. Increased occurrences of minimal to mild degeneration of the olfactory epithelium that lined the dorsal septum and dorsal medial aspects of the dorsal turbinates (ethmoturbinates) primarily at nasal levels II, III, and IV (Young, 1981) were found compared to the control group. The olfactory epithelial degeneration was characterized by disorganization and generally one or more of the following features: regenerative hyperplasia, individual cell necrosis, atrophy, and increased presence of Bowman's glandular elements and cysts within olfactory epithelium. Despite these olfactory epithelial changes, the exposure-induced lesions did not have any inflammatory response. The incidence of nasal lesions was less in high-exposure F_1 rats than in F_0 rats. No other exposure-related microscopic findings were noted in the 500-ppm group.

At the scheduled necropsy of the F_1 females, mean numbers of primordial follicles per section (Bolon et al., 1997) were 5.0 and 5.1 for females in the control and 500-ppm groups, respectively. The mean numbers of corpora lutea per section (Bucci et al., 1997) were 9.2 and 10.1 for females in the same respective groups. The mean numbers of primordial follicles and corpora lutea for all examined animals were unaffected by test article exposure.

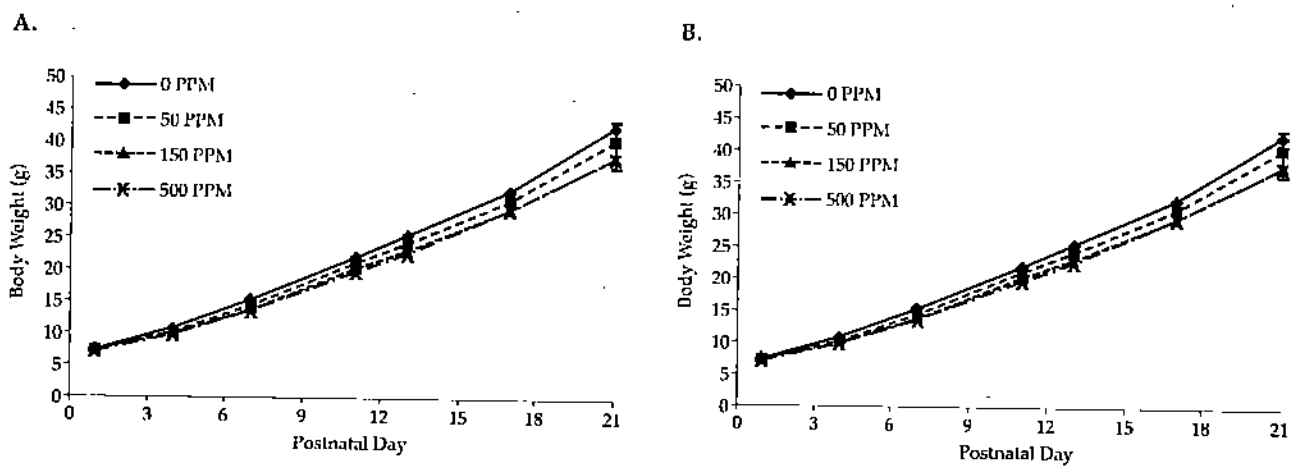
In addition, reproductive tract organs for low- and mid-exposure group adult animals that did not mate or produce offspring were examined microscopically. No treatment-related effects were found.

Offspring Evaluations (Tables 2, 3)

The mean number of F_1 and F_2 pups born, live litter size, percentage of males per litter at birth, and postnatal survival between PND 0 (relative to number born), 0-1, 1-4 (pre-selection), PND 4 (post-selection) to 7, 7-14, 14-21, and from birth to PND 4 (pre-selection) and PND 4 (post-

FIGURE 2

Mean bodyweights of male (A) and female (B) F_2 offspring through weaning.



selection) to PND 21 were unaffected by styrene exposure. The numbers of F_1 and F_2 pups found dead, euthanized in extremis, and/or missing, as well as the general physical condition of all F_1 pups in this study, were unaffected by styrene exposure.

Mean F_1 male and female pup body weights were unaffected by parental exposure to styrene. Mean F_2 pup body weight gains and mean body weights in the 500-ppm group were decreased (6.8-13.3%) throughout the pre-weaning period (PND 0-21). The mean male and female F_2 pup body weight changes in the 150-ppm group were similar to the control group during PND 1-4, but decreased PND 4-21 (only males significantly different from control). Mean body weights and mean body weight gains in the 50-ppm group F_2 males and females were unaffected by maternal exposure to styrene (Fig. 2).

Offspring Pathology

No macroscopic findings that could be attributed to parental exposure with the test article were noted at the scheduled necropsy of F_1 or F_2 pups euthanized on PND 21. Mean organ weights (absolute and relative to final body weight) in the styrene-exposed F_1 males and females examined at the PND 21 necropsy were similar to the control group.

Statistically significant ($p<0.01$) reductions in mean absolute pituitary, thymus, and uterine weight occurred in F_2 female offspring of dams exposed to 500 ppm. Because these pups had reduced body weight, compared to controls, and the relative organ weights were not reduced, the reductions in female pituitary, thymus, and uterine weight were attributed to growth retardation, not to direct effects on these organs. Similarly, a reduction in absolute, but not relative, pituitary weight in F_2 female offspring of dams exposed to 150-ppm styrene was considered due to growth retardation. Statistically significant ($p<0.01$) reductions in mean absolute and relative pituitary weight occurred in F_2 male offspring of dams exposed to 500 ppm (Table 5). Because the relative pituitary weight was decreased in males, this was attributed to a test article effect on the pituitary, not just growth retardation.

DISCUSSION

The parental systemic toxicity of styrene reported in this study was similar to that previously reported in rats following long-term inhalation exposure (Cruzan et al., 1997, 1998). Findings included degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates of F_0 and F_1 animals in the high-exposure group (500 ppm; nasal tissue was not examined in the low- and mid-exposure groups), decreased mean body weights in the mid-exposure group (F_0 and F_1 males and F_1 females) and high-exposure group (F_0 and F_1 males and females), and

TABLE 5

Organ Weights of F_2 Offspring Not Chosen for Neurotoxicity Evaluation[a]

	Group (ppm)			
	0	50	150	500
Males				
Brain (g)	1.441	1.418	1.376	1.378
(g/100 g BW)	3.427	3.507	3.557	3.671
Spleen (g)	0.180	0.180	0.169	0.158
(g/100 g BW)	0.418	0.432	0.426	0.406
Testis, Right (g)	0.093	0.096	0.086	0.087
(g/100 g BW)	0.220	0.232	0.230	0.226
Testis, Left (g)	0.094	0.095	0.088	0.086
(g/100 g BW)	0.221	0.231	0.226	0.221
Thymus (g)	0.193	0.182	0.163	0.169
(g/100 g BW)	0.452	0.441	0.411	0.436
Pituitary (g)	0.0038	0.0037	0.0034	0.0025
(g/100 g BW)	0.009	0.009	0.009	0.007*
Females				
Brain (g)	1.393	1.365	1.360	1.331
(g/100 g BW)	3.461	3.568	3.660	3.863*
Spleen (g)	0.173	0.176	0.165	0.149
(g/100 g BW)	0.419	0.440	0.427	0.412
Uterus (g)	0.057	0.050*	0.050	0.047*
(g/100 g BW)	0.140	0.131	0.133	0.134
Thymus (g)	0.197	0.180	0.172	0.161*
(g/100 g BW)	0.480	0.459	0.452	0.451
Pituitary (g)	0.0041	0.0039	0.0033*	0.0031*
(g/100 g BW)	0.0109	0.010	0.009	0.009

[a]Terminated PND 21, litter as experimental unit.
*Significantly different from control, $p<0.05$

increased water consumption during gestation in the high-exposure group (F_0 and F_1 females) and during lactation in the mid- and high-exposure groups (F_0 females). In the previous chronic study, nasal lesions increased in incidence and severity with dose. A NOAEL was not found, but effects at 50 ppm were slight and not all animals were affected even after 24 months. Decreased body weight and increased water consumption were found at 500 and 1,000 ppm for 2 years.

Reproductive performance and offspring postnatal survival prior to weaning were not adversely affected by styrene exposure. Pre-weaning pup weights were unaffected by styrene exposure for the F_1 generation. Following direct exposure of the F_1 weanlings on PND 22, body weight reductions were observed in the 500-ppm group that led to the reduced mean body weights in this group throughout the generation. In contrast to the F_1 generation, pre-weaning F_2 pup weights were reduced in both the 150- and 500-ppm groups (approximately 10 to 13% on PND 21). The weights of the F_2 pups continued to be reduced following weaning in the 150- and 500-ppm groups selected for neurobehavioral evaluation in the developmental neurotoxicity component (Cruzan et al., 2005, this issue). In addition, there were slight delays (generally not statistically significant) in the acquisition of developmental landmarks (Cruzan et al., 2005, this issue) that were suggestive of an overall pattern of slight developmental delay in the 500-ppm group. These pre-weaning developmental endpoints are highly correlated with pup body weight (Lochry, 1987) and are consistent with the reduced body weights seen in this group. Effects may have been greater in the F_2 offspring than the F_1 because exposure of the F_1 parents was started at a younger age (PND 21 vs. PND 50) and pre-mating toxicity was more evident in F_1 parents than F_0 parents.

This study confirmed previous observations of slight body weight effects of styrene exposure at 500 ppm or greater in rats and degeneration of nasal olfactory epithelium (Cruzan et al., 1997, 1998). It further demonstrated a lack of styrene effects on gonadal function, reproductive performance, and offspring survival. This enhances the conclusions of the previous 3-generation reproduction study of styrene in drinking water (Beliles et al., 1985). In addition, it supports the lack of effects on testes and ovaries reported in the subchronic studies of styrene (Cruzan et al., 1997, 1998, 2001; NCI, 1979; Roycroft et al., 1992), and disagrees with testicular pathology and decreased sperm counts reported by Srivastava et al. (1989).

The No-Observed-Adverse-Effect Level (NOAEL) for parental toxicity in this study was 50 ppm and the NOAEL for reproductive toxicity was ≥ 500 ppm.

Uncited References

Adams, 1986; Ashby and Lefevre, 2000; Jelnes, 1988; Kallman, 1994; Roycroft et al., 1992

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Developmental Neurotoxicity Study of Styrene by Inhalation in Crl-CD Rats

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This study was conducted to assess potential adverse functional and/or morphological effects of styrene on the neurological system in the F_2 offspring following F_0 and F_1 generation whole-body inhalation exposures. Four groups of male and female Crl:CD® (SD)IGS BR rats (25/sex/group) were exposed to 0, 50, 150, and 500 ppm styrene for 6 hr daily for at least 70 consecutive days prior to mating for the F_0 and F_1 generations. Inhalation exposure continued for the F_0 and F_1 females throughout mating and through gestation day 20. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117, and 300 mg/kg/day (divided into three equal doses, approximately 2 hr apart). Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5 and continued through weaning of the F_1 or F_2 pups on postnatal day (PND) 21. Developmental landmarks were assessed in F_1 and F_2 offspring. The neurological development of randomly selected pups from the F_2 generation was assessed by functional observational battery, locomotor activity, acoustic startle response, learning and memory evaluations, brain weights and dimension measurements, and brain morphometric and histologic evaluation. Styrene exposure did not affect survival or the clinical condition of the animals. As expected from previous studies, slight body weight and histopathologic effects on the nasal olfactory epithelium were found in F_0 and F_1 rats exposed to 500 ppm and, to a lesser extent, 150 ppm. There were no indications of adverse effects on reproductive performance in either the F_0 or F_1 generation. There were exposure-related reductions in mean body weights of the F_1

Based on the results of this study, an exposure level of 50 ppm was considered to be the NOAEL for growth of F_2 offspring; an exposure level of 500 ppm was considered to be the NOAEL for F_2 developmental neurotoxicity.

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and F_2 offspring from the mid and high-exposure groups and an overall pattern of slightly delayed development evident in the F_2 offspring only from the 500-ppm group. This developmental delay included reduced body weight (which continued through day 70) and slightly delayed acquisition of some physical landmarks of development. Styrene exposure of the F_0 and F_1 animals had no effect on survival, the clinical condition or necropsy findings of the F_2 animals. Functional observational battery evaluations conducted for all F_1 dams during the gestation and lactation periods and for the F_2 offspring were unaffected by styrene exposure. Swimming ability as determined by straight channel escape times measured on PND 24 were increased, and reduced grip strength values were evident for both sexes on PND 45 and 60 in the 500-ppm group compared to controls. There were no other parental exposure-related findings in the F_2 pre-weaning and post-weaning functional observational battery assessments, the PND 20 and PND 60 auditory startle habituation parameters, in endpoints of learning and memory performance (escape times and errors) in the Biel water maze task at either testing age, or in activity levels measured on PND 61 in the 500-ppm group. Taken together, the exposure-related developmental and neuromotor changes identified in F_2 pups from dams exposed to 500 ppm occurred in endpoints known to be both age- and weight-sensitive parameters, and were observed in the absence of any other remarkable indicators of neurobehavioral toxicity. Based on the results of this study, an exposure level of 50 ppm was considered to be the NOAEL for growth of F_2 offspring; an exposure level of 500 ppm was considered to be the NOAEL for F_2 developmental neurotoxicity. *Birth Defects Res B* 74:221-232, 2005. ©2005 Wiley-Liss, Inc.

INTRODUCTION

The reproductive and developmental effects of styrene (CAS no. 100-42-5) have been extensively reviewed by Brown et al. (2000). Although few details are provided, Vergieva et al. (1979) reported no dose-related effects on body weights or offspring behavior when rat dams were exposed via inhalation to 163 ppm styrene 4 hr/day 5 days/week on gestational days 2–16 or to 47 ppm on gestational days 2–21. Zaidi et al. (1985) reported that gavage treatment of rat dams with 200 mg/kg/day styrene throughout gestation had no effect on the number of pups born per litter, pup body weight, protein content of the brain, or striatal dopamine receptors of pups. In contrast, Kishi and coworkers have conducted two studies on the effects of prenatal styrene exposure. In the first study (Kishi et al., 1992, 1995), pregnant Wistar rats were exposed via inhalation to 0 (14 litters), 60 (3 litters), or 293 ppm (7 litters) styrene 6 hr/day on gestation days 7 to 21. There was no effect on gestation length or average litter size, but pup weights in both styrene-exposed groups were reduced compared to the controls. Neurobehavioral evaluation was conducted on 5 control litters, 2 litters exposed at 60 ppm and 5 litters exposed at 293 ppm. They reported differences in a number of developmental landmarks, as well as differences in open field activity, rotarod activity, and operant conditioning response for some but not all tested intervals. In the second study (Katakura et al., 1999, 2001), pregnant female rats were exposed to 0 (ad libitum feed, 14 litters), 0 (pair-fed to 300 ppm group, 12 litters), 50 (9 litters), or 300 (14 litters) ppm styrene by a static inhalation system. Compared to the pair-fed controls, exposure to 300 ppm styrene resulted in increased neonatal death, decreased pup weight on PND 21, increased time to lower, but not upper, incisor eruption, an increased time to development of air righting reflex, and decreased homovanillic acid in the cerebrum.

Effects on the nervous system have been reported for workers in the reinforced plastics industry. Neither the presence of effects nor the level at which the effects were observed have been reported consistently. Effects reported in some studies include central nervous system (CNS) depression, slower nerve conduction velocity, and decreased color discrimination. Some studies report effects at concentrations as low as 10 ppm, while others claim no effects at concentrations as high as 100 ppm (reviewed in IARC, 2002). In laboratory animals, CNS depression was reported following single exposures to 1,300 ppm for 4 hr. Changes in dopamine and related metabolites have been reported in the brain of rats exposed to 750 ppm styrene for 3 days. Increases in glial fibrillary acidic protein in the sensorimotor cortex and hippocampus were reported in rats exposed via inhalation to 320 ppm styrene continuously for 3 months (reviewed in IARC, 2002).

Because of the conflicting and limited animal data on the effects of styrene on development of the neurological system, a developmental neurotoxicity study was conducted via whole-body inhalation exposure using current regulatory guidelines. This was accomplished by evaluating dams from the F_1 generation and F_2 offspring from a two-generation reproduction study (Cruzan et al., 2005, this issue). In most reproduction studies conducted by the inhalation route, exposure is stopped on day 20 of gestation and not reinstated until lactation day 5 to minimize stress on the offspring from the more than 6-hr separation that would occur during the inhalation exposure of the dam. Because high concentrations of styrene affect

the CNS and significant development of the CNS occurs during the first few days after birth in rats, dams were treated orally during lactation days 1–4 at doses estimated by PBPK modeling to mimic a 6-hr inhalation exposure.

MATERIALS AND METHODS

Study Design

Four groups of F_0 and F_1 male and female Crl:CD rats (25/sex/group) were exposed to vapor atmospheres of styrene at 0, 50, 150, or 500 ppm for 6 hr daily (7 days/week) for at least 70 consecutive days prior to mating. Females were paired with males on a 1:1 basis for 14 days or until evidence of mating was observed. The F_0 and F_1 females continued inhalation exposure throughout mating and gestation through gestation day 20. On lactation days 1 through 4, the F_0 and F_1 females received styrene in virgin olive oil via oral gavage at dose levels of 66, 117, and 300 mg/kg/day (divided into three equal doses, administered approximately 2 hr apart). The doses were calculated to mimic the blood level of styrene at 2, 4, and 6 hr in a 6-hr inhalation exposure at the target concentration based on the PBPK model of Sarangapani et al. (2002). Inhalation exposure of the F_0 and F_1 females was re-initiated on lactation day 5 and continued through the day prior to euthanasia. Offspring were weaned on lactation day 21; exposure of F_1 pups began on PND 22. F_2 pups were weaned on PND 21 and not directly exposed to styrene. Neurobehavioral and neuropathological evaluations were conducted in F_2 offspring through PND 72. The details of the two-generation study have been published in the accompanying reproductive toxicity report (Cruzan et al., 2005, this issue).

Test Material

Styrene monomer (inhibited), CAS No. 100-42-5, was provided by Chevron Phillips Chemical Company LLP, St. James, LA. The purity and stability of the styrene were verified by gas chromotography with flame ionization detection. When present in the chromatograms, the percentage of benzene, ethylbenzene, styrene oxide, and styrene dimers was also determined. Results obtained indicated the styrene was at least 99.9% pure.

Exposures

Gas chromatographic analyses of chamber atmospheres demonstrated average exposures of 0, 50, 151, and 499 ppm for F_0 styrene concentrations and 0, 50, 153, and 501 ppm for F_1 styrene concentrations.

Animals and Animal Husbandry

Male and female Crl:CD® (SD)IGS BR rats from different barrier colonies were received from Charles River Laboratories, Inc., Raleigh, North Carolina, on July 24, 2001. The methods for acclimation, assignment to treatment groups, animal husbandry, and animal welfare are described in the accompanying two-generation reproductive toxicity study (Cruzan et al., 2005, this issue).

Reproduction Study

All animals were observed twice daily (at least 7 hr apart) for moribundity and mortality, appearance, behavior, and pharmacotoxic signs (prior to exposure/gavage dosing for the F_0 and F_1 animals). Individual F_0 and F_1 male body weights were recorded throughout the study. Further details are provided in the two-generation study (Cruzan et al., 2005, this issue). After a minimum of 70 days of exposure, each female was housed overnight in the home cage of a randomly chosen male for up to 14 days. After mating, the animals were separated and the female was housed in an individual plastic cage with nesting material. All females were allowed to deliver naturally and rear their young to weaning (PND 21). To reduce variability among litter size, 10 F_1 and F_2 pups of equal sex distribution, if possible, were randomly selected from each litter on PND 4. Pups were individually sexed on PND 0, 4, 7, 14, and 21. F_1 pups were individually weighed on PND 1, 4, 7, 14, and 21; F_2 pups were individually weighed on PND 1, 4, 7, 11, 13, 17, and 21. The following investigations were used to assess the maturation of the selected F_1 and F_2 pups: pinna detachment, surface righting response, hair growth, incisor eruption, eye opening, balanopreputial separation, and vaginal patency (Adams, 1986). After litter standardization, one F_2 pup/sex/litter (total of 20 pups/sex/group) was assigned to one of two subsets (Subset A and B) for neurobehavioral and neuropathological assessments, and an additional one F_2 pup/litter (total of 10 pups/sex/group) was assigned to Subset C for neuropathological assessment.

Neurobehavioral Testing

Functional Observational Battery (FOB) (Moser, 1991; Irwin, 1968; Gad, 1982; Moser et al., 1988; Haggerty, 1989; O'Donoghue, 1989) testing was performed on all F_1 dams on gestation days 6 and 12 and on lactation days 10 and 21; FOB testing was performed on 20 F_2 pups/sex/group (Subset A) on PND 4, 11, 22, 45, and 60. Testing (Table 1) was performed by the same trained technicians, whenever possible, who did not know the animal's group assignment. Chatillon Model DPP-1.0 kg or DPP-2.5 kg (as appropriate for the age of the animal) pull-push strain

TABLE 1

Parameters of Functional Observational Battery

Ease of removal from cage	Ease of handling animal in hand
Lacrimation / chromodacryorrhea	Salivation
Piloerection[a]	Fur appearance[a]
Palpebral closure[a,b]	Respiratory rate/character
Red/crusty deposits	Mucous membranes/ eye[a,b]/skin color
Eye prominence[a,b]	Muscle Tone
Mobility[a]	General body posture
Convulsions/tremors	Gait[a]
Grooming[a]	Arousal
Bizarre/stereotypic behavior	Urination /defecation
Pupillary response[a,b]	Backing
Forelimb/hindlimb grip strength[c]	

[a] Not assessed on PND 4 due to stage of development.
[b] Not assessed on PND 11 due to stage of development.
[c] Assessed on PND 22, 45, and 60 only.

gauges (AMETEK Test and Calibration Instruments Division, Largo, FL) were used for testing fore- and hindlimb grip strength during post-weaning FOB sessions. The test was conducted such that animals were allowed to grasp the pull strain gauge with their forepaws. The observer then pulled the animal until the grip was lost and the hindpaws grasped the push strain gauge and then let go with the continued movement. One pull and push was considered a single trial, and three consecutive trials were conducted on each testing day. The group mean of the averaged trials was reported in grams.

The locomotor activity of the same 20 pups/sex/group assigned to FOB asessments (Subset A) was monitored on PND 13, 17, 21, and 61. Locomotor activity was measured using the SDI Photobeam Activity System (San Diego Instruments, San Diego, California) in a room equipped with a white noise generation system set to operate at approximately 70 dB(A). Each chamber consisted of a series of infrared photobeams surrounding a clear plastic, rectangular cage. Four-sided black enclosures surrounded the clear plastic boxes and decreased the potential for distraction by extraneous environmental stimuli. Each chamber was calibrated before each testing session. The testing of treatment groups was done according to replicate sequence; no more than 24 animals were tested during a single session. Each test session was 60 min in duration and consisted of 12 5-min intervals. Each session recorded ambulatory (sequential interruption of two or more photobeams) and total (interruption of a photobeam) activity.

An acoustic startle response test was performed on the same 20 rats/sex/group (Subset A) on PND 20 and 60 using the SR-Lab Startle Response System (San Diego Instruments, San Diego, CS) in a room equipped with a white-noise generation system set to operate at approximately 70 dB(A). Each isolation chamber was composed of a wood core covered with a laboratory-grade plastic laminate and measured 15 x 16 x 23 inches. Each cabinet was equipped with an internal light, a fan, two viewing lenses, and a complete white-noise generation system. The animal was placed in a cylindrical enclosure of appropriate size, which was then placed into the isolation cabinet. Each enclosure was equipped with a motion sensor, which was calibrated prior to each day's testing. The testing of treatment groups was done according to replicate sequence; no more than 8 animals were tested during a single session. Each test session consisted of a 5-min acclimation period with an approximate 65 dB(A) broadband background white noise. The startle stimulus for each trial was an approximate 115 dB(A) mixed-frequency noise burst stimulus, approximately 20 msec in duration. Each test session consisted of 50 trials, with an 8-sec intertrial interval. Startle response data were analyzed in five blocks of 10 trials each. Concurrent with the onset of the startle burst, force data were collected every millisecond for 100 msec. The greatest force data recorded during that 100 msec was considered the maximum response amplitude (V_{MAX}) and was reported in mV. The time at which the V_{MAX} was observed was considered the latency (from the onset of the startle burst) to the maximum response amplitude (T_{MAX}) and was reported in msec. The average of all 100 response recordings was considered the average response amplitude (V_{AVG}) and was reported in mV.

Swimming ability and learning and memory were assessed for 20 rats/sex/group using a water-filled eight-unit T-maze similar to that described by Biel (1940). Those animals tested on PND 62 were the same as used above (Subset A), while those animals tested on PND 24 were used for this test only (Subset B). Animals were placed in the maze and were required to traverse the maze and escape by locating a platform that was hidden

2 cm (adjusted before each trial) beneath the surface of the water. The amount of time required to traverse the maze and the number of errors for all learning and memory trials were recorded. Each testing interval consisted of three phases that were conducted over 7 consecutive days. Phase one was an evaluation of swimming ability and motivation to escape from the maze and was performed in four consecutive trials on the first day of the Biel maze procedure by measuring the time required for the rat to swim the length of a straight channel. Phase two of the Biel maze procedure evaluated sequential learning on days 2–6. Animals were allowed two trials per day for two days to solve the maze in path A. Animals were then allowed two trials per day for three consecutive days to solve the maze in path B, which was the reverse of path A. For Phases two and three, the minimum intertrial interval was 1 hr. Phase three, day 7, probed the animal for its memory to solve the maze when challenged in path A. Each animal was allowed two trials to solve the maze in path A. Biel maze data were evaluated as the mean time to escape over all trials for each of the three phases (i.e., swimming ability and motivation, sequential learning and memory) of the Biel maze procedure. Also, the numbers of errors committed were evaluated for phases two and three.

Neuropathology

On PND 21, 10 F_2 pups/sex/group (Subset C) were perfused in situ and the brains processed for microscopic examination. On PND 72, 10 F_2 pups/sex/ group (selected from Subset A) were perfused in situ and central and peripheral nervous system tissues were processed for microscopic examination. The brain and central nervous system tissues were embedded into paraffin; peripheral nervous system tissues were embedded into plastic. At minimum, four coronal sections of the cerebrum and a mid-sagittal section of the cerebellum/pons/medulla and two transverse sections of the remaining half of the cerebellum/pons were prepared. The prepared tissues were sectioned at 4–8 μm, mounted on glass microscope slides and stained with hematoxylin and eosin. A simple, non-blinded morphometric analysis of the brains from these offspring was performed. Two coronal sections of the cerebrum and one midsagittal section of the cerebellum/pons/medulla were used for morphometry. Sections were homologous between animals. Specific levels analyzed were defined as follows: Level 1 was a coronal section taken approximately halfway between the base of the olfactory bulbs and the optic chiasm. This level was just rostral to the point where the corpus callosum bridges across the hemispheres and was characterized by a good representation of the caudoputamen and the presence of the opening of the rostral medial aspect of the lateral ventricle. Level 3 was a coronal section taken just rostral to the attachment of the pituitary gland (infundibular stalk) characterized by a slight separation between the hemispheres of the rostral hippocampus such that CA1 pyramidal neurons from each hemisphere formed only a slight depression before meeting medially. Rostrally there was no depression, and posteriorly there was a more pronounced depression of the medial lines of CA1 neurons between hemispheres. Level 5 was a midsagittal section of the cerebellum and brainstem. Levels 1, 3, and 5 correspond to figures 11, 32, and 79 of the adult rat brain as depicted by Paxinos and Watson (1998). Measurements were as follows:

- *Level 1:* Total bilateral height of the hemisphere measured just at the beginning of the lateral ventricle, and bilateral vertical thickness of the hemisphere measured at the apex of the corpus callosum and parallel to the height of the hemisphere.
- *Level 3:* Bilateral radial thickness of the frontoparietal cortex; bilateral vertical height of the hemisphere between the layers of hippocampal pyramidal neurons measured along a line that passed through the termination of the dorsal limb of the medial dentate hilus; bilateral height of the medial dentate hilus measured between the termination of the ventral limb perpendicular to the layer of pyramidal neurons, and bilateral length of the ventral limb of the dentate hilus.
- *Level 5:* Thickness of the caudal brainstem, toward lobule no. 9 (caudal to the cerebellar peduncle), measured at the stalk of the cerebellum and perpendicular to the ventral border of the pons, and distance across the base of cerebellar lobule no. 9, measured perpendicular to the white matter tract through the middle of the lobule.

These linear measurements were made using a computer imaging system (Pax-It™, Midwest Imaging Systems, Inc., Fraklin Park, IL). The average from the two hemispheres of the coronal sections, and single measurements from the mid-sagittal section of the cerebellum and brainstem, were used in calculations. All brain sections were also examined by light microscopy for any qualitative changes.

Statistical Methods

Analyses were conducted using two-tailed tests (except as noted otherwise) for a minimum significance level of 5%, comparing each test article-treated group to the con-

trol group. Statistical procedures used in the reproductive phase are covered in detail in the accompanying report and are not described here (Cruzan et al., 2005, this issue). F_2 mean day of acquisition of preweaning/post-weaning developmental landmarks data were analyzed for heterogeneity of variance (Levene, 1960) and normality (Royston, 1982). If the data were homogeneous and normal, a parametric one-way analysis of variance (ANOVA) was used to determine intergroup differences (Snedecor and Cochran, 1980). If the results of the ANOVA were significant ($p<0.05$), Dunnett's (1964) test was applied to compare the control group versus all treatment groups. If the data were not homogeneous and normal, the data were analyzed by the Kruskal-Wallis (1952) nonparametric ANOVA test to determine the intergroup differences. If the ANOVA revealed statistical significance ($p<0.05$), the Mann-Whitney U-test (Kruskal and Wallis, 1952) was used to compare the test article-treated groups to the control group.

Pup weights through weaning were analyzed separately by sex by analysis of covariance (ANCOVA), with pups weights nested within the litter, with the litter size as the covariate. The number of pups born was used as the covariate. The following assumptions were made regarding the ANCOVA: homogeneity of regression slopes, linear relationship between the pup weights and number of pups born, and additive group and regression effects. Histopathologic findings in the test article-treated groups were compared to the control group using a two-tailed Fisher's Exact test (Steel and Torrie, 1980). The following FOB data: group mean counts of backings, groomings, urinations and defecations and group means of forelimb and hindlimb grip strength, along with ambulatory counts measured in the locomotor activity assessment, average response in the acoustic startle assessment and Biel maze data (mean times to escape in the straight channel, learning and memory phases and the mean errors in the learning and memory phases) were subjected to a parametric one-way analysis of variance (ANOVA) to determine intergroup differences. If statistically significant differences were indicated by the ANOVA, Dunnett's test was used to compare the control and treated groups. FOB parameters, which yielded scalar and descriptive data, were analyzed by Fisher's Exact Test (Steel and Torrie, 1980). Intrasession total counts measured in the locomotor activity assessment and intrasession peak response and latency to peak response measured in the acoustic startle assessment were analyzed by the univariate repeated measures ANOVA (ReMANOVA, SAS, 1999-2001) to determine the presence of an interaction effect of treatment group by time using a Geisser-Greenhouse adjusted F-statistic. If a significant interaction effect of treatment group by time was indicated by the ReMANOVA, Dunnett's test was used to compare the control and treated groups at each within-session interval. In addition, the ReMANOVA was used to determine the presence of a main effect of treatment. If a significant main effect of treatment was indicated by the ReMANOVA, Dunnett's test was used to compare the control and treated groups. Repeated measures statistical analyses were performed by BioSTAT Consultants, Inc., Portage, MI. All analyses performed by BioSTAT Consultants, Inc. were conducted with the SAS System software (version 8.2).

RESULTS

Parental and Reproduction Effects

Briefly (see Cruzan et al., 2005, this issue), body weight gain was slightly reduced in F_0 males and females at 500 ppm, and F_1 males and females at 150 and 500 ppm during the premating exposure periods. There was no effect on bodyweight or bodyweight gain at 50 ppm in either the F_0 or F_1 exposure periods. There was no effect of styrene at any exposure level on body weight gain or feed consumption during gestation in either the F_0 or F_1 dams. At 500 ppm, there was increased water consumption during gestation in both F_0 and F_1 dams. There was no effect at 150 or 50 ppm. There was no effect of styrene exposure on body weight gain or food consumption during lactation in either the F_0 or F_1 dams. Exposure of F_0 and F_1 females had no effect on mean estrous cycle length or the mean numbers of days between pairing and coitus. Styrene exposure had no effects on F_0 or F_1 spermatogenic endpoints (mean testicular and epididymal sperm numbers, sperm production rate, sperm motility, and sperm morphology). The mean lengths of gestation were unaffected by styrene exposure. Exposure to styrene did not affect F_0 or F_1 male or female mating index, male or female fertility index, mean number of pups born, the number of former implantation sites, or the number of unaccounted sites. At the scheduled necropsy of the F_1 females, the mean numbers of primordial follicles and corpora lutea were unaffected in females exposed to 500 ppm of styrene. The mean number of F_1 and F_2 pups born, live litter size, percentage of males per litter at birth and postnatal survival were unaffected by styrene at all exposure levels evaluated. Mean F_1 male and female pup body weights were unaffected by parental exposure to styrene. The No-Observed-Adverse-Effect Level (NOAEL) for parental toxicity was 50 ppm and for effects on reproduction was 500 ppm.

TABLE 2

Bodyweight (g) of F_2 Offspring of Rats Exposed to Styrene in the F_0 and F_1 Generations

	ppm			
PND	0	50	150	500
Males				
1	7.4	7.6	7.3	6.9*
4	10.7	10.5	10.5	9.8
7	15.2	14.6	14.2	13.5*
13	25.4	24.2	23.1*	22.7*
21	42.6	40.3	38.2*	38.0*
35	134	129	127	120
49	260	247	248	236
70	388	378	372	360
Females				
1	7.1	7.0	6.9	6.4*
4	10.1	10.0	10.0	9.0*
7	14.3	13.9	13.7	12.4*
13	24.3	23.3	22.6	21.2*
21	40.5	39.1	37.4	35.4*
35	120	113	112	108
49	179	171	171	170
70	248	235	233	233

*Statistically different from control, $p < 0.05$.

F2 Body Weights

Mean F_2 pup body weight gains and mean body weights in the 500-ppm group were decreased (6.8-13.3%) throughout the pre-weaning period (PND 0-21). Body weights in these F_2 offspring remained reduced though PND 70 (Table 2). Mean male and female F_2 pup body weight changes in the 150-ppm group were similar to the control group during PND 1-4, but were reduced on PND 7-21. Following weaning, body weights in the F_2 offspring of parental animals exposed to 150 ppm of styrene were slightly lower than the controls. Mean body weights and mean body weight gains in the 50-ppm group F_2 males and females were unaffected by parental exposure to styrene throughout the pre-weaning and post-weaning periods.

Developmental Landmarks

Styrene exposure did not affect pinna detachment, surface righting response, hair growth, incisor eruption, and eye opening in F_1 male and female pups. Mean ages of acquisition of vaginal patency (group means between 34.3 and 36.3 days) and mean body weights (between 108 and 114g) on the day of acquisition were unaffected by styrene exposure in F_1 females.

In F_1 males, the mean ages of acquisition of balanopreputial separation were 45.6, 44.6, 45.7, and 47.0 days in the 0-, 50-, 150-, and 500-ppm groups, respectively. The differences from the control group were not statistically significant, and were within the laboratory's historical control data range (41.6—49.0 days). Mean body weights (grams) on the day of acquisition were 223, 214, 209, and 211 in the 0-, 50-, 150-, and 500-ppm groups, respectively. The slight increase in age at acquisition of balanopreputial separation was judged to be related to the lower body weight at 500 ppm.

In F_2 males and females, there were subtle indications of a delay in the acquisition of developmental landmarks, which accompanied the decreased body weights (Table 3). In general, mean ages of acquisition were not statistically significantly increased, but more high exposure animals acquired the landmark later than the controls. This included pinna detachment, surface righting ability, initiation of hair growth, eye opening, incisor eruption ($p<0.05$). The mean day of balanopreputial separation in the 500-ppm males occurred at 47.2 days vs. 45.3 days in the control group and 44.8 days in the laboratory's control database. While these delays were slight, the animals with the later occurrences were from the females with the lowest body weight and were usually among the lightest animals in the group. The delayed acquisition of these landmarks, in the presence of reduced body weight in offspring indirectly exposed to 500-ppm styrene, was suggestive of a slight developmental delay. Vaginal patency in the F_2 females was unaffected by styrene exposure.

Functional Observational Battery (FOB)

Exposure of F_1 dams to styrene had no effect on FOB observations on gestation days 6 and 12 or lactation days 10 and 21. No styrene-related effects were seen in FOB observations in F_2 offspring on PND 4, 11, or 22. On PND 45 and 60, forelimb and hindlimb grip strength was decreased in the offspring of parental animals exposed to 500 ppm styrene (Fig. 1). These offspring also weighed less than the control animals at these ages. Despite greater % differences in body weight in animals on PND 21 than on PND 42 or 63 (ages that most closely match test ages), the absolute differences in mean body weight between the 500-ppm group animals and their age-matched controls was much greater in the older animals. Since grip strength is positively correlated with absolute

TABLE 3

Days of Acquisition of Developmental Landmarks of F_2 Offspring of Rats Exposed to Styrene in the F_0 and F_1 Generations: Males and Females Combined[a]

	Landmark							
	Pinna detachment		Surface righting		Incisor eruption		Hair growth	
Treatment (ppm)	% on pnd4	Mean age (s.d.)	% on pnd5	Mean age (s.d.)	% on pnd10	Mean age (s.d.)	% on pnd13	Mean age (s.d.)
0	98	4.0 (0.07)	90	5.1 (0.10)	97	9.3 (0.30)	92	11.8 (0.82)
50	99	4.0 (0.03)	94	5.1 (0.11)	99	9.4 (0.38)	89	12.2 (1.31)
150	98	4.0 (0.07)	91	5.1 (0.14)	95	9.5 (0.42)	99	11.9 (0.83)
500	92	4.1 (0.22)	82	5.2 (0.18)	81	9.8 (0.57)*	81	12.5 (1.12)

	Landmark							
	Eye opening		Vaginal opening			Preputial separation		
Treatment (ppm)	% on pnd17	Mean age (s.d.)	% on pnd36	Mean age (s.d.)	BW	% on pnd51	Mean age (s.d.)	BW
0	100	15.1 (0.95)	100	33.5 (1.26)	109.3	100	45.3 (1.48)	222.3
50	99	15.6 (0.72)	80	34.2 (2.72)	106.4	95	46.1 (3.73)	219.6
150	100	15.4 (0.68)	90	34.0 (2.29)	104.9	89	46.1 (3.06)	217.4
500	98	15.5 (0.98)	89	34.1 (2.48)	100.7	90	47.2 (4.07)	216.9

[a] For each landmark, the first column is the percent of animals in the group that have developed that landmark on the age indicated; the second column is the mean (s.d.) days for development of the landmark in the group. For vaginal opening and balanopreputial separation, the mean body weight at the age of acquisition of the landmark is also included.
* Statistically significantly different from control, $p<0.05$.

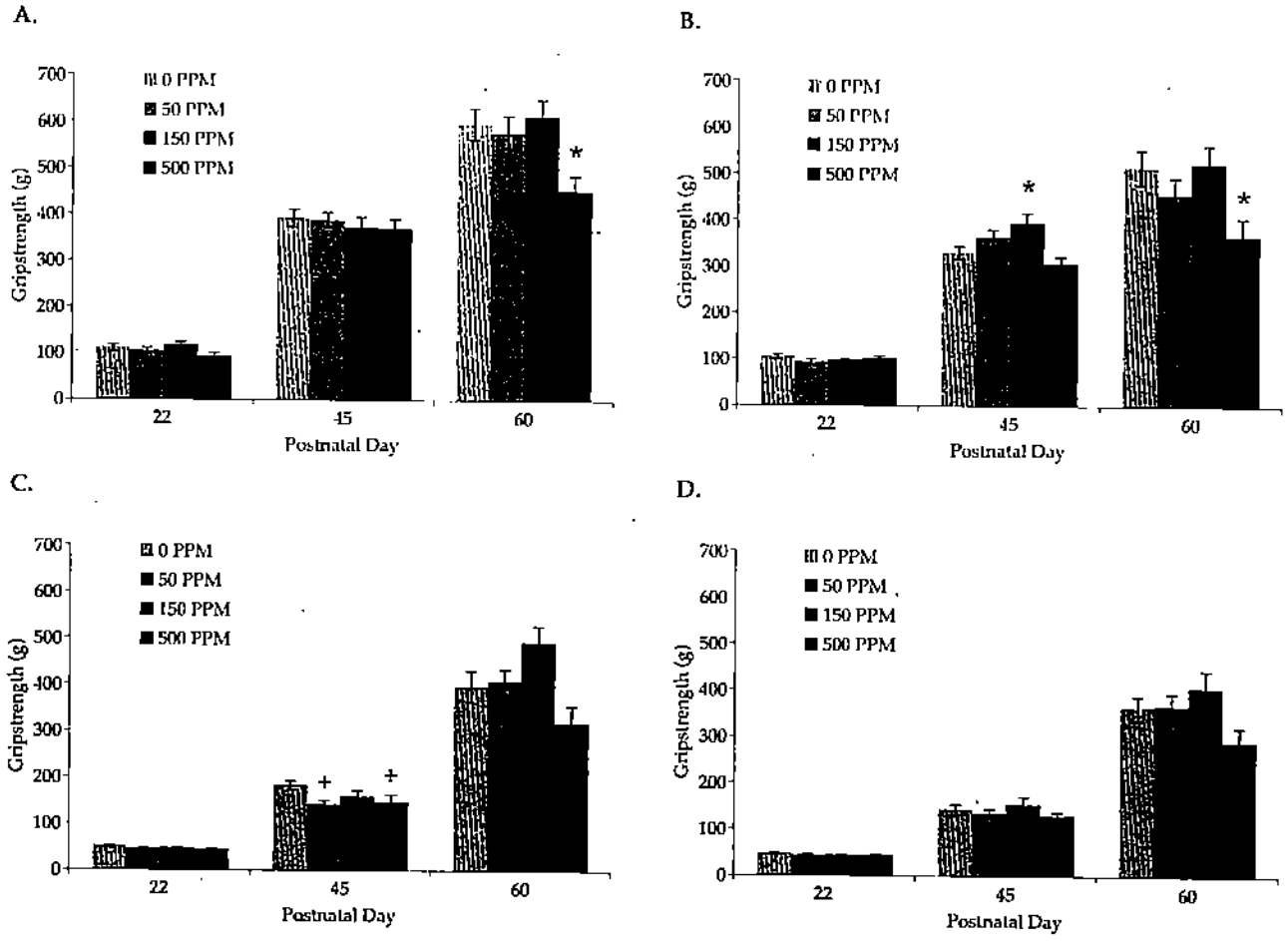
body weight (Maurissen et al., 2003) and given the slight delays in the acquisition of several developmental landmarks in this group, the decreased forelimb and hindlimb grip strength was a further indication of a slight developmental delay. No other FOB parameters were affected on PND 45 or 60.

Locomotor Activity

Although there were no statistically significant differences from the control group within the activity sessions conducted on PND 13, 17, and 21 (Fig. 2A,B), there was a slight shift in the ontogeny of locomotor activity in F_2 males and females in the 500-ppm group that, in the presence of reduced mean body weights in this group, was suggestive of an exposure-related developmental delay. Campbell et al. (1969) characterized the ontogeny of locomotor activity in the rat and showed that activity typically increases between PND 13 and 17 and then decreases between PND 17 and 21. Cumulative total and ambulatory locomotor activity in the 500-ppm group males and females were decreased slightly on PND 13. Cumulative total and ambulatory motor activity were increased in the male group on PND 17, but were decreased in the female group. By PND 21, mean motor activity in both sexes was increased slightly compared to the controls. On PND 61, motor activity in both sexes in the 500-ppm group was similar to the control group (Fig. 2C,D). Locomotor activity was similar to the control group for males and females in the 50- and 150-ppm groups at all ages tested. No changes in the distribution of within-session activity counts were apparent when the exposure groups were compared with the control group.

FIGURE 1

Grip strength (g)±SEM of F_2 offspring of rats exposed to styrene in the F_0 and F_1 generations. A: Males: forelimb. B: Females: forelimb. C: Males: Hindlimb. D: Females: hindlimb. Statistically different from control, $p<0.05$; *parametric test, +non-parametric test.



Auditory Startle

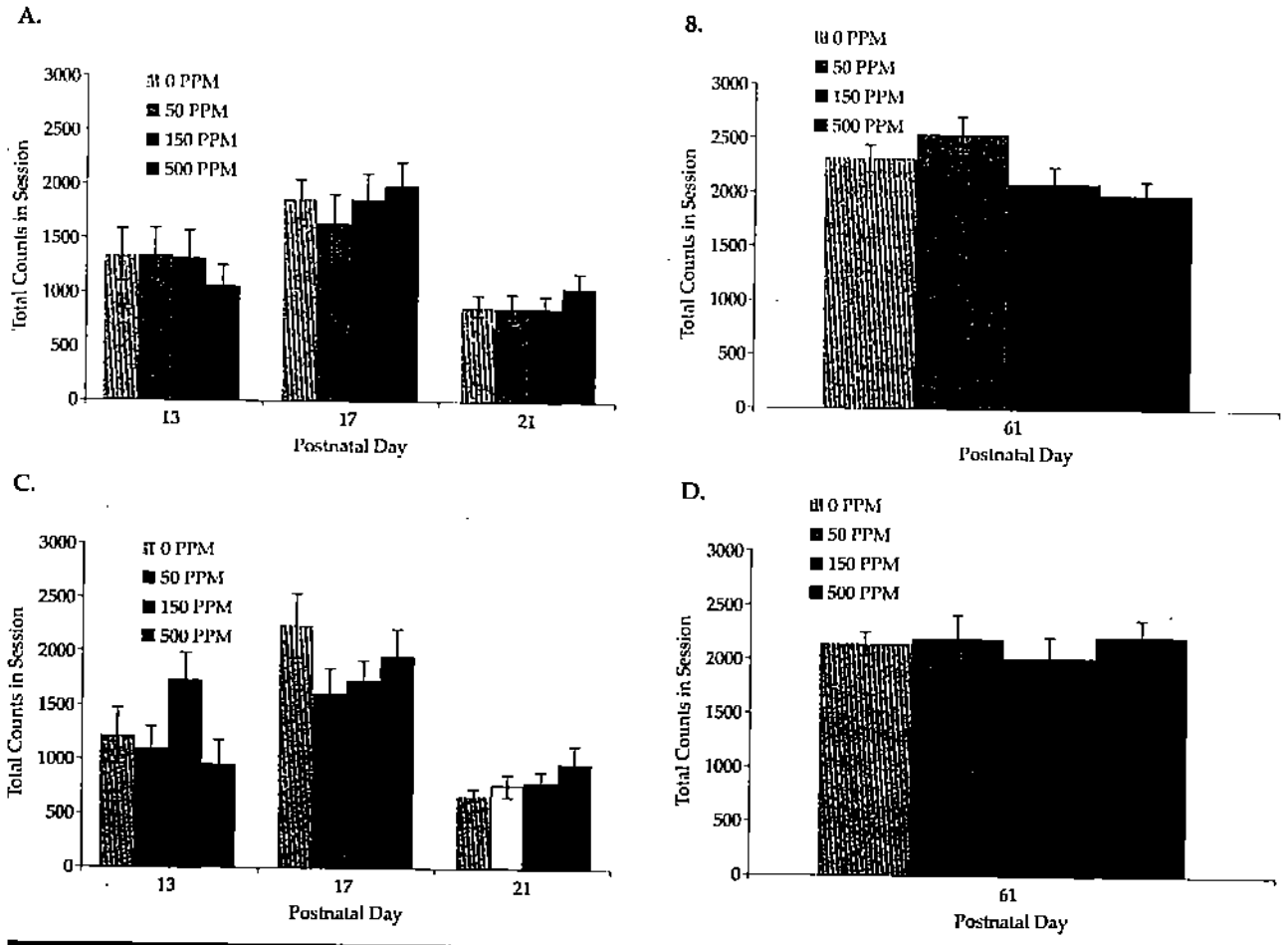
No statistically significant differences or exposure-related trends were apparent in the 50-, 150-, and 500-ppm group F_2 males and females on performance measured in the auditory startle test (Table 4). No changes in the ontogeny and distribution of within-session responses were apparent when compared with the control group.

Memory and Learning: Biel Maze Swimming Trials

On PND 24, swimming ability, as evidenced by an increase in the mean time to escape for the straight channel on the first day of Biel maze assessment, was slightly decreased in the 500-ppm exposure group males (10.58 sec vs. 7.53 sec for control group) and females (11.43 sec vs. 7.81 sec for control group) (Fig. 3). The increased escape times were suggestive of a slight exposure-related developmental neuromotor delay and were consistent with the reduced pre-weaning body weights, slight increases in the ages of acquisition of pre-weaning developmental landmarks, and the slight shift in the ontogeny of normal pre-weaning locomotor activity. There were no obvious changes in mean escape times during the swim test for animals of either sex in the 50- or 150-ppm groups on PND 24, or in any animals on PND 62.

FIGURE 2

Locomotor activity of F_2 offspring of rats exposed to styrene in the F_0 and F_1 generations; total counts in 60-min session. A: Males: PND 13, 17, 21. B: Females: PND 13, 17, 21. C: Males: PND 61. D: Females: PND 61.



Trials 1–4 (conducted on assessment days 2 and 3; PND 25–26 or PND 63–64) of the Biel maze were designed to measure learning and shorter-term memory of Path A, designated the forward path. Trials 5-10 (conducted on assessment days 4-6; PND 27-29 or PND 65-67) were designed to measure learning and shorter-term memory of Path B, designated the reverse path (the exact opposite of Path A). For all treatment groups tested beginning on either PND 24 or PND 62, the mean time to escape was relatively high in Trial 1 and decreased throughout repeated testing in the forward path. For the first trial in the reverse path (Trial 5), mean time to escape was much longer than for the first trial in the forward path but decreased throughout testing in the reverse path. No treatment-related effects on learning in either path direction were noted.

Trials 11 and 12 (conducted on assessment day 7; PND 30 or PND 68) were designed to measure long-term memory of Path A, the forward path, which was interrupted by trials 5-10 (Path B). For all exposure groups, the mean time to escape in Trial 11 was generally similar to trial 1 (the first trial of Path A) but relatively longer than Trial 4 (the last trial in the forward path during the learning portion of testing). For all exposure groups, mean time to escape for trial 12 was typically similar to Trials 3 and/or 4. As expected, since animals had already experienced

TABLE 4

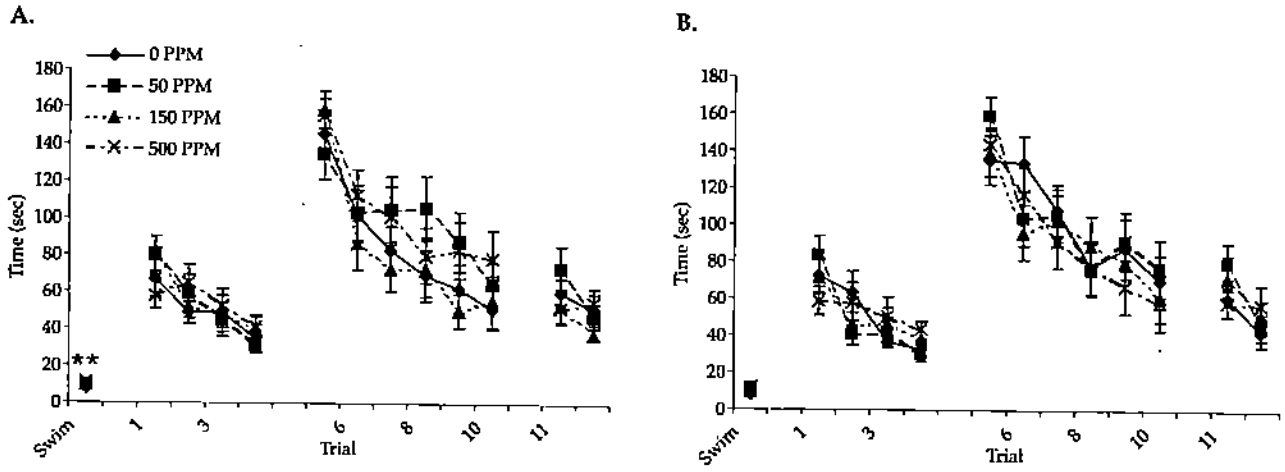
Startle Response of F_2 Offspring of Rats Exposed to Styrene in the F_0 and F_1 Generations [a]

	V_{max} (mV)		V_{ave} (mV)		T_{max} (msec)	
F_0 and F_1 exposure	Males	Females	Males	Females	Males	Females
PND 20						
0	97.3 ± 46.0	102.8 ± 58.5	20.3 ± 8.8	21.6 ± 11.8	27.2 ± 5.7	25.4 ± 3.7
50	86.0 ± 31.3	98.9 ± 52.8	18.0 ± 6.7	20.8 ± 10.7	28.8 ± 5.9	27.5 ± 5.5
150	85.7 ± 36.2	88.8 ± 40.0	18.5 ± 7.3	18.7 ± 8.3	28.0 ± 3.8	25.4 ± 4.1
500	92.9 ± 32.2	95.9 ± 39.5	19.4 ± 6.8	20.5 ± 7.8	27.4 ± 6.0	26.8 ± 4.0
PND 60						
0	134.0 ± 101.9	75.1 ± 41.6	29.2 ± 20.7	15.8 ± 8.4	34.4 ± 5.7	34.4 ± 4.5
50	171.7 ± 164.0	85.3 ± 49.9	38.0 ± 35.1	17.3 ± 8.5	33.0 ± 5.4	34.2 ± 5.0
150	159.7 ± 94.5	62.6 ± 34.0	34.7 ± 20.6	13.8 ± 6.9	31.1 ± 4.9	35.5 ± 4.2
500	128.5 ± 99.0	83.0 ± 46.6	27.8 ± 20.3	17.3 ± 8.7	32.9 ± 5.9	33.6 ± 4.3

[a] V_{max} is the maximum response to the startle stimulus. V_{ave} is the average response to the startle stimulus. T_{max} is the latency to the maximum response.

FIGURE 3

PND 24 Learning and memory (Biel Maze) of F_2 offspring of rats exposed to styrene in the F_0 and F_1 generations. A: Males. B: Females. Trial indicates the session trial (Swim represents the swimming ability testing conducted on the first day of evaluation). Trials 1—4 were conducted in the forward direction (Path A), Trials 5—10 were conducted in the reverse direction (Path B; exact opposite of the forward direction), and Trials 11—12 were conducted in the forward direction (Path A). Trials 1—10 tested learning and Trials 11—12 tested memory. **Statistically different from control, $p < 0.01$.



the forward path, the slope of the line between Trials 11 and 12 was steeper than the first four trials. No exposure-related differences in the mean times to escape and numbers of errors (data not shown) were observed in either sex at either age evaluated, indicating that there was no impairment of learning (Trials 1-10) or memory (Trials 11 and 12) following indirect exposure to styrene.

Neuropathology

No direct effects of styrene exposure on absolute brain weights of PND 21 or 72 male and female rats were noted (Table 6). However, brain weights relative to final body weights of 500-ppm group females were increased compared to control females in these same animals because mean final body weights were slightly decreased in the 500-ppm group females.

Mean brain lengths, measured at necropsy, in the 150- and 500-ppm group females evaluated on PND 21 were statistically significantly less than (4.0% for both groups) the control group (Table 6). Mean brain lengths in males and females of all three styrene groups were slightly increased when compared to the control group on PND 72. Therefore, the difference noted in PND 21 offspring was considered incidental in nature, since there were no correlative changes in other histomorphological endpoints in the females nor were there any changes in the males. Mean brain width in all exposure groups was similar to the control group value on PND 21 and 72.

No microscopic findings that could be attributed to parental exposure to styrene were noted in the 500-ppm group as a result of the qualitative neuropathologic examination of the brain on PND 21 or central and peripheral nervous system tissues on PND 72.

There were no histomorphologic changes in measurements of brain regions on PND 21 or 72 that could be attributed to parental exposure in the 500-ppm group (Table 5). In female rats of the 500-ppm group evaluated on PND 21, the mean height of the hemisphere on Level 1 (figure 11 of Paxinos and Watson, 1998) was slightly (6%) increased. However, the cortical thickness was not altered when compared to the control group, and the height of the hemisphere was not altered in offspring evaluated on PND 72. Therefore, the difference noted in PND 21 offspring was considered incidental in nature since there were no correlative changes in other histomorphological endpoints in the females nor were there any changes in the males. No other differences from control were observed in any measurement taken from rats on PND 21 or 72.

TABLE 5

Selected Brain Histomorphic Measurements of F_2 Offspring of Rats Exposed to Styrene in the F_0 and F_1 Generations

	Males		Females	
	0 ppm	500 ppm	0 ppm	500 ppm
Level 1				
Height of hemisphere (mm)				
PND 21	0.62 ± 0.05	0.61 ± 0.03	0.62 ± 0.04	0.66 ± 0.04*
PND 72	0.59 ± 0.03	0.61 ± 0.04	0.61 ± 0.04	0.65 ± 0.04
Cortical thickness (mm)				
PND 21	0.16 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.16 ± 0.01
PND 72	0.15 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01

* Statistically significantly different from control, $p<0.05$.

DISCUSSION

As reported in the accompanying two-generation reproduction study (Cruzan et al., 2005, this issue), the parental systemic toxicity in this study was similar to that previously reported in rats following long-term inhalation exposure to styrene (Cruzan et al., 1997, 1998). Findings included degeneration of the olfactory epithelium that lines the dorsal septum and dorsal and medial aspects of the nasal turbinates of F_0 and F_1 animals in the 500-ppm group (nasal tissue was not examined in the 50- and 150-ppm groups), decreased mean body weights in the 150-ppm group (F_0 and F_1 males and F_1 females) and 500-ppm group (F_0 and F_1 males and females). Reproductive performance and offspring postnatal survival prior to weaning were not adversely affected by styrene exposure. Pre-weaning F_1 pup weights were unaffected by styrene exposure. Consistent with the lack of effect on pre-weaning body weights in the F_1 pups, no styrene-related effects were observed on the F_1 preweaning developmental landmarks. Following direct exposure of the F_1 weanlings beginning on PND 22, weight gain was reduced in the 500-ppm group, which led to reduced mean body weights in this group throughout the generation. As a result of the reduced body weight gain in the 500-ppm group F_1 males, a corresponding delay in the age of acquisition of the balanopreputial separation was observed. Clark (1998) and Ashby and Lefevre (2000)

TABLE 6

Brain Weight, Length, and Width of F_2 Offspring of Rats Exposed to Styrene in the F_0 and F_1 Generations

	Males				Females			
	0	50	150	500	0	50	150	500
Body weight (g)								
PND 21	43	41	40	39	41	39	36	36*
PND 72	385	377	372	360*	248	235	233	233
Brain weight (g)								
PND 21	1.55	1.64	1.62	1.59	1.54	1.56	1.52	1.57
PND 72	1.98	1.89	1.93	1.87	1.83	1.84	1.80	1.78
Brain Weight/ 100g body weight								
PND 21	3.86	3.91	4.23	4.02	3.88	4.08	4.14	4.65*
PND 72	0.49	0.50	0.49	0.53	0.73	0.77	0.79	0.79
Brain length (mm)								
PND 21	17.6	17.9	17.2	17.6	17.6	17.5	16.9*	16.9*
PND 72	22.6	24.2	25.4	25.6	22.2	23.8	23.6	24.4
Brain width (mm)								
PND 21	14.6	14.6	14.0	14.5	14.0	13.9	13.9	13.6
PND 72	15.1	14.9	15.2	14.8	14.9	14.7	14.5	14.6

* Statistically significantly different from control, $p<0.05$.

have previously reported that delays in this endpoint are observed in the presence of significant body weight reductions. Therefore, the delay in acquisition of balanopreputial separation was attributed to the reduced male body weight.

As direct exposure to styrene had a greater effect on F_1 rats than on F_0 rats, styrene had a more pronounced effect on the F_2 pups than on the F_1 pups. In contrast to the F_1 generation, pre-weaning F_2 pup weights were reduced in both the 150- and 500-ppm groups (approximately 10 to 13% on PND 21). The weights of the F_2 pups selected for neurobehavioral evaluation in the developmental neurotoxicity phase continued to be reduced following weaning in the 150- and 500-ppm groups. A statistically significant delay (approximately 1/2 day) in the mean age of acquisition of incisor eruption was observed for the F_2 pups in the 500-ppm group. Slight delays (not statistically significant) in pinna detachment, surface righting, hair appearance, eye opening, and balanopreputial separation were also noted in this group. The magnitude of the delays in each of these endpoints was slight and if they had occurred in isolation, the delays would have been attributed to biological variability.

There is not a one-to-one relationship between body weight and these values. However, on an individual animal/litter basis, there is a pattern that relates litters and weights relative to the group mean value. For example, one 500-ppm group female, which is the lightest animal in that group on PND 63, weighed 157g, 27% below the group mean of 213g and 31% below the control group mean of 227g. This female and her male sibling had mean hindlimb grip strength values that were 35-37% lower than the group means (187g vs. 286g for the female and 200g vs. 320g for the male) and 48-50% below the control group means (357g for females and 397g for males). The forelimb grip strength values for these animals were not dramatically different from the group means. However, siblings of these rats showed delayed pinnal detachment (3 of 9 on PND 5 or 6 were from this litter), surface righting (3 of 22 on PND 5 or 6 were from this litter), incisor eruption and eye opening (3 from this litter were among the last with open eyes), as well as a female PND 24 swim

time of 16.96 sec (compared to 11.43 sec for the group and 7.81 sec for control females), acquisition of balanopreputial separation on PND 51, and acquisition of vaginal patency on PND 42. From a locomotor activity standpoint, this female and her male sibling showed a significant delay in development, with activity that was nearly nonexistent (and the lowest for the females) on PND 13 and that peaked on PND 21 rather than PND 17. So, both male and female pups from this litter contribute to the delay in several of the endpoints monitored, and this litter is just one example from the group as a whole.

Thus, when evaluated in toto, the slight delays in the acquisition of these parameters were suggestive of an overall pattern of slight developmental delay in the 500-ppm group. These pre-weaning developmental end-points are highly correlated with pup body weight (Clark, 1998; Lochry, 1987), and the delays in these endpoints were consistent with the reduced body weights observed in this group. Based on this profile, the effects of styrene on the growth and development of the F_2 pups were somewhat greater than those effects observed on the F_1 pups.

Further evidence of a slight developmental delay in the F_2 offspring included an apparent shift in the ontogeny of normal locomotor activity from PND 13 through 21, the reduced swimming ability (presented as longer straight channel escape times) in the water maze on PND 24, and reduced grip strength on both PND 45 and 60 in F_2 offspring of F_0 and F_1 rats exposed to 500 ppm styrene. The ontogeny of swimming ability, the pre-weaning locomotor activity profile, and the correlation of body weight with grip strength in rats have been well characterized (Adams, 1986; Kallman, 1994). The general developmental profile in this group demonstrated a pattern of delay that included changes only in age- and weight-sensitive endpoints throughout the entire F_2 generation; there were no indications of functional or morphologic effects suggestive of selective neurotoxicity. Therefore, based on the known correlation between body weight and these endpoints, the profile of changes observed in the 500-ppm group of the F_2 generation was attributed to a slight developmental delay as a result of parental styrene exposure. The NOAEL for growth of F_2 offspring was 50 ppm.

Kishi and coworkers, using a few litters of rats, concluded that styrene exposure of parents caused deficits in neurological development. The current study, performed according to accepted guidelines with greater statistical power to detect effects, confirms some of their observations, but does not support their conclusions of a direct effect on neurological development. In the first study (Kishi et al., 1992, 1995), they reported (using 5 control and 5 exposed litters) that inhalation exposure of pregnant Wistar rats at 293 ppm styrene on gestation days 7–21 resulted in offspring with decreased open field activity, rota-rod activity, and operant conditioning response. The study reported herein found no significant effects on locomotor activity or learning and memory in offspring following direct exposure of the F_0 and F_1 generations to 500 ppm styrene throughout growth, mating, gestation, and lactation. In a second study, Katakura et al. (1999, 2001) reported increased neonatal mortality, delayed incisor eruption and delayed air righting reflex in offspring of dams that had been exposed to 300 ppm on gestation days 7 to 21. The authors did not indicate whether there were body weight differences from control at the time these endpoints were determined. In the study reported herein, styrene exposure had no effect on neonatal survival. Surface righting and incisor eruption in off-spring of the 500-ppm group were slightly delayed, as noted above, but were judged to be secondary to reduced body weight.

Based on the guideline study reported herein, no specific effect on nervous system development was observed at exposures up to 500 ppm styrene.

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Olfactory Function in Workers Exposed to Styrene in the Reinforced-Plastics Industry

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Background
Impairment of olfactory function in humans has been associated with occupational exposure to volatile chemicals. To investigate whether exposure to styrene was associated with olfactory impairment, olfactory function was examined in workers with a minimum of 4 years exposure to styrene in the reinforced-plastics industry (current mean exposure: 26 ppm, range: 10-60 ppm; historic mean dose: 156 ppm-years, range: 13.8-328 ppm-years) and in a group of age- and gender-matched, unexposed controls.

Methods
Olfactory function was assessed using a standardized battery that included tests of threshold sensitivity for phenylethyl alcohol (PEA), odor identification ability, and retronasal odor perception. Odor detection thresholds for styrene were also obtained as a measure of specific adaptation to the work environment.

Results
No differences were observed between exposed workers and controls on tests of olfactory function. Elevation of styrene odor detection thresholds among exposed workers indicated exposure-induced adaptation.

Conclusions
The present study found no evidence among a cross-section of reinforced plastics industry workers that current or historical exposure to styrene was associated with impairment of olfactory function. Taken together with anatomical differences between rodent and human airways and the lack of evidence for styrene metabolism in

The present study found no evidence among a cross-section of reinforced plastics industry workers that current or historical exposure to styrene was associated with impairment of olfactory function. Taken together with anatomical differences between rodent and human airways and the lack of evidence for styrene metabolism in human nasal tissue, the results strongly suggest that at these concentrations, styrene is not an olfactory toxicant in humans.

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human nasal tissue, the results strongly suggest that at these concentrations, styrene is not an olfactory toxicant in humans. *Am. J. Ind. Med.* 44:1-11, 2003 ©2003 Wiley-Liss, Inc.

INTRODUCTION

One of the potential consequences of occupational exposure to respirable chemicals, the impairment of olfactory function, has been noted in the medical literature for more than 100 years [Mackenzie, 1984]. Because the function of the olfactory system is to detect ambient chemicals, the receptors must constantly interact with chemical stimuli, some of which are potentially toxic. Olfactory receptors are located on specialized neurons that extend into the nasal cavity and are therefore uniquely and consistently exposed to the external environment. Numerous animal studies have shown that olfactory neurons are the only CNS neurons that are continuously replaced throughout the adult vertebrate lifespan, even in healthy animals housed in clean environments [e.g., Loo et al., 1996], suggesting that normal olfactory function is inherently damaging to the neurons. Exposure to higher levels of pollutants, such as can be found in some industrial environments, might therefore be hypothesized to surpass the regenerative capacity of the olfactory system and lead to dysfunction.

While not, by itself, life-threatening, olfactory dysfunction can have serious consequences for the detection of many olfactory warning signals (e.g., smoke, spoiled food and gas leaks) [Cowart et al., 1997] and can have significant impact on nutritional status, eating satisfaction, and many other issues related to quality of life [Breslin et al., 1997]. In addition, a worker whose olfactory abilities are impaired may be at greater risk from exposure-related injuries due to the loss of an early warning system for chemical exposure.

Two lines of evidence suggest that occupational chemical exposure may impair olfactory function. First, animal toxicological studies have demonstrated selective and dose-dependent histopathologic alterations in the nasal cavity from experimental exposures to a diverse range of chemical substances, (e.g., methyl bromide, chlorine, isobutyraldehyde, formaldehyde) [Jiang et al., 1983; Hurtt et al., 1988; Monticello et al., 1991; Abdo et al., 1998]. The precise nature and distribution of these chemically induced nasal lesions can vary considerably as a function of regional deposition of the inhaled substance and the local susceptibility of the nasal tissue. However, inhalation exposure studies suggest that the olfactory epithelium is particularly vulnerable to damage by inhaled compounds [Jiang et al., 1983; Genter et al., 1998] with a variety of non-neoplastic lesions of the olfactory neuroepithelium and damage to central olfactory structures, such as the olfactory bulb, associated with chronic exposure to a variety of chemicals [Ekblom et al., 1984; Odkvist et al., 1985; Feron et al., 1986; Rose et al., 1992].

Second, a number of studies of individuals and/or populations occupationally exposed to various chemicals have reported problems with their sense of smell or apparent decrements on certain measures of olfactory function. For example, an analysis of work history and olfactory ability among the 712,000 (20-79 year old) US and Canadian respondents to the National Geographic Smell Survey revealed that factory workers reported poorer senses of smell and demonstrated objective evidence of poorer odor detection ability, although this effect was pronounced among elderly individuals [Corwin et al., 1995]. Notably, among this sample, factory workers reported the highest rates of olfactory decrements secondary to chemical exposure and head injury.

Amoore [1986] identified more than 100 airborne substances that were reported to disrupt olfactory function following either acute or chronic exposures, including organic solvents, metals, inorganic non-metallic compounds, and dusts. Much of this evidence is based on single case studies or anecdotal observations in occupational environments [Emmett, 1976; Amoore, 1986; Prudhomme et al., 1998], and relies on subjective reports of olfactory function or limited olfactory testing. Although several investigators have examined olfactory function in larger groups of chemically exposed workers and reported associations between exposure and olfactory function, the relationship is far from straightforward. For example, decrements in performance on the University of Pennsylvania Smell Identification Test (UPSIT), a 40-item test of olfactory identification ability, have been observed among some, but by no means all, workers exposed to organic solvents [Sandmark et al., 1989; Schwartz et al., 1990, 1991]; among workers exposed to acrylate and methacrylate vapors, small decrements in function were only observed for non-smokers [Schwartz et al., 1989]. In brief, although many chemicals have been implicated as causative agents in olfactory dysfunction [Amoore, 1986], considerable variation has been observed both in the type and degree of impairment associated with occupational exposures. Moreover, in the case of the few published epidemiological studies, well-matched control groups and/or assessments of current and historical chemical exposures are largely lacking.

Recent inhalation studies in animals have found that exposure to as little as 20-50 ppm styrene results in non-

neoplastic, histopathological changes in the olfactory epithelium of rodents [Cruzan et al., 1997, 1998]. However, because of structural differences in the nasal passages between rodents and humans, and differences in the biochemistry of the nasal/olfactory tissue, and because the most common laboratory animals models are obligate nasal breathers, these changes might not occur in humans in comparable environments. Moreover, because animal toxicological studies have rarely incorporated functional measures, very little is known about the relationship between the type and severity of olfactory epithelial damage and the extent to which olfactory function is altered. Thus, the question of how to relate the observed structural damage in rodent olfactory epithelium to humans, and the functional consequences of such damage for either species, remains unknown. The goal of this study was to examine the effect of exposure to styrene under conditions that permitted a careful assessment of the olfactory function of both occupationally exposed workers and of a matched group of unexposed controls.

Styrene is a clear colorless liquid with a characteristic pungent odor. The primary use of styrene is in the production of polymers and copolymers, including polystyrene, styrene-butadiene-rubber, styrene-butadiene-latex, and a variety of different resins. Styrene monomer is combined with polyester resins and serves as a cross-linking agent in the manufacture of numerous fiberglass-reinforced products (bathtub and shower enclosures, boats, tanks, panels, etc.). The most significant occupational exposure to styrene vapor occurs in the reinforced-plastics industry, where exposure can occur both by inhalation and direct skin contact [Lemasters et al., 1985]. However, inhalation of styrene vapor is the major route of occupational exposure to styrene [Brooks et al., 1980] with the nasal epithelium as a point of entry. Thus, there is potential for adverse effects of occupational styrene exposure on nasal histopathology and olfactory function.

Our study was designed to evaluate whether repetitive exposure to styrene vapor at the upper range of concentrations likely to be encountered in the workplace [American Conference of Government Industrial Hygienists, 1998; Morgan, 1997] is associated with clinically significant olfactory dysfunction. To evaluate this, we measured olfactory function among workers who were occupationally exposed to styrene in the reinforced plastics industry, using a comprehensive battery of objective tests of olfactory function, developed and validated at the Monell-Jefferson Chemosensory Clinical Research Center. In addition to the potential for causing generalized olfactory dysfunction, continued or repetitive exposure to any odorous chemical will lead to olfactory adaptation, a compound-specific reduction in olfactory sensitivity to that chemical. As a measure of specific adaptation to the ambient chemical environment, we tested olfactory sensitivity to styrene vapor. All olfactory assessments were coupled with current and retrospective determinations of airborne styrene exposure for the workers [for details see Lees et al., 2003 (this issue)] and were compared with olfactory assessments performed on age- and gender-matched, non-exposed controls at each site, and with normative data obtained from healthy individuals.

MATERIALS AND METHODS

Overview of Olfactory Testing

We measured olfactory detection thresholds to two chemicals (styrene and phenylethyl alcohol) and odor identification ability using twenty chemicals in order to provide a comprehensive evaluation of olfactory function, similar to the clinical assessment used at the Monell-Jefferson Chemosensory-Clinical Research Center. Each type of test can reveal a different component of olfactory function: detection thresholds can provide assessments of peripheral olfactory function, whereas tests of odor identification are assumed to tap more central components of olfaction as well. Because performance on both types of tests of olfactory ability can be influenced by a variety of demographic factors, (e.g., age, gender, and education) and dysfunction can arise from a variety of non-toxic etiologies [e.g., nasal-sinus disease or head injury; Cowart et al., 1993], it is essential to compare the performance of the exposed cohort with the performance of a suitably matched control or referent group.

There are compelling reasons to include comprehensive olfactory evaluations and appropriately matched control groups into occupational evaluations. In studies that measured both peripheral (odor detection) and central (odor identification) olfactory function within the same exposed individuals, occupational exposure to cadmium was shown to impair both the detection and the identification component of olfactory function [Rydzewski et al., 1998]. It is instructive to note that when olfactory function in a similar group of cadmium-exposed workers was compared with a group of age- and gender-matched controls, only detection sensitivity differed between the two groups [Rose et al., 1992]. The present study, therefore, attempted to address previous shortcomings in the characterization of chemical-exposure effects on olfaction by coupling a thorough evaluation of past and present styrene exposures with a battery of clinical tests that included an assessment of both peripheral and central components of general olfactory

function (odor detection thresholds and odor identification tests, respectively) and a specific test of exposure-induced adaptation (detection thresholds for styrene).

If either historic or current exposure to styrene produced generalized olfactory damage, we would expect to find that the prevalence of olfactory dysfunction would be greater among workers with exposure to styrene than in the general population, as measured by our normative clinical database. However, due to the potential confounding effects of a variety of geographic, local environmental, and lifestyle factors, we also recruited and examined olfactory function in a group of referents who were similar to the workers with respect to socio-economic status, geographic location, and matched for age and gender. Comparisons between these groups were expected to reveal whether occupational exposure to styrene produces any olfactory loss or dysfunction above and beyond age- or health-associated effects found among workers of this socio-economic status, generally.

Site Selection

Workers in the reinforced-plastics industry were selected as the study group because the highest occupational exposures to styrene are reported to occur in this industry [Lemasters et al., 1985]. Solicitation letters were sent to approximately 30 candidate companies that manufactured reinforced-plastics and composite products, inviting them to participate in the study. Of those indicating interest in participating, we determined eligibility according to criteria described by Lees et al. [2002]. Based on these criteria, four facilities were selected to participate in the study: two factories engaged in the manufacture of fiberglass-reinforced shower and tub enclosures using an open-mold process, one factory that manufactured reinforced-fiberglass paneling, and one factory that produced a variety of reinforced-plastic products, including truck parts, sinks, and fan blades, using a closed-mold process.

Subjects

At each site, potentially eligible workers and controls were individually identified and recruited following a review of: (1) industrial hygiene surveys that indicated ambient styrene concentrations for each job title and (2) personnel records identifying individual work histories. Through the company's personnel office, pre-screened workers were notified of their potential eligibility for the study and invited to fill out a medical and occupational history questionnaire, the answers to which were used to determine their suitability for the olfactory study.

To adjust for the potentially confounding effects of a wide range of social, economic, and environmental factors, we also tested a comparison worker group, comprised of workers from the same geographical region and having similar socio-economic status but no chemical exposure.

All medical/occupational questionnaire responses were masked with respect to cohort/control group membership and reviewed by the director of the chemosensory disorders clinic and the occupational physician to screen for medical conditions and prior exposure to environmental agents or chemicals, *other than styrene*, that were known or suspected to affect olfactory function. Respondents were sorted into three categories: (1) eligible, (2) eligible but requiring additional clarification on certain aspects of their medical/exposure history, and (3) ineligible. Workers in the first two categories were then invited to participate in the study, with 94% of the eligible workers agreeing to participate.

All workers and control subjects provided informed consent for their participation in the study using the form that was approved by the Committee for Studies Involving Human Beings of the Institutional Review Board at the University of Pennsylvania. Volunteers were advised that they were free to withdraw at any time. Workers continued to receive full wages during the 1-2 hr of data collection. The control subjects who participated on their own time were compensated for their participation.

Sixty-two exposed workers and sixty-seven controls were tested in the study. On the basis of follow-up questions at the time of test, eight of the exposed workers and three control subjects were excluded because of pre-existing medical conditions or exposure to other chemicals potentially causative of olfactory dysfunction. Two more workers were excluded based on age and failure to understand/comply with test instructions. To match the number, gender, and age of subjects in the worker-group, fifteen control subjects were dropped from the analysis, resulting in fifty-two subjects in each group. The selection procedure for dropping control subjects was blind with respect to olfactory performance; at each site, subjects were included in the order of testing until the control group was matched to the workers on age (within one decade) and gender.

Odor Stimulus Preparation

Styrene monomer (Sigma-Aldrich) and phenylethyl alcohol (Sigma-Aldrich) were used as stimuli in the odor detection tests. Styrene was diluted into odorless, light, white mineral oil in an 18-step binary dilution series, beginning with a concentration of 20% v/v. Prior to use, the mineral oil was filtered through a column of silica gel to remove odorous contaminants. Phenylethyl alcohol was diluted into glycerol (Sigma) in a 19-step semi-log

TABLE 1

Stimuli Used in Odor Identification Test

Identification	Chemical/odorant	Identification	Chemical/odorant
Menthol	L-Menthol	Cloves	Eugenol
Rye bread	D-Carvone	Licorice	Anethol
Turpentine	Terpinolene	Spearmint	L-Carvone
Banana	Amyl acetate	Smoke	Guaiaicol
Almond	Benzaldehyde	Wintergreen	Methyl salicylate
Strong cheese	Butyric acid	Cinnamon	Cinnamaldehyde
Fish	Triethylamine	Coconut	Octalactone
Peanut butter	Ethylpyrazine	Root beer	Safrol
Vinegar	Acetic acid	Rose	Phenylethyl alcohol
Vanilla	Vanillin	Strawberry	C-16 Aldehyde

dilution series, starting with a concentration of 100% v/v. The dilutions were placed into clean, 280 ml glass bottles, fitted with a flip-top cap into which a Teflon nosepiece was inserted. Each bottle contained 10 ml of the stimulus. For each subject, a fresh set of bottles, containing 10 ml of diluent only, served as blanks. When volunteers inhaled from the sniffing port, they sampled from the headspace inside two bottles (one sniffing port per nostril). In a typical sniff by adult males at rest, 500 ml of air is drawn through the nostrils. Thus, drawing the headspace from the two glass bottles (total volume 540 ml) should have provided an adequate volume of stimulus, undiluted by incoming air.

Banana, butterscotch, coffee, lemon, and peppermint, alcohol-free extracts (Frontier) were used as stimuli in the retronasal olfactory assessment. One-half milliliter of each extract was pipetted onto a cleaned, black, polyethylene snap cap (Wheaton) and covered with parafilm until ready for testing. One set of odorants was prepared for each participant and discarded after use.

The stimuli for the odor identification test consisted of twenty odorants that were presented in cleaned, boiled, 250 ml polypropylene squeeze bottles with flip caps (Wheaton). Each bottle contained a total of 5 ml of odorant or odorant and diluent combined. Table 1 lists the odorants used and presents the correct odor label for each stimulus.

New threshold series and odor identification stimuli were prepared for each week of testing and analyzed via gas chromatography immediately after the series was prepared to ensure reliability across and within series.

Testing Procedure

All olfactory tests were administered in a styrene-free environment (as established by area samples using active-sampling technology to be below the analytical limit of detection, <1 ppm styrene). All exposed workers were tested during their regular 8- or 10-hr work-shift (day, evening, or night). Workers assumed their regular duties for a minimum of 1 hr and a maximum of 6 hr prior to the test. Except for workers at one site, all had worked full shifts on at least the two days immediately prior to their olfactory test. Scheduling constraints at one site meant that some workers had only worked one consecutive day prior to their test. Testing of controls and workers was alternated as much as possible during each day of testing.

All volunteers were tested individually in a single session that lasted approximately 1½ hr. Subjects were asked to refrain from smoking, eating, chewing candy, or drinking a beverage in the hour prior to the test. The session consisted of multiple assessments. First, odor detection thresholds were obtained for two compounds, phenylethyl alcohol—a measure of general olfactory function, and styrene—a measure of specific adaptation to the work environment. Next, a retronasal odor identification test was administered followed by an odor identification assessment.

Medical History and Screening Questionnaire

Prior to the olfactory evaluation, each volunteer's responses to the medical history questionnaire were again reviewed with the tester; when necessary, follow-up questions elicited additional information about specific nasal, allergy or sinus problems, head or facial injuries and surgeries, exposures to environmental agents, and medications used that may contribute to or cause olfactory dysfunction.

Olfactory Detection Thresholds

Olfactory detection thresholds were obtained by using an objective, two alternative, forced choice, modified staircase method, a procedure that is the method of choice for most clinical and experimental applications, since it pro-

vides a reliable threshold measure with a relatively small number of trials [Wetherill and Levitt, 1965; Dalton and Wysocki, 1996]. Thresholds for styrene and PEA were obtained sequentially, with the order of test counter-balanced across subjects. On each trial, the subject was presented with two pairs of bottles. One pair consisted of two blanks and the other pair consisted of one blank and the appropriate dilution step of styrene or PEA. As determined from a pilot study, the starting dilution step for styrene was step 8 and for PEA was step 11.

On receiving the set of bottles, volunteers inserted the nosepieces into each nostril and took a normal sniff from the air in the headspace. After sniffing from both pairs, volunteers were asked to identify which pair contained the odorants. Presentation of increasing or decreasing concentrations continued until the individual had achieved five reversals (a reversal is defined as two correct identifications followed by one incorrect one, or one incorrect identification followed by two correct ones, at a given concentration step).

Retronasal Odor Identification

Each of the five stimuli (peppermint, lemon, coffee, banana, and butterscotch) was presented twice retronasally and twice orthonasally in a pre-determined, semi-random order, as described by [Pierce and Halpern, 1996]. On each trial, the subject chose one of the five stimulus names from a given list.

Odor Identification Test

Each odorant was presented twice resulting in a forty-item test in which each presentation was associated with a choice of four labels. Each odorant name appeared twice as a correct response, twice as a near-miss, and four times as a far-miss. For example, amyl acetate (banana odor) was presented with the following labels: banana (correct), cloves (incorrect), strawberry (near-miss), smoke (incorrect). The volunteer was required to sniff from the bottle and choose from among the four options, the label that best fit the odor. Due to the inherent difficulty in the task, subjects were permitted to take more than one sniff prior to choosing the answer.

Criteria for Clinical Abnormality

The classification of olfactory dysfunction or loss among the workers tested was based upon previously established clinical criteria for the diagnosis of olfactory disorders [Cowart et al., 1997]. Each of the odor threshold measures and the identification tests were examined separately and in combination. Among the exposed workers, elevation of the styrene threshold (relative to the controls) when the PEA threshold and odor identification tests fell within normal limits was regarded as a measure of occupational adaptation to styrene, but not a clinical abnormality per se. If subjects reported odor quality distortions on the questionnaire, a primary diagnosis of dysosmia (distortions in odor quality) was assigned when (1) the PEA threshold fell within normal limits and (2) the odor identification score fell below criterion performance. A primary diagnosis of hyposmia was assigned when PEA threshold fell above the concentration step of 0.01% v/v (step 7) and an odor identification score below 31/40 for males and 34/40 for females (reflecting a gender difference in the normative sample). These test scores were used as the basis for a diagnosis of hyposmia or anosmia. Generally, the upper (or lower) 2.5th percentile is regarded as the cutoff point for chemosensory abnormality [Feinstein, 1985]. Due to age-related declines in olfactory sensitivity observed among normative subjects beginning in the sixth or seventh decade, these criteria are based on scores obtained by subjects under 50 years of age; to avoid the necessity of age-adjusting scores in this study, the majority of our study group was comprised of individuals between 21-50 with only 5 individuals in each group who were between 50 and 60 years of age.

The retronasal odor identification test provided another unique measure of olfactory dysfunction, likely to be functionally evident in the diminished perception of everyday food flavor. Based on the limited normative sample, an identification score less than 8 out of 10 on either orthonasal or retronasal presentation was indicative of clinical abnormality.

Assessment of Current and Past Occupational Exposure to Styrene

Establishment of a relationship between occupational exposure to styrene and any observed olfactory dysfunction rests critically on the estimates and assessments of current and historical exposure to styrene among the exposed workers. Thus, personal exposure profiles were created for each of the styrene-exposed workers who participated in this study based on individual or surrogate sampling data from the facility, where available [for details about exposure reconstruction see Lees et al., 2003]. Current exposures to styrene were determined by full-shift, personal air sampling, and biological monitoring of each enrolled worker.

Since any effect of styrene on the human olfactory system might result either from the direct local effect of the air concentration acting on the nasal epithelium or from the effect of absorbed styrene, carried by the blood stream, we also evaluated current styrene exposures through urinalysis for metabolites of styrene (mandelic acid and

phenylglyoxylic acid) [Gotell et al., 1972; Mizuuma et al., 1993]. For each worker, pre- and post-shift urine samples were collected on the day prior to the one in which they were tested for olfactory function. For each control subject, a single urine sample was obtained at the time of their olfactory test.

TABLE 2

Demographic Characteristics of Participants

	Total no.	Females	Age mean & range	Smokers	Styrene exp. duration (mean years & range)
Workers	52	6	37.6 (21-60)	35	12.5 (4-41)
Controls	52	6	36.7 (21-57)	27	0

Data Analysis

Thresholds for PEA and styrene were calculated by taking the mean of the dilution steps representing the last four reversals in each test. If the subject was able to smell the stimulus at the weakest concentration available, they were assigned the next dilution step (e.g., step 19 for styrene and step 20 for PEA). Scores on the retronasal trials testing olfactory function were given one point for each correct answer out of a total possible of ten. The odor identification test was scored by assigning one full point for every correct answer and one-half point for every near-miss response.

To evaluate differences among the styrene-exposed workers and the control group on the clinical tests of olfactory function, separate, one-way Analysis of Variances (ANOVAs) were performed on the PEA thresholds, the retronasal scores and the odor identification scores. Potentially confounding variables were identified on the basis of prior research and included age, years of education, and smoking status (pack years); these were then used as covariates in the analysis. To evaluate differences among the workers and controls on styrene sensitivity, a one-way ANOVA was performed on the individual styrene thresholds. Multiple linear regression was used to evaluate significant differences, if any, among groups on any of the olfactory measures.

RESULTS

The demographic characteristics of each group are presented in Table 2.

Current Workplace Environmental Exposures

The results of the personal air sampling during the two workshifts prior to olfactory testing are presented in Table 3, for each study site. Workers were currently exposed to a range of styrene concentrations, with many exposure measurements considerably less than 30 ppm and a few exposure measurements in excess of 50 ppm. The highest air concentration measurements were confined to three individuals wearing respiratory protection, but the use of respirators ensured that their actual styrene exposures were considerably less than those measured.

In order to adjust the air exposure data, a respiratory protection factor (RPF) was determined for a group of workers at one site who were currently using respirators [Lees et al., 2002] and an RPF of 5 was subsequently used.

With the exception of Site 4, the measurements of current exposures were generally consistent with historic exposure measurements which had been made over the last ten or more years. During air sampling at Site 4, production (and, therefore, ambient concentrations) were significantly reduced from historic norms, which accounted for this difference.

Urine samples were analyzed for the presence of the styrene metabolites, mandelic acid and phenylglyoxylic acid, corrected by measured creatinine levels. Creatinine determination was performed by colorimetric spectroscopy. Table 4 shows the pre- and post-shift means and ranges of both metabolites for the workers tested. As expected, the levels of styrene metabolites for all control subjects were below the limit of detection (0.02 g/L).

TABLE 3

Mean and Standard Deviations of Airborne Styrene Concentrations by Study Site

	Site 1	Site 2	Site 3	Site 4	Avg.
Day 1 (ppm)	58.8(34.6)	18.2(23.3)	*	12.6(9.1)	20.6
Day 2 (ppm)	65.5(44.7)	15.8(15.8)	14.1(9.2)	11.3(6.9)	24.5

*Air not sampled on day 1 at this site.

TABLE 4

Means and Ranges of Urinary Metabolites of Styrene

	MA Pre-shift	MA Post-shift	PGA Pre-shift	PGA Post-shift
Workers	0.10(0.0-0.51)	0.62 (0.0-6.98)	0.07(0.0-0.47)	0.17 (0.0-2.25)

All values for mandelic acid (MA) and phenylglyoxylic acid (PGA) are g/g creatinine corrected.

TABLE 5

Means and Ranges of Historic Exposures to Airborne Styrene for Individual Workers (n = 52)(in ppm)

	Mean exposure	Cumulative mean exposure	Peak Year exposure
Workers (all sites)	13.25(3.5-31)	156(13.8-328)	26(5.2-76.5)

Historic Workplace Environmental Exposures

Individual historic styrene exposure profiles were developed for every study participant using job title-based estimates of annual average styrene exposure for each year of employment. After accounting for respirator use, a cumulative "effective" estimate of styrene dose (calculated as the sum of annual average exposures and expressed in terms of ppm-years) was also established. Annual average exposures for workers at the individual sites basically mirrored styrene in air concentrations reported in the previous section except that the calculation of effective exposures (to account for the historic use of respirators by a small number of individuals), dramatically reduced the upper end of the exposure distribution (see Table 5).

While both current and historic exposure measurements focused on styrene, a subset of workers in some of these facilities were exposed to a variety of other chemicals. In addition to a wide range of catalysts and other additives to the styrene resins (e.g., brominated flame retardants) used over the last two decades, laminators (gunners and rollers) historically used large quantities of acetone to clean rollers and chopper guns. Although the level of exposure to acetone is unknown, its use has been discontinued within the last 5 years at all sites studied except Site 3. In addition, Site 3 uses a small amount of methyl methacrylate in the production of panels. Air concentrations of methyl methacrylate ranged from 0 to 28 ppm (mean = 6.7 ppm) when measured in the late 1980s. More recent measurements are not available.

Evaluation of Olfactory Function: Exposed Workers Vs. Controls

The group means, standard deviations and ranges of scores for the three clinical tests of olfactory function are presented in Table 6. Separate, one-way ANOVAs performed on the phenylethyl alcohol thresholds, the retronasal test and the odor identification scores showed no significant differences between the performance of the styrene-exposed workers and the matched controls on any of the tests of olfactory function.

In marked contrast to the performance on the clinical olfactory assessments, odor detection thresholds for styrene were significantly different among the exposed and unexposed groups, $F(1,100) = 16.69$, $P = 0.00001$. On an average, the styrene threshold for exposed workers was almost four dilution steps (i.e., 32-fold) higher than for the unexposed control subjects (10.6 vs. 14.3, workers and con-

TABLE 6

Performance on the clinical tests of olfactory function

Test	Workers Mean (s.d.)	Workers Range	Controls Mean (s.d.)	Controls Range
PEA threshold (dilution step 0-20)	16.67 (4.5)	5.5-20.0	16.66 (4.8)	1.5-20.0
Retronasal ID (number correct of 10)	8.11 (1.8)	2.0-10.0	8.05 (2.0)	1.0-10.0
Odor ID (number correct of 40)	34.80 (2.6)	27.0-39.5	35.75 (3.0)	23.0-40.0

trols, respectively). Stratifying the groups by age (in decades) revealed that this difference was not uniform across all workers in our sample. As shown in Figure 1, the youngest workers and controls showed small, but significant differences in their sensitivity to styrene during their 3rd (n=26) and 4th (n=36) decades. However, the greatest reduction in sensitivity to styrene was shown by the workers who were in their 5th (n=28) and 6th (n=14) decades.

Although there is a small, but significant correlation between the age of the workers and the duration of exposure to styrene (r=0.31), the marked decline in sensitivity to styrene in older workers cannot be accounted for by the duration of their past exposure to styrene. When styrene thresholds for the exposed-worker population were regressed upon the variables of age, exposure duration, and other exposure factors (ppm-years, peak-year), age was the only significant predictor of styrene thresholds, and was still significant, even after adjusting for the effects of exposure duration ($P<0.03$). Importantly, as shown in the inset graph depicting PEA thresholds as a function of age for both groups, there were no age-associated changes in sensitivity to PEA among workers or controls.

Evaluation of Olfactory Function: Incidence of Clinical Abnormality

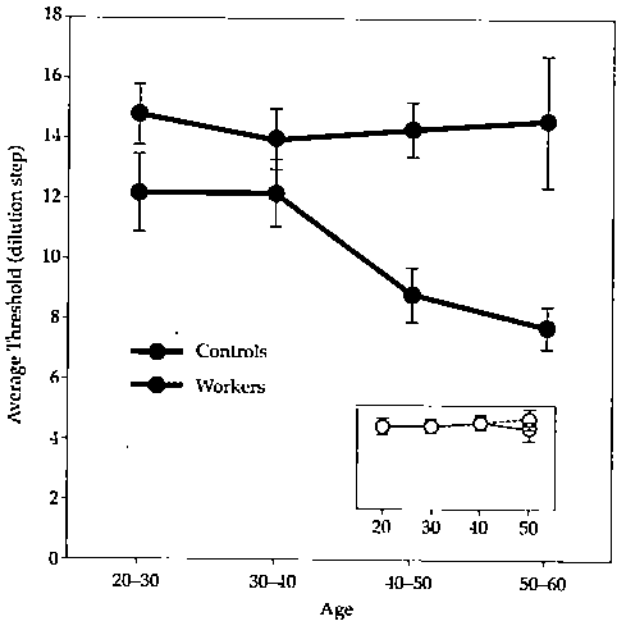
To evaluate the incidence of clinical dysfunction (hyposmia, dysosmia, or anosmia) among either exposed workers or controls, we compared each subject's threshold for PEA and their score on the odor identification test with the cut-off scores for olfactory function as determined by the normative sample obtained from the Monel-Jefferson CCRC database. No significant differences in test performance were found between the exposed workers and the controls.

Although a few more workers than controls reported disturbances of some sort on olfactory function, the only diagnosed abnormality of olfactory function among the entire sample was hyposmia, of varying severity, and the frequency of observed abnormality did not differ between groups. Moreover, a test of proportions revealed that the prevalence of olfactory dysfunction (as measured by the PEA threshold and odor identification test) was not higher among either group than would be expected to occur in the general population (2.5%; z=1.31, $P=0.08$).

Although scores on the retronasal olfactory evaluation were lower than expected in both workers and controls when compared to our normative population data, this outcome may have arisen from non-exposure related factors, such as dietary experience or cultural practices: odor identification tests are known to be influenced by previous exposure to odorants [Doty et al., 1996]. Our normative sample was drawn predominantly from an urban environment whereas participants in this study were drawn from relatively rural environments.

FIGURE 1

Styrene thresholds and PEA thresholds (inset graph) as a function of occupational exposure and age (in decades).



DISCUSSION AND CONCLUSIONS

Exposure to styrene vapor, in a cross-sectional analysis of workers in the reinforced-plastics industry, did not appear to be associated with any adverse effect on olfactory function. Performance on the tests of peripheral (PEA threshold) and central (odor identification test) olfactory function revealed no significant differences between the workers exposed to styrene and the comparison group of unexposed controls. Given the widespread use and validation of these or similar tests to diagnose and identify olfactory dysfunction in many clinical settings [e.g., Cain et al., 1988], we are confident that these evaluations have been able to reveal any patterns of generalized deficits in olfactory ability among the workers in this sample. Moreover, the lack of any observed effect on objective olfactory function in the present study correlates well with the workers' self-reported olfactory performance. Only a few workers reported problems with

their sense of smell and of these complainants, only one individual showed evidence of any abnormality on objective olfactory evaluation.

Occupational exposure to styrene affected sensitivity to styrene, but this effect is expected and consistent with specific, exposure-induced effect of adaptation—which can be observed in a variety of field settings and also in the laboratory following even brief exposures to volatile chemicals [Ählstrom et al., 1986; Dalton and Wysocki, 1996; Wysocki et al., 1997]. The present study revealed, however, that this exposure-related decrement was more pronounced among older workers than younger ones. Given that (1) all workers had been working in their regular position (and exposed to styrene vapor) for a minimum of one hour prior to olfactory testing and that (2) neither age nor exposure duration were significant predictors of variation in any of the other olfactory measures, a plausible explanation for the age-related effect on styrene thresholds among the workers is that occupational olfactory adaptation (in this case to styrene) is both more profound and more persistent among older workers than younger ones. Age-related differences in the kinetics of olfactory adaptation have been noted previously when Stevens et al. found that the rate and degree of adaptation accelerated while recovery decelerated with age [Stevens et al., 1989]. Although exposure-induced adaptation effects appear attenuated or reversible following cessation of exposure [Ählstrom et al., 1986; Gagnon et al., 1994; Mergler and Beauvais, 1992], the time course of recovery after long-term exposure is unknown. The more pronounced decrement in styrene sensitivity shown by the older workers may need to be taken into account in any situations where early detection of the ambient chemical is critical (i.e., detection of respirator failure).

Although there is recent evidence of histopathologic lesions in olfactory epithelium of rodents following exposure to styrene at levels similar to those experienced by many of the workers in this study, we found no evidence that such exposures in humans produced impairments in olfactory function. Since we did not examine the nasal or olfactory epithelium of the worker participants, we cannot determine from the present study whether exposure to styrene in humans results in changes in the nasal epithelium that are similar to those observed in animal toxicological studies. However, if such changes are present, they do not appear to have a functional impact on any aspect of olfactory ability as measured in this study.

How can we reconcile the failure to observe a relationship between long-term styrene exposure and adverse effects on olfactory function as demonstrated by the current study with the presence of significant olfactory lesions in experimental animals exposed at similar concentrations? Comparative studies of nasal uptake and metabolism in rodents and humans have identified several factors to account for discrepancies, and suggest that estimates of olfactory risks based on exposures to rodents may not be relevant to humans.

The first factor is the presence of significant anatomical differences between the nasal passages of rats, mice, and humans that result in alterations in the volume and patterns of airflow. Thus, one explanation for the discrepancy between animal studies and the current functional assessment is that the inhaled styrene vapor may deposit in the nasal passages of humans, but unlike in rodents, high levels of deposition do not occur in areas that subserve olfaction. Evidence for this possibility comes from experimental data [Morgan and Monticello, 1990] and transport models of airflow that have been used to predict and model the deposition of inhaled chemicals in the nasal cavities of rodents [Kimbell et al., 1997] and humans [Keyhani et al., 1997]. Based on anatomical casts of the upper airways in both species, computer models reveal strikingly different patterns of airflow through the nasal compartment that result in substantial differences in chemical deposition and concentration in the olfactory regions [Morgan and Monticello, 1990]. Taken together with the fact that rodents are obligate nose breathers, these differences in structure and airflow would mean that exposure to the same airborne concentration of styrene would result in different doses and patterns of nasal deposition for humans and rodents.

Alternatively (or additionally), biochemical differences in the nasal tissue of rodents and humans could account for the disparity between the olfactory damage seen in styrene-exposed rodents and the lack of functional effects on humans exposed to styrene vapor. In the case of styrene, the primary metabolic pathway is the oxidation by cytochromes *P*-450 to two enantiomeric forms of styrene oxide [Bond, 1989]. When this metabolic process is prevented by pre-exposure to the cytochrome *P*-450 enzyme inhibitor (5-phenyl-1-pentyne), the development of olfactory lesions in rodents following exposure to styrene does not occur [Green et al., 2001]. This strongly suggests that the lesions found in rodent olfactory tissue are induced by the primary metabolite of styrene, styrene oxide, and not by exposure to styrene per se.

Evidence that this metabolite may not be present in human nasal epithelium exposed to styrene comes from a recent investigation that compared metabolic activity of styrene in vitro in rat, mouse, and human nasal respiratory tissue and found important differences in the metabol-

ic activity of styrene across these species [Green et al., 2001]. Specifically, rat and mouse nasal respiratory fractions were found to contain high concentrations of the two cytochrome *P*-450 isoforms necessary for the conversion of styrene into styrene oxide, whereas human nasal fractions did not. Species differences in the nasal metabolism of other chemicals that produce rat nasal tumors have been recently reported as well [Green et al., 2000], suggesting that the relevance of nasal tumorigenesis studies in rodents for human risk prediction may need to be evaluated on a chemical-by-chemical basis. Both anatomical and metabolic differences could explain differences in the toxic effects of styrene on the olfactory epithelium in man and rodents.

One limitation of the present study is the potential self-selection bias of the workers that were tested. Classically, it is held that workers with perceived exposure-related health effects select out of high-exposure jobs and are thus unavailable for study. Evaluation of the remaining participants in a cross-sectional study may lead to serious underestimation of the effects under study. In the present study, however, it is equally possible that olfactory dysfunction status had an effect on exposure status, but in an opposite direction. That is, in a reversal of the 'healthy worker effect', workers with poorer olfactory ability may be more likely to remain in high-exposure job positions than workers with good olfactory ability. If this were the case, although worker complaints about chemical odor/irritation have been reduced, we would have also expected to find a higher prevalence of olfactory dysfunction/lowered sensitivity among the worker population than the unexposed controls, which we did not observe.

Another limitation of a cross-sectional study is the inability to measure changes in olfaction from a pre-exposure or baseline level. We cannot, for example, rule out the possibility that the styrene-exposed workers, we tested, had better-than-average olfactory ability prior to styrene exposure. If this were the case, however, the comparison of the performance of the styrene-exposed workers with a carefully matched control group and the normative sample from our database strongly suggests that (1) both the exposed workers and the control group had roughly equivalent olfactory ability prior to workplace exposure, and the exposed workers gradually lost sensitivity to styrene alone and (2) exposure to styrene does not have effects on general olfactory function. Nevertheless, there may be subtle changes that occur in any chemically exposed population that could only be observed in undertaking a longitudinal study where workers who exhibit normal olfactory ability were enrolled prior to any exposure and, serving as their own controls, are assessed periodically across the course of their occupational exposure.

Much of the prior evidence for olfactory dysfunction subsequent to chemical exposure has been derived from anecdotal reports and case studies [Emmett, 1976; Prudhomme et al., 1998]. In the present study, each worker's personal exposure history was developed and documented through careful examination of work records, industrial hygiene surveys, plant records, and personal medical/occupational history interviews. Moreover, the performance of styrene-exposed workers was compared with the performance of an appropriately matched referent group and with the normative measures of performance from our clinical database. Equally important, the olfactory assessments used in the current study were logical, comprehensive, and known to be capable of detecting even subtle forms of olfactory dysfunction.

In summary, despite observations in animal studies that exposure to styrene at or below currently acceptable workplace limits produced lesions in the olfactory epithelium of rodents, the present study found no evidence among a cross-section of reinforced-plastics workers that current or historical exposure to styrene was associated with impairment of general olfactory function. Taken together with animal and human anatomical and metabolic data, the current results strongly suggest that styrene at these exposure levels is not an olfactory toxicant in humans.

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Exposure Assessment for Study of Olfactory Function in Workers Exposed to Styrene in the Reinforced-Plastics Industry

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Background
This study was undertaken in conjunction with an evaluation of the olfactory function of 52 persons exposed to styrene vapors to provide quantitative styrene exposure histories of each subject for use in the interpretation of the results of olfactory function testing.

Methods
Current and historic exposures were investigated. Historic exposures were reconstructed from employment records and measurements of styrene exposure made in the subject facilities over the last 15 years. Current exposures were estimated for every exposed subject though personal air sampling and through pre- and post-shift measurements of urinary metabolites of styrene.

Results
The study population had been employed in the reinforced-plastics industry for an average of 12.2 ± 7.4 years. Their mean 8-hr time weighted average (TWA) respirator-corrected annual average styrene exposure was 12.6 ± 10.4 ppm; mean cumulative exposure was 156 ± 80 ppm-years. The current respirator-corrected 8-hr TWA average exposure was 15.1 ± 12.0 ppm. The mean post-shift urinary mandelic and phenylglyoxylic acid (PGA) concentrations were 580 ± 1,300 and 170 ± 360 mg/g creatinine, respectively and were highly correlated with air concentrations of styrene.

Conclusions
This quantitative exposure evaluation has provided a well-characterized population, with documented exposure histories stable over time and in the range suitable for the purposes of the associated study of olfactory function.

Without quantitative measures of exposure, findings and conclusions are of limited usefulness. The use of exposure information in this manner is critical for the development of defensible assessments of the risk of exposure to occupational and environmental agents and, in turn, to the standards setting process.

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INTRODUCTION

The establishment of a relationship useful for quantitative risk assessment between occupational exposure to a chemical and any observed health effect rests critically on the estimates of exposure to that chemical among the exposed workers. This study was conducted to provide quantitative styrene exposure information for a group of workers who were the subjects of a simultaneous study examining the possible olfactory dysfunction resulting from this exposure [Dalton et al., 2003]. As the olfactory study was designed to differentiate between the olfactory effects of chronic and acute exposures, accurate estimation of short- and long-term exposures was of concern. Short-term (current) exposures were estimated through measurement of the concentration of styrene in air in the workers' breathing zones during the two shifts immediately prior to olfactory testing and through the concurrent measurement of urinary biomarkers of exposure. Chronic exposures over the subjects' employment histories were reconstructed from job histories and historic exposure-monitoring records. For subsequent analytical purposes, the short-term exposure measurements were unique to the subject; historically reconstructed chronic exposures were, of necessity, based on a population (job title) pooling of exposure information and were unique to a job title. In all cases, data used for historic exposure reconstruction were restricted to the facility of origin.

BACKGROUND

Styrene is a clear colorless liquid with a highly characteristic pungent odor, detectable at concentrations >0.32 ppm [Amoore and Hautala, 1983] and highly irritating at concentrations >100 ppm [Stewart et al., 1968]. Recent production data indicate that 11.9 billion pounds of styrene were produced in the US in 1999 [Chemical and Engineering News, 2000]. The primary use of styrene is in the production of polymers and copolymers, including polystyrene, styrene–butadiene-rubber, styrene–butadiene-latex, and a variety of different resins. Styrene monomer is combined with polyester resins and serves as a cross-linking agent in the manufacture of numerous fiber-reinforced products. Except for those products requiring special performance characteristics, for example, impact resistance, fiberglass is the reinforcement of choice. Although many production processes are enclosed and worker exposure to styrene is low, workers involved in open production processes such as the manufacture of bathtub and shower enclosures, boats, tanks, and panels experience substantially higher exposures [LeMasters et al., 1985].

Although animal data and human laboratory data [Berode et al., 1985; Pezzagno et al., 1985; Wieczorek, 1985; Petreas et al., 1995; Nylander-French et al., 1999] suggest that styrene may be readily absorbed through the skin, in the workplace exposure to styrene is primarily via inhalation. Brooks et al. [1980] suggest that the polyester resin/styrene complex is sufficiently different from pure styrene test exposures "that percutaneous absorption of styrene is not an important route of exposure in the reinforcedplastics industry" [Brooks et al., 1980].

Reinforced plastics are produced by a variety of manufacturing methods using open, semi-open, and closed production processes. Because they produce vapor exposures in the range of interest for the study of olfactory effects, the open production processes are of particular importance to this study. Exposures related to two open production methods (manual spray-up and lay-up methods), one semi-open method (panel production), and one closed production method (matched die molding) were studied.

The spray-up manufacturing method often entails the highest exposures [LeMasters et al., 1985]. In this method, a pigmented gel coat of styrene–polyester resin is initially sprayed onto an open mold (male or female). The gel coat represents the finished side of the product. Next, a styrene–polyester resin, an initiator, and short (5–8 cm) strands of fiberglass fibers (roving) are sprayed simultaneously onto the mold through the use of a hand-held chopper gun. The components mix in the air; on the mold, the mixture is hand rolled to consolidate the material (eliminate voids), to ensure conformance with the mold, and to provide for complete wetting of the fibers [Strong, 1989]. Excess material is trimmed from the product by hand before it is allowed to cure, usually at room temperature.

Manual lay-up methods may also entail high exposures to styrene [LeMasters et al., 1985]. In this method, successive layers of a woven reinforcing fabric, frequently fiberglass, are placed on a mold; each layer is manually saturated with resin as the item is fabricated. The manual lay-up operations included in this study were limited to the fabrication of large fan blades using sheet molding compound (SMC) "pre-pregs;" wet or in-process impregnation methods were not used in any of the facilities studied. Pre-pregs are fibrous materials, usually woven fabrics, that have been saturated or pre-impregnated with resin and allowed to cure slightly at a location remote from the manufacturing process. They are transported to the fabrication site (in this case elsewhere in the manufacturing facility) where they are manually cut and applied to a mold in sequential layers to build up the item being fabricated. After fabrication is complete, the piece is allowed to cure in an autoclave at elevated pressure and elevated temperature.

The semi-open production process studied involved the production of fiberglass-reinforced styrene polyester panels. In this continuous highly automated process, styrene resin is spread to the appropriate thickness onto a sheet of cellophane unwound from a large roll. Chopped fiberglass fibers are allowed to fall onto the resin before the mixture is passed between sets of rollers, which compress the mixture and apply a top (finish) cellophane laminate to the sheet. The product is then passed through an oven where it is cured. The resulting continuous sheets, up to thousands of feet in length, are either wound into rolls or cut to convenient lengths.

The closed production process studied involved matched die molding of bulk molding compound (BMC). In this process, chopped fibers, resin, initiators, and fillers are combined to form a dough-like mixture called a pre-mix. A weighed amount of pre-mix is manually placed in the lower half of a matched die mold. The upper portion of the mold is then pressed against the BMC, forcing it to spread in conformance with the cavity between the dies. The pressure, temperature, and time the dies are together are specified for the subsequent curing process.

There is a modest literature summarizing worker styrene exposures in the reinforced-plastics industry [Schumacher et al., 1981; Ikeda et al., 1982; Crandall and Hartle, 1985; LeMasters et al., 1985; Andersson et al., 1994]. Little additional information has appeared in the published literature in the last decade. In one of the most extensive reports, summarizing approximately 1,500 exposure measurements collected between 1974 and 1981 at 36 facilities, styrene exposures were grouped by industry product category [Lemasters et al., 1985]. As expected, fabrication processes involving open methods (primarily hand lay-up and chopper gun application) were found to have the highest exposures. Other methods were found to be associated with substantially lower exposures. Mean styrene exposure (time not specified but assumed from context to be 8-hr time weighted average [TWA]) in boat fabrication was estimated to be 82 ppm, that in truck part fabrication was 61 ppm, that in tub and shower fabrication was 47 ppm, that in yacht fabrication was 47 ppm, and that in tank and pipe fabrication was 24 ppm. All other process/product exposures, with the exception of die molded truck and automobile parts were associated with mean exposures less than 20 ppm. Chopper gun operators were consistently among the most highly exposed across all studies.

Interestingly, historical exposures (20 years ago) reported in the literature did not seem to vary substantially from those concentrations measured as a part of this study. An additional historical perspective, primarily from the boat fabrication industry, indicated that only a little more than a third of the exposed employees wore approved respiratory protective equipment (RPE) [Schumacher et al., 1981].

US and international exposure limits for styrene have undergone a steady decline over the last 30 years. In the US, the threshold limit value (TLV®) for styrene, a guideline published by the American Conference of Governmental Industrial Hygienists (ACGIH), has declined from 100 ppm as an 8-hr TWA prior to 1975, to 50 ppm in 1975, and finally to 20 ppm in 1997 [American Conference of Governmental Industrial Hygienists, 1997]. The permissible exposure limit (PEL), a regulatory limit promulgated by the US Occupational Safety and Health Administration (OSHA), officially remains at 100 ppm [Occupational Safety and Health Administration, 2000], although memoranda of understanding have been signed with the major users of styrene, voluntarily reducing the PEL to 50 ppm [Occupational Safety and Health Administration, 1996]. The most recent TLV reduction was adopted based on evidence related to the prevention of peripheral and central nervous system effects and to further minimize the potential for irritation [American Conference of Governmental Industrial Hygienists, 1997].

In addition to exposure limits based on air concentrations of styrene, ACGIH has developed recommended maximum concentrations of urinary metabolites of styrene, termed biological exposure indices (BEI) [American Conference of Governmental Industrial Hygienists, 1996]. The recommended post-shift BEI for mandelic acid (MA) concentration is 800 mg/g creatinine and that for phenylglyoxylic acid (PGA) is 240 mg/g creatinine. (Note that these BEI's were adopted in 1986 and have not been revised to reflect reduction of the TLV in 1997.) MA represents approximately 85% and PGA approximately 10% of the styrene excreted [American Conference of Governmental Industrial Hygienists, 1996]. Although both metabolites appear in the urine shortly after the start of exposure, an examination of the kinetics of metabolism suggests that the MA value reflects a rapid metabolic pathway ($t_{1/2}$≈4–8 hr) and the PGA value reflects a somewhat slower pathway ($t_{1/2}$≈8–16 hr) [American Conference of Governmental Industrial Hygienists, 1996]. For this reason, the German practice is to sum these components with a combined (MA+PGA) recommended value of 600 mg/g creatinine, termed the Biologischer Arbietsstoff-Toleranz Wert (BAT) [Deutsche Forschungsgemeinschaft Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area, 2000].

SELECTION OF FACILITIES FOR STUDY

The facilities studied were selected based on a stringent set of criteria. These criteria are fully enumerated in the companion paper [Dalton et al., 2003]. Of critical importance to the exposure assessment process, facilities were selected based on the stability of the manufacturing process and process control technology over the preceding 10 years, worker exposures of 30–50 ppm over this same period, the availability of personnel records from which individual worker job histories could be extracted, information on historic respirator use, and the existence of at least a modest amount (three surveys) of suitably documented and identifiable historic air monitoring data for styrene.

Based on these criteria, four sites were selected for study. All were located in the eastern United States and employed a variety of manufacturing methods, although all sites used similar low styrene content (<40%) polyester resin systems. Sites 1 and 2 produced fiberglass-reinforced bathtubs and shower enclosures through standard manual spray-up techniques using a chopper gun. The resin system and chopped fiberglass roving were sprayed onto male molds and cured at room temperatures. Production was a completely manual, labor-intensive process involving gel coating, barrier coating, and two laminating steps with associated rolling and trimming. Despite high volume styrene use at these two sites, exposures were fairly well controlled through the use of extensive ventilation systems.

Site 3 produced sheet products through a "printing press method." The styrene-containing resin was doctored onto a web, sprinkled with chopped fiberglass roving and compressed by passing through a series of rollers. A continuous sheet of fiberglass-reinforced plastic either 54 or 108 inches wide was produced and cured at elevated temperature in an enclosed ventilated oven. There were three production lines. This process was highly automated with only a few workers in the immediate vicinity of the uncured resin. The "press" and curing ovens were nearly fully enclosed and ventilated.

Site 4 had the fewest workers of the four facilities studied and was the only site with a single shift production schedule. All products were manufactured at this site either through hand lay-up of SMC or compression die molding of BMC. All die-molding operations were manually fed. The BMC and SMC used in these operations were produced on site. The SMC product was cured at an elevated temperature and elevated pressure, while the BMC products were cured at room temperature. Ventilation control of exposures was very limited at this site. At the time of the exposure survey and olfactory testing, the workforce had been reduced by more than 50% from historic levels due to a drastically reduced work load.

At the time of the exposure survey, systematic use of respirators for specified jobs was limited to Sites 1 and 2 only. In these cases, NIOSH-approved dual organic (charcoal) cartridge half-face masks were used. Prior to about 1986, respirator use was optional at all four sites and determined by individual worker preference. Based on observations during sampling, except for Site 1, very few individuals opted for the use of respirators.

The employment histories of the exposed subjects selected for the olfactory function study are summarized in Table 1.

TABLE 1

Work Experience of Styrene Exposed Subjects by Site

Employment variable	Site1	Site 2	Site 3	Site 4
Manufacturing method	Spray/ chopper gun	Spray/ chopper gun	Sheet "press"	Hand lay-up. and die molding
Product	Bath tubs and shower enclosures	Bath tubs and shower enclosures	Sheet products	Fan blades and die molded items
Number of exposed subjects	11	13	19	9
Mean employment (years)	10.1	10.1	9.5	23.4
Minimum employment (years)	3	5	4	15
Maximum employment (years)	16	14	31	41

ASSESSMENT OF HISTORIC EXPOSURES

Methods

All sites selected for inclusion in the study had records of a series of personal measurements of worker styrene exposures covering a period of time roughly contemporary with the employment of the study subjects. All estimates of historic exposure were derived from these data and from the exposure measurements collected as part of this study immediately preceding the olfactory function evaluations. All reconstructions of worker exposures were site-specific, i.e., only data from that facility were utilized and there was no assumed relationship in exposures between the sites.

Control subjects were selected from manufacturing locations which did not use styrene or other known or suspected olfactory toxicants and were located nearby for three of the four study sites. Historic exposure reconstruction for the control subjects was limited to questionnaire responses concerning past employment and past styrene exposure; anyone indicating a history of styrene or other olfactory toxicant exposure was excluded from the study. Those not indicating such a history were assumed to have no historic styrene exposure. At Site 2, control subjects were selected from persons employed by the same company, but working at a location remote from operations involving styrene. Air monitoring was conducted and urine samples collected at Site 2 to confirm the non-exposure status of the control population (see below).

For exposed subjects, styrene exposure reconstruction was accomplished through the integration of personnel records indicating job history at the facility and historic exposure measurements from the same facility. A protocol for the utilization of the existing data was developed and followed to provide a uniform approach to estimating historic exposures. Historic exposure estimates were based on historic measurements for the specified job title and year. The arithmetic means of the air concentration measurements fitting the specified facility-job title-year criteria were calculated and the arithmetic mean 8-hr TWA calculated. (Both 8-hr TWA samples and 4-hr TWA samples, treated as an 8-hr TWA, were used to calculate historic exposures; the small number of 15 min peak samples collected at two facilities were not used for this purpose.) Exposure estimates for facility-job title-year combinations without directly corresponding exposure data were calculated according to the protocol using other specified similar job titles and/or exposure data from other specified years, as appropriate. Similar job titles were defined based on similarity of exposure levels and were derived from exposure measurements, from interviews with facility personnel, and from researcher observations.

An exposure matrix based on job title and year was developed directly from the styrene monitoring data and from exposure estimates derived from the monitoring data. A separate exposure matrix was developed for each facility.

Historic styrene exposures of the study subjects were estimated by combining information from the exposure matrices and individual work histories derived from employment records. Based on a study subject's job titles and years of employment, annual average exposure estimates were selected from the appropriate cells of the facility exposure matrix. If a worker changed job titles within a year, a weighted annual average exposure was calculated based on the exposure estimates for those jobs held during the year and the portion of the year that they were held. In addition to estimates of annual mean styrene exposure, lifetime cumulative styrene exposures (ppm-years) were calculated for each exposed subject by summing the annual average exposure estimates over all years exposed to styrene.

Although an attempt was made to avoid selecting subjects with a history of respirator use, 13 subjects who had used respirators at some point in their employment history were included in the study population. Some of these individuals had ceased using respiratory protection prior to this study; several subjects were currently using respiratory protection. Since the overall purpose of the exposure assessment effort was to provide information for the evaluation of olfactory function tests on the workers, it was necessary to determine the actual concentrations of styrene inhaled by the workers. It was, therefore, necessary to account for the (presumed) reduction in inhalation exposure while respiratory protection was in use. Exposures for job titles that were known to be associated with respirator use were calculated by dividing the measured concentration of styrene in air by an arbitrary factor of five to derive an "effective exposure" estimate. Although the respirator customarily used by this population has an assigned protection factor of ten, a protection factor of five was selected for the purposes of this study to account for poorer fit due to assumed more lax historic fit-testing procedures and to account for work periods when the respirator was not worn.

TABLE 2

Summary of Historic Exposure Measurements by Site

Measurement Parameter	Site1	Site 2	Site 3	Site 4
Manufacturing method	Spray/ chopper gun	Spray/ chopper gun	Sheet "press"	Hand lay-up and die molding
Date of earliest measurement	5/25/1988	7/30/1987	7/19/1988	6/14/1994
Number of days with measurement	579	472	41	4
Total number of measurements	753[a]	644[a]	68	22

[a]Includes 8-hr, 4-hr, and 15-min samples.

RESULTS

Key descriptors of the exposure data used to reconstruct historic exposures are presented in Table 2. Exposure-monitoring programs have been in place at Sites 1 and 2 since the mid- to late-1980s. As a part of these programs, the exposures of individuals employed in higher exposure jobs are measured on an ongoing basis. The data from these sites consist primarily of 4- and 8-hr air samples, which are statistically indistinguishable by job title, and a limited number of 15 min samples (not used for calculations, but indicating higher peak concentrations). Data from Site 3 were also derived from a formal exposure-monitoring program similar to that in place in Sites 1 and 2, but of smaller scale. Exposure data known to have been collected between 1990 and 1996 at Site 3 could not be located. Exposures at Site 4 were estimated from the results of four plant-wide sampling surveys (three conducted by the facility's insurance carrier and one conducted by OSHA).

As described, these exposure data were consolidated

FIGURE 1

Frequency distribution of historic annual average effective styrene exposures of US study population (n=604*); *excludes values for three subjects with unclear dates in job history.

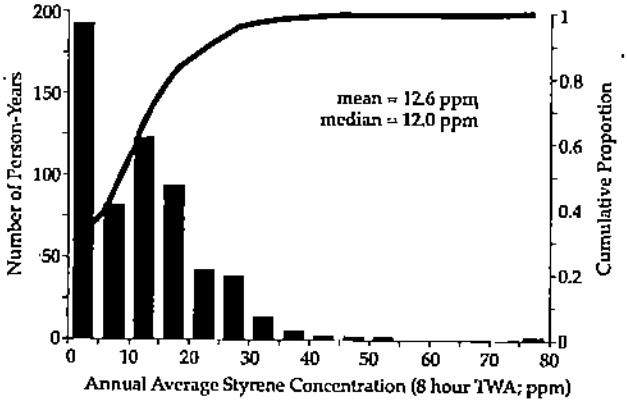


FIGURE 2

Frequency distribution of cumulative effective styrene exposures of US study population (n=49*); *excludes values for three subjects with unclear dates in job history.

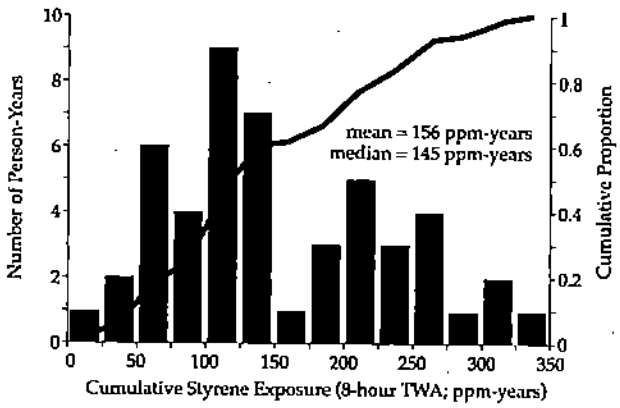


TABLE 3

Summary of Respirator-Corrected Historic Exposure Estimates Used for Styrene Exposed Subjects by Study Site, 8-hr TWA*

Exposure parameter	Site1	Site 2	Site 3	Site 4
Manufacturing method	Spray/ chopper gun	Spray/ chopper gun	Sheet "press"	Hand lay-up and die molding
Number of exposed subjects	11	13	16•	9
Person-years of observation	111	130	153	210
Number of job titles (total)	9	12	6	5
Mean annual exposure, all jobs (ppm)	17	13	16	7
Median annual exposure, all jobs (ppm)	16	12	16	7
Maximum annual exposure, all jobs (ppm)	55	41	77	18
Minimum annual exposure, all jobs (ppm)	<1	<1	<1	<1
Mean cumulative exposure, all jobs (ppm-years)	170	132	157	170
Median cumulative exposure, all jobs (ppm-years)	145	122	131	176
Maximum cumulative exposure, all jobs (ppm-years)	313	289	310	328
Minimum cumulative exposure, all jobs (ppm-years)	20	30	52	52

*TWA, time weighted average.

•Three exposed subjects (30 person-years) excluded from these analyses due to indeterminant job change dates.

in exposure matrices developed for each facility and specifying annual mean 8-hr TWA exposure by job title and year. A summary of the exposure metrics used to interpret the olfactory function findings [Dalton et al., 2003] is presented by site as Table 3. In addition, the distribution of annual average historic exposures and the distribution of cumulative historic exposures are presented as Figures 1 and 2, respectively.

ASSESSMENT OF CURRENT EXPOSURES

Methods

Air

Personal air samples were collected for each exposed subject during the two shifts (one shift at Site 3) immediately prior to olfactory assessment. Personal and/or area samples were collected for control subjects at Site 2 where subject proximity to manufacturing processes involving styrene, i.e., with a potential for inadvertent exposure, indicated a need to confirm their non-exposure status. Because olfactory testing was conducted at or near the facilities studied, area samples were also collected in each of the rooms used for olfactory assessment during testing. Duplicate air samples and field blanks were collected throughout the course of the exposure evaluation for quality control purposes.

Air samples were collected and analyzed in conformance with the National Institute for Occupational Safety and Health (NIOSH) Method 1501 [National Institute for Occupational Safety and Health, 1994] using coconut shell charcoal sorbent tubes and personal sampling pumps calibrated to a flow rate of 50–75 ml/min, depending on the anticipated concentration of styrene and shift length. After collection, sample tubes were capped and stored in a cool environment prior to shipping. The samples were shipped and analyzed as a single batch using gas chromatography with a flame ionization detector. All chemical analyses were conducted by Clayton Environmental Consultants (Novi, MI). This commercial laboratory is fully accredited under the American Industrial Hygiene Association's Industrial Hygiene Laboratory Accreditation Program (IHLAP) for the gas chromatographic method used for analysis of these styrene samples.

Biological

Pre- and post-shift urine samples were collected in conjunction with air sampling on the day before the olfactory assessment. Paired urine samples were collected from all exposed study subjects. Urine samples were not collected from unexposed controls with the exception of four control subjects at Site 2 whose proximity to the manufacturing process suggested the need to confirm their non-exposed status. All samples were collected in 40-ml vials and immediately frozen on dry ice. The samples were shipped at the end of each week to the laboratory where they were kept frozen at –10°C until analyzed. MA and PGA were analyzed by a method employing anion exchange chromatography with suppressed conductivity detection derived from Murer et al. [1994]; creatinine was analyzed by a standard test kit based on a colorimetric spectroscopic method (IL Test Creatinine; Instrumentation Laboratory Company, Lexington, MA). All MA and PGA concentrations are reported as creatinine-corrected concentrations. Approximately 20% of the urine samples were split for subsequent blinded duplicate analysis. All samples were analyzed as a single batch by National Medical Laboratories (Willow Grove, PA).

All exposed workers and control subjects provided informed consent for their participation using a form that was approved by the Committee on Human Research of the Johns Hopkins University School of Hygiene and Public Health (now the JHU Bloomberg School of Public Health) and by the Committee for Studies Involving Human Beings of the University of Pennsylvania. Volunteers were advised

FIGURE 3

Distribution of current measured 8-hr time weighted average (TWA) effective styrene concentrations for US study population (n=99).

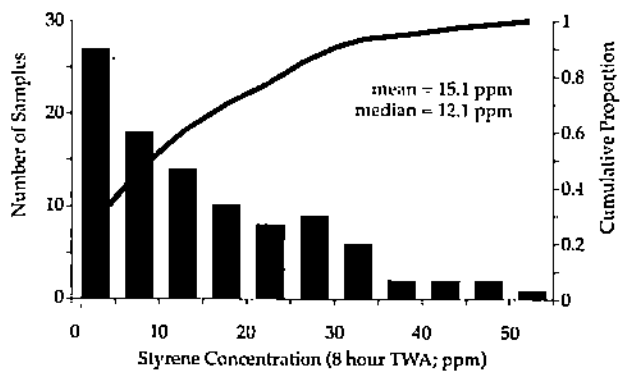


TABLE 4

Summary of Actual and RPE-Corrected Estimates of Styrene Exposure, 8-hr TWA

Parameter	Site1		Site 2		Site 3		Site 4	
Manufacturing method	Spray/chopper		Spray/chopper gun		Sheet "press"		Hand lay-up and gun die molding	
Samples (n)	22		31		24		22	
Samples with RPE (n)[a]	10		2		3		0	
Job titles (n)	9		12		6		5	
Mean time (hr:min)	7:25		8:08		10:18		7:13	
Concentration	Actual	Effectb	Actual	Effect[b]	Actual	Effectb	Actual	Effectb
Mean (ppm)	55.0	24.1	18.2	13.9	16.8	14.8	9.2	9.2
Median (ppm)	35.2	24.8	7.4	7.4	13.5	12.0	6.4	6.4
Minimum (ppm)	11.6	4.9	0.1	0.1	3.4	2.7	3.9	3.9
Maximum (ppm)	140.3	45.1	92.7	45.1	50.9	50.9	28.1	28.12

[a]Samples collected on persons wearing respiratory protective equipment (RPE).

[b]Effective exposure calculated by dividing air concentration by five for persons wearing RPE.

that they were free to withdraw at any time. Workers continued to receive full wages during the period of data collection. The control subjects who participated on their own time were compensated for their participation.

RESULTS

Air

The results of air sampling are summarized in Table 4 and the distribution of air sampling results is presented as Figure 3. Note that in addition to all exposed subjects, the exposures of several additional persons are included at each site. These samples were collected from persons who were subsequently disqualified or otherwise excluded from olfactory portion of the study, but still represent valid measures to characterize the population's exposure. Concentrations of styrene in air are presented as 8-hr time weighted averages. Samples were collected over the entire working period. At Sites 1, 2, and 4, the working period corresponded closely to 8 hr and the resulting TWA's are nearly identical to actual measured concentrations. At Site 3, the sampling and work period averaged approximately 10 hr; the resulting 8-hr TWA is, therefore, proportionally larger than the actual measured styrene concentrations.

Table 4 illustrates the effect of adjusting individual inhalation exposures to account for use of RPE. As explained earlier, this adjustment was required for interpretation of the olfactory testing results. For the most part, this adjustment affected only persons with the highest exposures (more likely to wear RPE), but this was not always so. At Site 1, styrene air concentrations were highest, but RPE use was also greater than at other sites. Adjustment of air concentrations lowered "effective" exposure most dramatically at Site 1, with smaller adjustments at Sites 2 and 3. Adjustment made mean and median exposures more comparable across the sites.

Between-day variability of styrene concentrations was moderate. Although the data are not presented, the mean absolute value of the differences in paired (same worker, same job) styrene air concentrations between day 1 and day 2 was 35.8%. On the other hand, the difference between the overall mean styrene concentrations for day 1 and day 2 was only 1%. These differences indicate a very stable population mean exposure, while the variability in styrene exposure for an individual worker is moderate. Although not statistically significant, there was a slight tendency for day 2 concentrations to be higher than day 1 concentrations.

Consistent with reports in the published literature, exposure of sprayers (Sites 1 and 2) was highest across all job titles and sites, averaging approximately 100 ppm (job title-specific exposure estimates not presented). Persons with this job title, however, were most likely to wear RPE, substantially reducing actual inhalation of styrene. At Sites 1 and 2 there was a wide range of air concentrations of styrene across jobs; adjustment of exposures to account for RPE use made the range of exposures at these sites more homogeneous. Exposures at Site 3 were relatively homogeneous, averaging about 20 ppm, largely because the production process at this site was highly automated, eliminating the intense contact with styrene present in the more manual processes. At Site 4, exposures were uniformly lower than at other sites, probably because of greatly reduced production, but still ranged up to 28 ppm.

Nineteen duplicate air samples collected at the four sites had a mean difference of 2.1% and a mean absolute difference of 5.0%. This variability is well within the published expected variability of the sampling and analytical method. A paired *t*-test showed means of the duplicates not to be statistically different.

Urinary Biomarkers of Exposure

Pre- and post-shift urine samples were collected from all of the exposed subjects on the day prior to olfactory testing; the results of urinalysis of the exposed workers are summarized in Table 5. Urine samples were also collected from the unexposed control subjects at Site 2. Concentrations of styrene metabolites in all urine samples from these control subjects were below the analytical limits of detection (generally approximately 10–20 mg/g creatinine for both MA and PGA, depending on the creatinine concentration), confirming their non-exposure status.

Twenty-nine urine samples were split immediately after collection and submitted for blinded analysis of MA, PGA, and creatinine. Overall results showed excellent reproducibility. Examination of the creatinine concentrations, however, showed clear outlier values for two of the duplicate samples (423 vs. 3,238 and 1,000 vs. 1,803 mg/ml). These values, in turn, affected the calculation of creatinine-corrected MA and PGA concentrations. Excluding these two outlier values, the mean absolute value of the differences between duplicate creatinine analyses was 3.0%. The mean difference for MA analyses was 4.1% and for PGA was 5.7%. Correlation coefficients for duplicate MA, PGA, and creatinine analyses were r^2=0.99, 0.99, and 0.98, respectively. For both samples excluded from these analyses, the apparent outlier values were for the duplicate analyses. The primary samples used for statistical analyses elsewhere were consistent with the other primary samples for the study subjects.

TABLE 5

Summary of Pre- and Post-Shift Creatinine-Corrected Urinary Metabolite Concentrations (mg/g creatinine) in Styrene Exposed Workers

	Site1		Site 2		Site 3		Site 4	
Analyte and concentration	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Samples (n)	11	11	13	13	19	19	9	9
MA (mean)	210	1,740	90	320	80	260	30	190
MA (median)	190	670	40	100	60	210	30	150
MA (min)	20	200	<10	<10	<10	<10	<10	50
MA (max)	510	6,980	390	1,020	170	1,040	90	350
PGA (mean)	130	490	60	90	60	80	30	90
PGA (median)	90	160	50	60	40	60	30	70
PGA (min)	40	80	<10	<10	<10	<10	<10	20
PGA (max)	470	2,250	270	290	210	370	120	210
Manufacturing method	Spray/chopper gun		Spray/chopper gun		Sheet "press"		Hand lay-up and die molding	

MA, mandelic acid; PGA, phenylglyoxylic acid.

DISCUSSION

Historic Exposures

Historic worker exposures reconstructed from plant records are fairly consistent with the results of previously published studies, both in the magnitude of exposure and in the patterns of exposure between jobs. This strengthens confidence in the methods used to estimate historic exposures and, importantly for the general applicability of the olfactory study, indicates that the sites chosen for study are fairly representative of the reinforced-plastics industry as a whole.

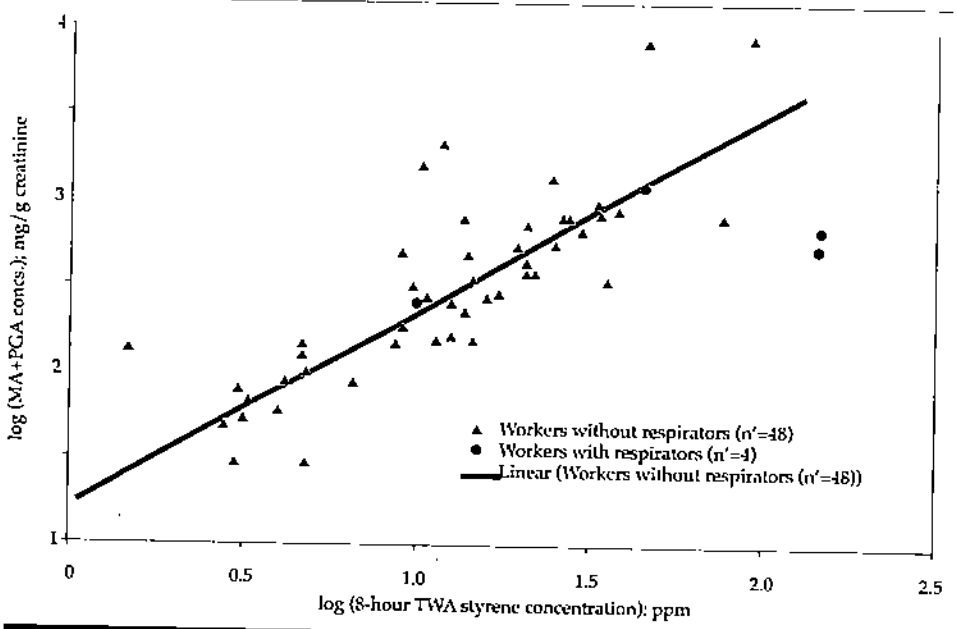
Both mean historic annual exposures and cumulative historic exposures conformed generally to the expected log-normal distribution. The mean annual respirator-corrected historic exposure of the 52 study subjects encompassing 634 person-years of exposure, was 12.6 ± 10.4 ppm, expressed as an 8-hr TWA. Mean exposures were very consistent between Sites 1, 2, and 3, while the mean historic exposure at Site 4 was approximately half that of the other sites. Maximum exposures at Sites 1, 2, and 3 were also similar, with Site 4 having a maximum exposure concentration substantially less than half of that for the other sites. Mean cumulative exposures over the study subjects' working histories was 156 ± 80 ppm-years. As opposed to the annual mean exposures, cumulative exposures were highly consistent across all sites. This resulted largely from the coincidence of longer exposure duration at the site with the lowest exposure and vice versa. When compared to olfactory testing results, however, this difference better allowed for the examination of a possible dose-rate effect.

Current Exposures

The results of air sampling undertaken immediately prior to testing of subjects' olfactory function indicate styrene exposures generally consistent with those estimated from historic records. Taken as a whole, the exposure measures and estimates indicate fairly consistent exposure over time, at least for the last 15 years. Because an estimate of actual inhalation exposure was required for the purposes of the olfactory testing, for the several subjects who wore respirators during air sampling, exposures presented in this paper are reduced to account for the reduction in exposure attributable to the respirator. The mean exposure reported in this paper, 15.1 ppm, therefore, understates actual air concentrations of styrene. Actual styrene concentrations measured (n=99) exceeded the current 20 ppm TLV in 39% of the samples, exceeded the current 50 ppm OSHA consent level in 11% of the samples, and

FIGURE 4

Relationship between airborne styrene and post-shift urinary metabolite concentrations by worker respirator use status for US study population.



exceeded the current 100 ppm PEL in 4% of the samples.

As expected, urinary metabolite concentrations showed a consistent increase between pre-shift and post-shift samples. In all cases, mean MA and PGA concentrations were substantially higher than the corresponding median value, indicating a skewed distribution with high values characteristic of the expected log-normal distribution. Measures of urinary metabolite concentration indicate roughly comparable styrene exposures at Sites 2, 3, and 4. Based on urinary metabolite measures, exposures at Site 1 appear to be substantially higher than at the other sites. At Site 1, the mean and median metabolite values are strongly influenced by a substantial number of very high values. Mean post-shift MA and PGA concentrations at Site 1 exceed the recommended BEI's; the corresponding values at Sites 2, 3, and 4 are well below the BEI's, although single maximum values at Sites 2 and 3 exceed the BEI's. Combined urinary metabolite concentrations (MA+PGA) exceeded the BAT at Site 1 only.

MA and PGA urinary biomarkers of exposure were found to be highly correlated with measured air concentrations (r^2=0.78 for log MA concentration vs. log styrene concentration and r^2=0.51 for log PGA concentration vs. log styrene concentration). Persons wearing RPE were omitted from this analysis. These correlations confirm for this population the previously reported conclusion [Ramsay et al., 1980; Pezzagno et al., 1985; Guillemin and Berode, 1988] that MA and PGA are good predictors of recent exposure as measured by air concentration of styrene. There is, unfortunately, no biomarker of long-term (months or years) styrene exposure.

Figure 4 presents the relationship between (log) air styrene concentration and (log) sum of urinary MA and PGA concentrations. As above, the four persons wearing RPE are excluded from the calculation of the regression coefficient. The correlation coefficient (r^2=0.75) for this analysis is comparable to that for MA and substantially better than that for PGA individually. This difference may be a function of the different kinetics of metabolism for MA and PGA and the timing of sample collection. In addition, Figure 4 presents the data points representing the four persons wearing RPE. As expected, the points for two of these individuals fall below the regression line, indicating the protective effect (i.e., metabolite concentration less than expected for a given air concentration of styrene) of wearing RPE. Interestingly, the MA+PGA concentration falls on the regression line for the other two individuals, indicating little, if any, protective effect from wearing RPE.

The use of respirators was not a major factor in exposure measurement or estimation except for Site 1. Because of a variety of factors, nearly half of the workers tested at Site 1 wore RPE during their work shift. Actual inhalation exposures were estimated for use in interpretation of the olfactory test results based on the division of styrene air concentrations by an arbitrary factor of five. The apparent disparity between estimated RPE-corrected exposures and metabolite concentrations at Site 1 suggests that, on average, the respiratory protection factor may not be as

high as assumed. At the very least, it is apparent that there may be a wide range of respiratory protection factors (and resultant effective exposures) across individuals, suggesting possible mis-assignment of effective exposures for at least a few workers.

CONCLUSIONS

The historic and current estimates of styrene exposure were consistent with each other and consistent with other published reports. Historic exposures were generally consistent across the four study sites, while there were indications from both air sampling and urinary metabolite measures that current exposures at Site 1 are somewhat higher than at the other three sites. While the respirator-adjusted population mean exposure was approximately 13 ppm, the overall range of exposures studied (1–28 ppm) was consistent with the primary objectives of population selection for the olfactory study and provided a range of current and historic exposures against which the olfactory function testing results could be evaluated.

A thorough understanding of subject exposure is crucial to the interpretation and understanding of observed health outcomes in general, and specifically with reference to this study, to the interpretation of olfactory function. The addition of quantitative measures of exposure allows the quantitative determination of an exposure–response relationship between exposure and disease. This is an important addition in that, unlike other measures of exposure common to epidemiologic studies, it is directly useful for risk analysis and regulatory purposes. Quantitative assessment of subject exposure is essential to this understanding and should be a standard part of such studies.Without quantitative measures of exposure, findings and conclusions are of limited usefulness. The use of exposure information in this manner is critical for the development of defensible assessments of the risk of exposure to occupational and environmental agents and, in turn, to the standards setting process.

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Neuropsychological Effects of Styrene Exposure: Review of Literature Published 1990 – 2003

REPORT TO THE STYRENE INFORMATION AND RESEARCH CENTER

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PART I

Critical Review of Studies since 1990

INTRODUCTION

Following a literature search conducted by Leyla Bagirzadeh, 20 papers relevant to the effects of styrene on the human nervous system and published since 1990 were located. These excluded effects on the visual and auditory systems as these were to be covered in other reviews, but included studies of mortality from diseases of the nervous system (3) and of neurochemical functioning (3). The remaining 14 papers had as outcomes neurophysiological measures, including EEG, somatosensory evoked potentials and nerve conduction studies (4), neurobehavioural testing (8) or symptoms only (2). Two studies reported results in more than 1 paper and these are considered together in the reviews attached.

The format of each review reflects some common difficulties in interpreting these studies. Having described the aim, design, subjects and outcome measures in each study, the question of exposure then merits a separate paragraph. This is for at least 3 reasons. First, given that the main interest of the review is the effects of styrene at the lower levels found since 1990, it is important to recognise that many of the studies include workers exposed in previous decades when exposures will have been much higher. Second, for all studies except perhaps for mortality and neurophysiological changes, it is important to distinguish between effects resulting from acute exposure (which may not reverse completely during a weekend break) and those that may be attributed to repeated exposure over many years (and which may not be reversible). Third, studies differ in their ascertainment of exposure. While urinary metabolites will reflect individual work habits and exposures and as such are ideal for investigation of dose-response relationships, if there are important differences in metabolism then results from such analysis may be difficult to extrapolate to exposure standards expressed in respirable concentrations. The role of PPE, important in exposure assessment, is only mentioned (and then in passing) in a handful of these papers. Although efforts are made in Part 1 of this report to interpret the data in terms of a 'no effect' level, only one paper explicitly analyses data in this way.

Studies published since 1990 are, in general, of high quality and avoid many of the pitfalls of the earlier studies of neurobehavioral effects. The conclusions drawn by the authors are usually appropriately modest and point to the relatively slight effects that can be attributed to styrene in the populations studied.

The paragraph on exposure is immediately followed by ones outlining the statistical methods used and the broad results obtained. This is followed by a paragraph on confounding and on the extent to which attempts have been made — and with what success — to allow for this. Many of the early studies, and some included here, gave inadequate thought to issues of study design that would increase the internal validity of the report. The final two para-

graphs of each review report briefly the authors' main conclusions and those of the reviewers.

Although there are issues of validity and interpretation that are common to most of the studies reviewed, their relative importance differs with the design and outcome used. The overview that follows considers in turn the 4 main outcomes outlined above. Within each section studies are considered in order of publication date, the earliest study being discussed first.

1.0 MORTALITY

1.1 Three studies have been reported, from Denmark (Kolstad et al. 1995), from the European styrene cohort (Welp et al. 1996) and from the US cohort (Wong and Trent, 1999). This review considers only deaths from diseases of the nervous system, although each of the 3 papers considered other outcomes also.

The pattern of results from the papers is inconclusive. The Danish study, published first, drew appropriate attention to an apparent increased risk of neurodegenerative diseases, based on a small number of deaths. Subsequent analysis of the European cohort produced results that were consistent with the Danish study but it is not clear that the 2 were independent. The Danish study was based on workers from 386 reinforced plastic companies. The European cohort included 287 Danish plants. In discussion of this latter paper, Wong and Trent report that the excess of deaths from nervous system disease was seen particularly in the Danish workers and was not evident in those from the UK, the next largest group in this European cohort. The analysis of the US cohort showed no evidence of any excess although little detail was given of the internal analysis which had proved particularly useful in the Danish and European cohorts. In each of the 3 reports, no excess of nervous system disease was seen in comparison to external referent groups.

A number of comments can be made about the adequacy of these 3 studies to identify any underlying risk of degenerative nervous system disease associated with styrene exposure. First, all 3 cohorts are young, with few deaths reported. It remains plausible that exposure to styrene, as with other solvents, may contribute to disorders of the central nervous system that become apparent much later in life. Second, mortality is a very crude indication of nervous system degeneration. Third, should further follow-up be carried out on 1 or more of these cohorts, and an excess of degenerative disease of the nervous system found, the absence of information on lifestyle confounders, particularly alcohol, may complicate interpretation.

1.2 Implications for Assessment of "No Effect"
All 3 cohorts included subjects who were employed during periods (certainly before 1970 and perhaps later) where exposures may well have exceeded current limit values. Given that no consistent effect has been observed, extrapolation is difficult.

2.0 NEUROPHYSIOLOGICAL MEASURES

2.1 Five papers were reviewed under this heading. 1) Matikainen et al. (1993) examined EEG, 2) Murata et al (1991) and Stetkarova et al. (1993) used somatosensory evoked potentials, 3) Pierce et al. (1998) measured the P300 auditory event related evoked potential and 4) Murata et al. (1991) and Yuasa et al. (1993) measured peripheral nerve conduction parameters. Murata et al. (1991) also interpreted variation in R-R interval from electrocardiograph traces as a measure of autonomic nervous system function.

This group of studies is somewhat difficult to review. The approach is valuable, in that it provides measures of nervous system function that are independent of the level of collaboration of the subject. The body of evidence on neurotoxicity also suggest that, with the possible exception of EEG recordings, any effects seen are not likely to represent simply acute effects that may be reversible within hours. The five groups reporting, two from Japan, and one each from Finland, Czechoslovakia and the United States, come from institutes with a good experience in work of this sort.

The results, however, give a mixed picture of the toxicity of styrene. The evaluation of EEG in a group of 100 workers, chosen in ways not specified, found no excess of abnormal EEG on visual assessment but, using an automated analysis, found that the group with the highest cumulative exposure had an excess of recordings judged abnormal and an increase in total EEG power in the a and b bands, particularly in the frontal and temporal regions of the brain.

Two studies measured somato-sensory evoked potentials. The results reported by Murata et al. (1991) showed no significant differences in somato-sensory evoked potentials between the exposed and comparison groups. In contrast, the results from Stetkarova et al. (1993) appeared to show slowing in central conduction time and prolonged latency of cortical evoked potentials in the styrene exposed group. Pierce et al. (1998) found no change in auditory evoked potential in 44 healthy volunteers exposed to styrene in an exposure chamber.

The study by Murata et al. (1991) reported a reduced R-R coefficient of variation in the exposed, which they

interpreted as a significant change in the autonomic nervous system in the styrene exposed group.

A number of studies have also measured peripheral nerve conduction as a possible marker of styrene toxicity. Murata et al. (1991) reported significant slowing of sensory conduction velocity but not of motor conduction in the median nerve. In contrast, Yuasa et al. (1996) reported a greater (and significant) slowing of motor rather than sensory conduction in the ulnar nerve, with significant slowing also in the peroneal (motor) nerves but not in the sural (sensory) nerve. Stetkarova et al. (1993) also reported that peripheral conduction velocity was slower in styrene exposed workers compared to a comparison group on stimulation of both the median and tibial nerves.

On balance, the results of these studies do suggest some minor electro-physiological differences between the exposed and comparison groups. While it might be that measurement of confounders was imperfect or incomplete, this seems unlikely to fully explain the differences and bias, in selection of subjects or in observation or reporting, also seems an insufficient explanation. A real difficulty does arise from the number of measures taken, which might be expected to lead to some positive results from chance variation. In addition there may also be a degree of publication bias in that papers without at least one positive finding fail to reach the journals. However, although effects are probably real, they are not entirely consistent and the clinical significance is questionable. All subjects appeared to be healthy workers.

2.2 Implication for Assessment of "No Effects"

The results discussed here may be of little clinical significance but, particularly in relation to nerve conduction studies, are consistent with some earlier observations. All the studies, however, contain subjects with a history of high past exposures. Since effects on the peripheral nervous system are assumed to reflect chronic exposures, with uncertain reversibility in the absence of complete cessation of exposure, the relation of these studies to current low exposures is unclear.

3.0 BEHAVIOURAL TESTING

3.1.1 Cross shift design (acute effects)

Two studies involving exposure of subjects under experimental conditions were identified. Pierce et al. (1998) observed no effect of styrene exposure on a digit recognition test. Ska et al. (2003) similarly reported no effect on a number of tests including simple reaction time and symbol digit matching.

Three studies considered cross shift (acute) effects. Letz and colleagues (1990) found no relation between exposure during the shift and scores on the continuous performance test (of complex reaction time) or on a test of hand-eye performance. Scores on the symbol digit test at end of shift were related to styrene in air corrected for respirator use (by a method not specified) and to urinary mandelic acid. Although the data are not presented, it appears that those exposed to >50 ppm have the lowest symbol digit scores. Jegaden and colleagues (1993) conducted a similar study in a French naval shipyard. The analysis across shift was not reported adequately but there appears to have been no significant difference between changes across the shift in the exposed and comparison groups. Significant pre (and post) shift differences were observed which the authors interpret as chronic effects but could equally reflect either inadequate adjustment for confounding or incomplete clearance of styrene and its metabolites from the previous day's exposure. Edling and colleagues (1993) found no change in test score over shift between the exposed and comparison groups but within the exposed group there was a relation between complex reaction time and various measures of exposure during the shift including eight hour time weighted average. No effect was seen on the symbol digit test, the only test found by Letz and colleagues (1990) to have an exposure-related cross shift change.

On balance, and in light of previous studies, it seems likely that small differences are detectable across shift in test scores of workers exposed to styrene at levels found 10-15 years ago. Such changes are not seen with experimental short exposures to relatively low concentrations.

3.1.2 Implications for Assessment of "No Effect"

Letz explicitly says that most of the effects are found in those exposed to more than 50 ppm on the day (this data is not presented). Jegaden and colleagues measured exposure on the day of testing and the highest level of concentration was 55 ppm with a mean of 23 ppm; no notable cross shift effects were found. Edling's exposures were very low (8.6 ppm on average and nobody exposed to more than 15.4 ppm). He was, however, able to measure excursions greater than 50 ppm and found that greater number and duration of excursions above 50 ppm were related to poor scores on the complex reaction time test. Taken together these three surveys do suggest that at the exposures below 50 ppm, and without excursions above this level, cross shift change in test score may not be detectable using current techniques.

3.2.1 Reversibility on Reduction or Removal of Exposure

Only two studies reported the effects of reducing or

removing exposure on behavioural test scores. These were Mergler et al. (1996) and, debatably, Viaene et al. (1998 and 2001). The study reported by Mergler and colleagues is particularly interesting. In this, three workplaces were assessed and baseline measurements made of scores of performance on neurobehavioural tests. Two years later the exposure and neurobehavioural tests were repeated, following interventions at one of the three companies to improve working conditions. The overall exposures were low and did not change essentially but it appears that those whose mandelic acid concentrations increased over time did worse on the second occasions, and importantly, those whose mandelic acid concentrations were reduced did better on at least some tests. This study has a number of weaknesses (see the detailed comments in Part II) but the design is extremely attractive. In contrast the study reported by Viaene and colleagues (1988 and 2001) has such a weak design that it is not possible to draw any conclusions about the reversibility of effects. The authors would appear to deduce that the better test scores seen for people laid off three years earlier would reflect such a reversal, but no baseline data are available.

3.2.2 Implications for Assessment of "No Effect"
Both these studies involve workers exposed to relatively low levels of styrene, certainly less, on average, than 50 ppm. For different reasons, discussed in the detailed comments, neither can provide good data on which to reach conclusions.

3.3.1 Cross Sectional Studies of Neurobehavioural Effects
Only one study, that of Tsai and Chen (1990) from Taiwan, was found that presented simply cross sectional data comparing behavioural tests in an exposed group and a non-exposed comparison group. This study used an internal comparison group of workers from within the same six manufacturing plants in Taiwan and information given on potential confounders suggesting they were in fact very well matched. This rather thorough study, in plants where the mean concentration was 22 ppm, found worse performance on the continuous performance test (of complex reaction time test) and on vibration perception where the threshold was high in both the hand and foot for the exposed. The duration of employment was 8.3 years and it is not clear whether high levels of exposure had pertained in the past.

Three studies already mentioned could also be interpreted as contributing to this design. In the study of neurophysiological changes reported by Murata there was a second paper, by Yokoyama and colleagues (1992), which included information on neurobehavioural testing. In this study of 12 styrene exposed workers and 11 non-exposed steel workers, the exposed workers had a mean duration of exposure of 5 years and current exposure of around 26 ppm with a maximum eight hour TWA of 77 ppm. It is not known whether there had been higher exposures in the past. In comparison to the comparison group the exposed workers did less well on the picture completion test but there was no difference in scores on the digit symbol test. It does not appear that the comparison group was particularly well matched, but statistical adjustment for confounders was attempted. The Jegaden study (1993) has been discussed. Here the exposed group did significantly worse than the comparison group on a test of simple reaction time, complex reaction time and digit span. The authors suggest this was likely a result of chronic exposure. In the preceding year, mean exposures had been approximately 30 ppm with perhaps 20% of exposures more than 50 ppm. There are, however, a number of difficulties in interpretation of this study. Finally in this section, Viaene and colleagues (2001) compared scores of the currently exposed and the previously exposed with a comparison group of never exposed. The scores on the symbol digit substitution and digit span forward test appeared to be worse than those who had been exposed to styrene.

3.2.2 Implications for Assessment of "No Effect"
Because of the difficulties in interpreting such studies of chronic effects with an external comparison group, rigorously conducted negative studies are likely to be more convincing of a "no effect" level than positive studies where the adequacy of adjustment for confounding or residual effects are in question. Of these four studies, the Tsai and Chen (1996) study is perhaps most convincing but it is impossible to say whether the observed effects on the continuous performance test and on vibration perception threshold reflect current exposures of around 22 pm or past exposures that may have been considerably higher.

4.1 NEUROCHEMICAL FUNCTIONING

Three studies have been published. Checkoway et al. (1992), Bergamaschi et al. (1996) and Bergamaschi et al. (1997) looked at the interrelationship between styrene exposure, markers of neurochemical function, particularly Type B monoamine oxidase (MAO-B), dopamine-b-hydroxylase (DBH) and prolactin. Checkoway et al. (1992) looked also at serotonin and sigma receptor activity and concluded that monoamine oxidase B might be a useful marker or even an intermediate step between styrene exposure and CNS neurotoxicity. In this study MAO-B

activity and CNS symptoms were correlated. Bergamaschi and colleagues in a first study published in 1996 attempted to reproduce the MAO-B effect and also measured DBH and prolactin. They failed to demonstrate any very convincing differences between exposed workers and the comparison group on MAO-B, although this was significantly depressed in those with higher exposure (>25 ppm). DBH activity was decreased in the styrene exposed and prolactin increased in both men and women. A second paper by this group, Bergamaschi et al. (1997), presumably—but not certainly—had no overlap in subjects with those in the earlier paper. They found no difference between exposed workers and the comparison group on either DBH or MAO-B although prolactin levels were higher in styrene exposed workers. Reported symptoms were similar in the exposed and non-exposed groups but within the exposed group DBH was negatively related to the number of reported symptoms.

These studies are all interesting but interpretation of results is difficult and their importance in the understanding of styrene neurotoxicity has yet to be established. In comparing exposed to a non-exposed comparison group the problems of unmeasured confounding is substantial because the relevant confounders are unknown.

4.2 Implications for Assessment of "No Effect"
These studies do not, in their measurement of neurochemicals, contribute yet to this discussion.

5.1 STUDIES OF SYMPTOMS

Two studies, those of Geuskens et al. (1992) and Challenor and Wright (2000) use symptoms as the only outcome considered. Many of the other papers, however, also report symptom data and the most relevant of these are included in this discussion.

Geuskens et al. (1992) carried out a study to assess factors affecting exposure levels in large and small factories using styrene in the Netherlands. As part of that investigation, they included a symptom questionnaire which they had also used with a non-exposed population for another study. Details of this comparison group are not given. The hygiene assessment suggested that although the daily concentrations of styrene measured did not exceed 100 ppm generally, there was a substantial risk of doing so during some of the processes and peak exposures up to 1200 mg/m³ were seen. In the comparison of symptoms a group which they labeled as "irritation", including outcomes such as feelings of depression, and one labeled "pre-narcotic" were higher in the exposed workers. This study was not reported in sufficient detail to allow conclusions to be drawn about the validity of ascribing the excess symptoms to exposure.

Challenor and Wright (2000) chose to look only at the aggression scale of the Profile of Mood States. They compared exposed workers with an internal group of unexposed workers. With exposures up to about 66 ppm, styrene exposed workers, particularly those in the first few years at work, were more likely to have higher aggression scores than the comparison group.

Ten of the other studies report symptom data. Of these some were little more than clinical reports on the exposed workers while others used inappropriate measures (for example a measure of long standing personality rather than present state). Amongst those using appropriate and established questionnaires, the most frequent were the Swedish Q16 solvent questionnaire and the Profile of Mood States. In general the data are more convincing if they relate to changes within individual across time (Edling et al. (1993) Mergler et al. (1996)) or in a dose-response analysis comparing less exposed to the more highly exposed such as those of Matikainen et al. (1993) and Checkoway et al. (1992). No study in this group attempted to use questions designed to look at subtle changes over the course of a shift. Edling et al. (1993) did look at questions of acute irritation and found them related to exposure parameters but with no difference in the before and after shift responses. Ska et al. (2003) reported no change in mood or symptoms following experimental exposure with peaks to 100 ppm.

Although there is some variability in results, almost all the field studies did find a relation between exposure and symptoms. Three are of particular interest in discussing a "no effect" level. Edling et al. (1993) examined a group for whom the eight hour time weighted average was 8.6 ppm and found that exposed men reported more acute symptoms and more symptoms using the Q16 questionnaire, particularly of tiredness and headache. He also noted that the number of symptoms reported by the exposed men reduced when the Q16 was repeated after a break for the summer holiday, although this difference was not statistically significant. The change in number of symptoms between the beginning and end of shift was related to the 8 hr TWA for styrene. The authors concluded, and this seems reasonable, that exposures below 20 ppm produced, in their sample, an increased number of symptoms.

Tsai and Chen (1996), however, using Q16 and the profile of mood states in a cross sectional study, but with well matched internal controls, found no acute or chronic symptom significantly affected by exposure to styrene in

a study where the mean concentration was 22 ppm. The range of concentrations included values of up to 181 ppm as an 8 hr TWA. This study adapted the profile of mood states to be more relevant to the Chinese culture and on the three scales used no difference was seen. Other studies demonstrating a dose response relationship included Matikainen et al. (1993), Mergler et al. (1990) and Bergamaschi et al. (1997). Negative results were found by Yuasa et al. (1993) and Viaene et al. (2001).

On balance, the stronger study designs did tend to find that symptom reporting was related to exposure even at relatively low levels.

5.2 Implications for Assessment of "No Effect"

In the studies reported here many of the exposed subjects had worked at much higher levels than those measured on the day of testing. It is frequently not clear whether the worker is reporting current symptoms or symptoms ever experienced. In assessing the studies with clearly described methods, the study of Edling et al. (1993) is particularly of interest. It demonstrated a higher number of symptoms with higher concentration of styrene in the breathing zone (driven in part by two high symptom scores at around 12 ppm of exposure) but also data on excursions above 50 ppm related to skin irritation and unpleasant taste. These findings suggest that although TWA exposures may be kept below levels at which symptoms generally occur, peak exposures may still contribute to discomfort, if not necessarily to long term changes in neuropsychological health.

OVERALL CONCLUSION

Studies published since 1990 are, in general, of high quality and avoid many of the pitfalls of the earlier studies of neurobehavioral effects. The conclusions drawn by the authors are usually appropriately modest and point to the relatively slight effects that can be attributed to styrene in the populations studied. Nevertheless the conclusion is unavoidable that styrene has the potential to cause unpleasant symptoms, and probably changes in test performance, at levels of exposure close to 50ppm and that it should be treated as a potentially harmful substance that merits best preventative practice. None of the studies reviewed adequately addresses the question of debilitating illness in the elderly population of workers exposed to styrene over many years and this important question—for public health if not for regulatory and compensation agencies—remains to be answered.

PART II

Review of Individual Studies

Bergamaschi E, Mutti A, Cavazzini S. et al.

Peripheral markers of neurochemical effects among styrene-exposed workers.

Neurotoxicology 1996; 17:753-760.

AIM

MAO-B, dopamine-b-hydroxolase (DBH) and prolactin (PRL) were measured to evaluate possible changes in biochemical events in the central nervous system in workers occupationally exposed to styrene.

DESIGN

This was a cross-sectional study using 53 workers from a number of factories manufacturing fibreglass and reinforced plastics and a comparison group of 60 non-exposed workers from other industries in the same area.

EXPOSURE

Ambient monitoring suggested exposure from 5 to 100 ppm in exposed groups. Exposed workers had been employed for an average of 9.3 years (range from 1-22 years). Urinary metabolites (mandelic acid and phenylglyoxidic acid) were measured in a spot urine sample collected 15 hours after last exposure.

OUTCOME

A blood sample was drawn 15 hours after last exposure and used to determine DBH activity, MAO-B activity, and prolactin. These assessments were made for both exposed and comparison subjects.

ANALYSIS

Means were compared using Students t-test, analysis of variance and correlations to evaluate association between variables. Cut-off values were calculated as the 5th and 95th percentile of the comparison group and the probability of abnormally high values evaluated using a Cox regression analysis.

RESULTS

Styrene exposed workers had lower DBH activity and higher prolactin (in both men and women) than the comparison group. Mean MAO-B activity was somewhat higher in the comparison group. DBH activity was negatively correlated with the sum of the urinary metabolites (MA + PGA). No correlation was reported between the other biomarkers and these metabolites. The group of workers with the higher urinary metabolites had significantly lower MAO-B activity than the controls. Extreme values were then examined, with significantly more of those in the higher exposure group having abnormally low DBH activity and abnormally high values of prolactin. No abnormal values of DBH were found in those with the sum of metabolites (MA + PGA) less than 200 but the Cox's regression model showed increasing risk with higher values. In the analysis of prolactin, men and women were considered separately and women appeared to have changes at a lower exposure level than males.

CONFOUNDING

The analysis was stratified by gender for the prolactin analysis. Age, smoking and alcohol use were compared between exposed and comparison group and no difference found but they were not obviously included in the analysis. Though there was a wide range in duration of exposure (1-22 years) no account appears to have been taken of this in the analysis.

AUTHORS' CONCLUSIONS

DBH activity was significantly decreased amongst styrene exposed subjects but the reason for this is unclear. Most likely the decreased serum DBH activity could be an adaptive mechanism to a reduced turnover rate of dopamine at the neuronal level. Prolactin was increased amongst styrene exposed workers with a greater sensitivity of female workers, showing an increased risk for abnormally high prolactin at relatively low exposure levels.

REVIEWER'S CONCLUSIONS

The study does appear to show negative relation between exposure and DBH and a positive one with prolactin. The relation to MAO-B is weak.

Bergamaschi E, Smargiassi A, Mutti A, et al.

Peripheral markers of catecholaminergic dysfunction and symptoms of neurotoxicity among styrene-exposed workers.

Int Arch Occup Environ Health 1997; 69:209-214.

AIM

To investigate the inverse association of platelet and MAO-B with symptoms, to assess the relation between peripheral markers of catecholaminergic dysfunction and the relation to exposure and to compare prolactin with other markers of catecholamine metabolism.

DESIGN

This was a cross-sectional study using an external non-exposed comparison group

SUBJECTS

46 styrene-exposed male workers from a number of factories manufacturing reinforced plastics were recruited together with a comparison group of 30 blue collar workers, with no history of exposure to chemicals, from local industries.

EXPOSURE

Exposed workers had been employed for 0.2-29 years with a median of 6 years. Styrene exposure by ambient monitoring ranged from 5 to 120 ppm as a TWA with a median level 25 ppm. Urinary spot samples were collected on a Friday morning 15 hours after last exposure and analyzed for mandelic acid and phenylglyoxidic acid.

OUTCOME

The Swedish Q16 symptom questionnaire was completed on the Friday morning on which blood was drawn. The blood was analyzed for prolactin, MAO-B and dopamine b hydroxylase (DBH).

ANALYSIS

Results presented appeared to depend on the Mann Whitney U test, Fisher's exact test, linear regression and multiple logistic regression.

RESULTS

No difference was seen in the mean number of symptoms between exposed and control workers but, in the exposed group, a relation was found between the number of symptoms reported and urinary metabolites, with those with higher metabolites reporting more symptoms. This result was not reported in detail. Prolactin was higher in the exposed group and MAO-B lower. The most exposed had lower values of DBH than the non-exposed although a straight comparison of exposed and comparison groups showed no difference. When extreme values were calculated, with cut-off at the 5th of 95th percentile in the comparison group, differences were seen for prolactin; significantly more exposed than controls presented with abnormally high values. In the exposed group DBH was negatively correlated with the sum of urinary metabolites. No relation was found between any of the peripheral markers and exposure duration. It is reported, but no are details given, that none of the results was modified by age, drinking or smoking.

CONFOUNDING

The comparison group was older and more likely to report drinking alcohol than the exposed. It was reported that the effect of these confounders, together with cigarette smoking, were examined but no detail was given.

AUTHORS' CONCLUSIONS

Plasma prolactin was higher in styrene-exposed workers but its level was not related to the intensity of exposure to styrene. Although no difference was seen in DBH levels between exposed and the comparison group, the relation between urinary metabolites, symptoms and DBH suggests that the change in DBH may contribute to the appearance of the symptoms. The lower platelet MAO-B and plasma DBH might represent an adaptive mechanism to a dopamine depletion. Of the three peripheral markers of catecholaminergic dysfunction, plasma prolactin appears to be most sensitive.

REVIEWERS' CONCLUSIONS

The study tends to support the observation that prolactin was increased and DBH decreased in styrene-exposed workers and provides modest support for the suggestion that MAO-B is also reduced. It is not certain that the sample examined in this study is independent of that in the same authors' 1996 study and indeed some of the observations made may have been the same in the two review studies. The absence of any difference in symptoms between the exposed and comparison series may in part be due to systematic differences between the two (for example in age); this interpretation is supported by the positive relation between urinary metabolites and symptoms in the exposed group. The levels of exposure were relatively low (a median of 25ppm) but included some workers exposed as high as 120 ppm.

Challenor J, Wright D.

Aggression in boat builders: a search for altered mood states in boat builders exposed to styrene.

Occup Med (Oxford) 2000; 50:185-92.

AIM

To determine whether builders of glass reinforced plastic boats might be more aggressive or hostile than their counterparts in areas of a company where volatile organic compounds were not being used.

DESIGN

This was a cross sectional study comparing mood state in exposed and non-exposed personnel in one factory.

SUBJECTS

213 workers exposed to styrene and 114 non-exposed employees took part.

OUTCOME

A profile of mood states was completed, asking the subject to rate feelings during the past week, "including today". A supplementary questionnaire on job satisfaction, life events, stress, smoking and alcohol was also completed.

EXPOSURE

Exposure from records kept by the company between 1990 and 1996 gave a range of 4-150 ppm. Passive samples of 23 subjects was carried out two weeks after completing the study. The highest air concentration recorded was 66 ppm.

ANALYSIS

T-tests, Wilcoxon's rank sum tests and the Mann-Whitney U test were used to compare the means of the exposed and non-exposed groups.

RESULTS

The styrene exposed workers had scores on the aggression scale of the profile of mood states that suggested they were more aggressive than the non-exposed. This difference was marginally significant at the p=0.5 level. Further analysis suggested that this aggression was most evident in those who more recently started working with styrene.

CONFOUNDING

The text suggests that the styrene exposed group smoke more but have equivalent alcohol intake, but this information is not presented.

AUTHORS' CONCLUSIONS

GRP boat builders were found to have an increase in mood state aggression/hostility. The fact that the increased score for aggression/hostility is most evident in the earlier period supports the view that the finding may reflect a selection phenomenon and not an exposure phenomenon.

REVIEWER'S CONCLUSIONS

This study is essentially a descriptive one of a phenomenon noted in a single factory. It is not clear that it can be generalized to other situations.

Checkoway H, Costa LG, Camp J, Coccini T, Daniell WE, Dills RL.

Peripheral markers of neurochemical function among workers exposed to styrene.

Br J Ind Med 1992; 49:560-5.

AIM

To evaluate the utility of peripheral blood cell parameters of neurochemical function in styrene-exposed workers.

DESIGN

Cross-sectional design with an internal, non-exposed, comparison group.

SAMPLE

60 exposed workers from 3 fiberglass reinforced plastics plants in Washington State were studied, together with 18 non-exposed workers from one of the plants.

EXPOSURE

Exposed workers had been employed for a little under 4 years, on average. Exposure measurements were made mid-week, on Wednesday and Thursday, with full-shift passive dosimeters, from which 8h TWA were determined. Blood and urine samples appear to have been taken at an early point in the shift and were analyzed for styrene concentrations (blood) and urinary metabolites. Exposure groups were defined by blood styrene concentration. The mean exposure of the group with highest blood styrene was 58ppm (SD=11.1).

OUTCOME

Blood samples were also analyzed for Type B monoaminoxidase (MAO-B), serotonin and sigma receptor binder. CNS symptoms were collected by a self-completed questionnaire that is not described in more detail.

ANALYSIS

Correlations and comparisons of means between the exposure groups were the main analytical tools although a multiple logistic regression analysis was apparently

carried out (but not reported in detail) to assess possible confounding.

RESULTS

Those with higher blood styrene higher concentration reported a higher total number of symptoms, and a higher proportion in the middle and high blood styrene groups reported each of the 7 symptoms listed. The pattern apparently did not change after a allowance for age, sex, alcohol, smoking and duration of employment.

The authors report correlation coefficients between blood styrene concentration and peripheral markers of neurochemical function. These showed a significant, negative, relation between MAO-B and blood styrene, which was no longer quite statistically significant when adjusted for age, race, sex, years of employment, smoking, and alcohol intake. Serotonial uptake had a positive correlation which, at least on the rank order correlation, increased slightly after adjustment for confounders and was then shown as significant ($p<0.05$). The relation between symptoms and neurochemical markers was also examined. No relation was found between symptoms and serotonin or sigma receptor binding but MAO-B was inversely related to a number of CNS symptoms, having allowed for age, race, sex, years of employment, alcohol use and smoking.

CONFOUNDING

Key analyses were appropriately adjusted for potential confounders. It was notable however that those in the moderate and high blood styrene groups had much higher rates of smoking than in the non-, or low exposed groups.

AUTHORS' CONCLUSIONS

Results from this study indicate that MAO-B activity may be useful as a marker of effect of occupational exposure to styrene.

REVIEWER'S CONCLUSIONS

This appears to be a well-conducted, although essentially negative, study. The relatively high rate of symptoms in the groups with higher exposure is noted but as concentrations ranged as high as 160ppm it is difficult to generalize to current exposure levels. The suggestion that MAO-B may be involved as an intermediate step in styrene-induced CNS neurotoxicity does not seem well-founded.

Edling C, Anundi H, Johanson G. et al.

Increase in neuropsychiatric symptoms after occupational exposure to low levels of styrene.

Br J Ind Med 1993; 50:843-850.

AIM

To determine if exposures to low concentrations of styrene (below 20 ppm) are related to neurobehavioural effects or symptoms.

DESIGN

The assessment of neurobehavioural effects by psychometric testing was done using a cross shift design and an external non-exposed comparison group. Symptom data was collected on two occasions, once during the normal working year with a follow-up some two to five weeks since last exposure, at the end of the summer vacation.

SUBJECTS

The exposed group were 20 men working at a single plastics factory. The comparison group were 20 non-exposed men matched on age, working schedule and physical workload. They worked in nearby industries and had not been occupationally exposed to solvents.

OUTCOME

Three tests were used, a test of simple reaction time, color word vigilance and the symbol digit test. The color word vigilance test is a choice reaction time and the symbol digit test is a test of speed of processing. The outcome of interest was change in the test during the work shift. Symptoms were recorded using the Swedish 16 question solvent questionnaire. At the end of shift testing session a computerized questionnaire of 17 symptoms of acute effects including local irritation and central nervous system symptoms was completed.

EXPOSURES

Workers had been exposed to styrene for between 1-25 years (mean 9 years). Exposures were measured at 15 second intervals throughout the day using a back pack containing a mobile photo ionization detector. This was worn

during one working day, presumably on the day the behavioural testing was done. Passive air sampling was performed on the same day using carbon diffusion samplers placed in the breathing zone of each subject. Blood and urine samples were collected immediately before and after the work day.

Using passive dosimetry the mean eight hour time weighted average was 8.6 ppm (range 0.04-15.4 ppm). From the continuous monitoring device the number of excursions greater than 50 ppm could be measured and were detected for nine of the workers.

RESULTS

Exposed men reported more acute symptoms both at the beginning and end of shift but there was no change across shift. On the first recording of symptoms using the Q16 questionnaire the exposed men reported significantly more symptoms, particularly of tiredness and headache, than the referents. Although the number of symptoms reported by the exposed men reduced when this was repeated at the end of a break from exposure for the summer holiday, improvement was statistically not significant. The change in the number of symptoms between the end and beginning of shift was, in the exposed men, related to the eight hour time weighted average for styrene. The total number of excursions above 50 ppm was related to reports of skin irritation at the end of shift and peak exposures were related to sensations of an unpleasant taste.

On the performance test no difference was found on the test of simple reaction time between the exposed and referents either before or after the end of shift. On the color word vigilance test, a test of complex reaction time, the exposed did worse than the referents both before and after the test. The exposed did somewhat better, on both occasions, on the symbol digit test.

No relation was found between any of the cross shift exposure measures and simple reaction time or change in simple reaction time. The complex reaction time scores were related to time weighted average exposure, the number of excursions above 25 ppm and above 50 ppm and to the duration of excursions above 50 ppm within the exposed group. No dose-response data is reported for symbol digit test.

CONFOUNDING

The design considered a number of aspects of confounding. Half the workers completed the end of shift test before the start of shift, to allow for possible effects of learning. Physical workload was evaluated using a computerized electrocardiograph to compare physical workload between the exposed and referent subjects. Other workplace contaminants (acetone and toluene) were also measured. The exposed and referent group were thought to be similar on age, working schedule and physical workload but there is no discussion of other potential confounders such as educational level.

AUTHORS' CONCLUSIONS

This study showed no difference in the psychometric tests when men exposed to 0.4-50.4 ppm of styrene were compared with non-exposed referents. It suggests however that exposures below 20 ppm produced an increased number of neuropsychiatric symptoms.

REVIEWER'S CONCLUSIONS

This study is valuable for its investigation of the effects of variations during the working day on test score and symptoms. The cross sectional comparison of symptoms between the exposed and unexposed groups in different workplaces may have been biased by the knowledge of the purpose of the study. However the relation between the change in symptoms and average styrene concentration in the exposed appears to be a sound result.

Geuskens R, Klaauw M, Tuin J. et al.

Exposure to styrene and health complaints in the Dutch glass-reinforced plastics industry.

Ann Occup Hyg 1992; 36:47-57.

AIM

To describe exposures and level of health complaints in four large and 12 small plants using styrene.

DESIGN

To compare symptoms reported by workers exposed to styrene to those reported by a comparison group of unexposed men who had completed the questionnaire for another unpublished study.

OUTCOME

13 questions about health complaints were grouped into three factors: "irritation," "pre-narcotic symptoms" and "cognitive/locomotor disabilities." Irritation symptoms indicated itching of the skin, headache, sniffing and burning eyes and feelings of depression. Pre-narcotic symptoms included nausea, feeling drunk, dizziness and disturbance of equilibrium and cognitive/locomotor disabilities bilateral communication, impaired communication with colleagues and trouble with movement.

EXPOSURE

In the four large plants workers were grouped into four job categories and styrene concentration in the breathing zone of each worker was measured over three (or four) days in winter. The eight hour time weighted average was derived from two consecutive four hour samples. Short term measures were also made to determine peak concentrations. The geometric mean concentrations of the highest categories were between 97-212 mg/m³. In the small plants walk through surveys were carried out and the average concentration during moulding was estimated to range from 100-400 mg/m³. Health complaints were not collected in the smaller factories.

ANALYSIS

The mean score on each of the three grouped factors — irritation, pre-narcotic symptoms and cognitive/locomotor disabilities — were compared with those from the non-exposed comparison group using a student t-test.

RESULTS

The styrene exposed workers were more likely to report symptoms grouped by these authors under the title irritation. They were also more likely to complain of pre-narcotic symptoms. No difference was found in the category cognitive/locomotor disabilities.

CONFOUNDING

No description is given of the comparability of the exposed and control groups and it is not known to what extent confounding is a problem. The authors comment that "none of these differences can be explained by differences in work conditions, work hygiene or health risk factors between the two groups," but no data are given. It is also commented that the exposed workers often mentioned styrene and methylene chloride as the cause of their complaints but no information is given on the exposure to methylene chloride.

AUTHORS' CONCLUSIONS

The work related differences in irritation and pre-narcotic symptoms between the exposed and control groups are quite obvious and may be regarded as short term health effects. They appear to attribute them to peak levels which were observed in the study to be up to 2000 mg/m³.

REVIEWER'S CONCLUSIONS

This was essentially a study to determine levels of exposure and the symptoms reported are noted but not really interpretable.

Jegaden D, Amann D, Simon JF, Habault M, Legoux B, Galopin P.

Study of the neurobehavioural toxicity of styrene at low levels of exposure.

Int Arch Occup Environ Hlth 1993; 64:527-31

AIM

To determine whether neurotoxicity occurs when exposure to styrene is less than 50 ppm.

DESIGN

Cross shift design of exposed workers and a non-exposed internal comparison group.

SUBJECTS

30 moulders exposed to styrene in a French naval dockyard were identified. A non-exposed group from the same company was chosen, matched on ethnic group, age, sex, and intellectual and social cultural level.

OUTCOME

Three tests were used administered by computer and apparently developed for this study. They were tests of simple reaction time to a visual stimulus, complex reac-

tion time to a visual stimulus and a digit span test requiring the subject to memorize a list of numbers presented by the computer.

EXPOSURE

The mean duration of exposure to styrene was 5 years (range 1-14 years). Exposure on the day of testing was measured using passive diffusion badges, urinary metabolites (mandelic acid and phenylglyoxylic acid) at the beginning and end of shift and area monitoring using mass spectrometer. Long term exposure in the workplace in general was estimated from urinary samples collected each Thursday evening over a 43 week period from six men randomly assigned each week.

In this study the mean exposure to styrene was 23 ppm on the day of testing (range 4-55 ppm). At the start of shift (on a Monday after two days away from exposure) the mean urinary metabolites (mandelic acid and phenylglyoxylic acid) was 37.6 mg/g creatinine in the morning. It was noted only seven of the 30 moulders had no urinary metabolites at this point. The increase in urinary metabolites during the day was strongly related to the measured level of environmental styrene.

The authors estimate that during the 43 weeks of routine testing the mean exposure had been approximately 30 ppm of styrene with 20% of the urinary determinations suggesting exposures >50 ppm.

ANALYSIS

Both the between group and within group tests scores were compared. An analysis of duration and level of exposure on the day of tests is also mentioned but no details are given of the acute dose response effect.

RESULTS

On the test of simple reaction time exposure subjects performed significantly less well than the control group both in the morning and evening. This was also found for the test of complex reaction time and on the memorization of listed numbers (digit span). It appears that no significant difference in the change over shift was observed. No relation was reported between test scores and length of exposure amongst the exposed group.

CONFOUNDING

The design included two parallel versions of the tests to minimize any effects of learning but nevertheless the subjects (both exposed and comparison) did better on the tests at the end of the day than in the morning. Although the authors say that they had carefully matched the comparison group on intellectual and sociocultural level, it is not explained how they did this and the adequacy of matching remains a question. Other exposures at work were determined but the concentration of methylene chloride, the only one mentioned, was said to be insignificant.

AUTHORS' CONCLUSIONS

The authors conclude that, at the levels investigated, there were no acute signs of neurotoxicity but that the lower performance in the morning on psychometric tests indicated chronic effects of exposure.

REVIEWER'S CONCLUSIONS

It appears that no cross shift effect is demonstrated on the three tests used. However the conclusions to be drawn about acute effects are limited. First, evidence of changes (or absence of them) in relation to measured exposure during the day are not given. This seems a strange omission. Second, the poorer test scores in the morning have not been fully evaluated. It would have been appropriate to report the relation between the start of shift tests and metabolites measured at the beginning of shift which were present for 23 of the 30 moulders, suggesting considerable exposure during the previous week. Finally the conclusion of chronic effects is not justified in the absence of this pre-shift analysis and of good data demonstrating that the exposed and comparison groups were well matched on important confounders.

Kolstad H, Juel K, Olsen J, Elsebeth L.

Exposure to styrene and chronic health effects: mortality and incidence of solid cancers in the Danish reinforced plastics industry.

Occup Environ Med 1995; 52:320-327.

AIM

To study the occurrence of non-malignant diseases and solid cancers in workers exposed to styrene in the Danish reinforced plastics industry.

DESIGN

Cohort mortality study.

SAMPLE

53,847 male workers employed between 1964-1988 in companies producing reinforced plastic and alive on January 1, 1970 were found by linkage to a Danish national pension plan. 2,944 workers were in companies with unknown production and were excluded. 36,610 workers from 386 reinforced plastic companies and 14,293 workers from 84 companies in similar industries but without styrene exposure were included.

EXPOSURE

No data on individual exposure or job titles was available. Concentrations of styrene exposure within the Danish reinforced plastics industry had been monitored since the early 1960's and was said to be around 180 ppm during the 1960's and 54 ppm during subsequent decades. For this study, 2 dealers of plastic raw materials reviewed the production and classified the 470 companies into those in which more than 50% of workers in the company would have been involved in producing reinforced plastic, those with less than 50% of workers involved in the production and those where no worker was involved. These were designated as having high exposure probability, low exposure probability or unexposed. Date of first employment in a relevant industry and the duration of employment were estimated from pension fund records.

OUTCOME

Vital status was determined from the national population registry and underlying cause and up to 2 contributory causes of death were established from the national cause of death register. Degenerative disorders of the nervous system were the outcome of interest. This group included multiple sclerosis, parkinsonism and motor neuron disease together with other non-malignant diseases of the brain.

ANALYSIS

Years at risk were calculated from the start of employment or from the first year the company produced reinforced plastic. Standardized mortality ratios were calculated by comparing observed number of deaths with the expected numbers using age and calendar specific national rates. Poisson regression was used to estimate mortality rate ratios (MRR) using internal comparisons with the rates for workers unexposed to styrene. These models included, as well as exposure probability, age, year of first employment, duration of employment and time since first employment.

RESULTS

In all there were 24 cases with an underlying cause of death attributed to degenerative disorders of the nervous system, and 60 where there was an underlying or contributing cause. Of these 60, 7 were multiple sclerosis, 24 parkinsonism, 24 other diseases of the brain and 5 motor neuron disease. In comparison with national rates the SMR for degenerative disorders of the nervous system for those in styrene exposed industries was 0.94 (0.56-1.50) and for the comparison group 0.82 (0.30-1.69). In the Poisson regression those with high exposure probability had a MRR of 1.8 (compared with the unexposed comparison group) with a 95% confidence interval 0.8-4.3. The MRR was higher in those with longer duration of employment and greater time since first employment.

CONFOUNDING

The Poisson regression was adjusted for age, duration and date of employment but no information was available on factors such as alcohol intake that may be an important effect modifier. Use of internal controls may have avoided confounding by socioeconomic factors. No account was taken of workplace exposures to other chemicals.

AUTHORS' CONCLUSION

Degenerative diseases of the nervous system occurred more often amongst workers with a high probability of exposure to styrene than in comparable non-exposed workers. These findings could easily be explained by chance, and the lack of information on exposure was an important limitation.

REVIEWER'S CONCLUSION

Although the confidence intervals reported do not rule out a chance finding, the consistency of the pattern of excess of degenerative disorders of the nervous system using internal comparisons is worthy of comment. The rather crude estimate of exposure by the division of com-

panies into those with low and high probability of exposure will certainly have led to misclassification which may have underestimated any true effect. The lack of information on confounding factors is a concern. The cohort is young, with less than 9% mortality, and it may be too soon to detect long term effects on degenerative disorders.

Letz R, Mahoney F, Hershman D, et al.

Neurobehavioral effects of acute styrene exposure in fiberglass boatbuilders.

Neutoxicology and Teratology 1990; 12: 665-668.

AIM

The study was designed to investigate the acute effects of styrene exposure using tests from the computerized Neurobehavioural Evaluation System.

DESIGN

This was a cross shift design comparing changes within individuals in the styrene exposed group with similar changes in the non-exposed internal comparison group. Testing was carried out at three points during the day, early morning, near mid-day and at the end of the work shift.

SUBJECTS

118 subjects were tested at five fibreglass boat building companies in New England. The results reported are on 105 males with relatively complete data and no history of head trauma. All fibreglass laminators in each company were asked to participate and other workers partially exposed or not exposed were also recruited.

OUTCOME

Three computerized tests were used, the continuous performance test (a measure of reaction time and accuracy), the hand-eye coordination test (evaluating manual dexterity and coordination) and the symbol digit substitution test which is based on the digit symbol test of the Weschler adult intelligence scale and measures speed of processing.

EXPOSURE

Duration of exposure was, on average, 4.6 (SD 6.7) years. Personal monitoring pumps were used to measure exposure in the breathing zone over the shift. Urinary mandelic acid was measured from a spot urine sample obtained near the end of the work shift. The geometric mean of styrene exposure was 13.5 ppm and of mandelic acid 164 mg/g creatinine.

ANALYSIS

Regression analyses were carried out to assess the effect of exposure (8 hr time weighted average for styrene), mandelic acid and dichotomized exposure (cut point 50 ppm of styrene) on performance on the test at the end of shift session. Within subject analysis (using the subject's morning performance as a covariate) was also carried out but is not reported in detail. The multiple regression included other confounders (age, education and exposure duration).

RESULTS

There was no relation between performance, at the end of the day, on the continuous performance test or hand-eye performance and exposure indices. The end of shift score on the symbol digit test was related to styrene ppm corrected for respirator use and to urinary mandelic acid concentration. The start of shift scores on any test were not related to duration of exposure.

CONFOUNDING

The design and analysis appears to have allowed appropriately for the main confounders on neurobehavioural testing, namely age, education and alcohol use. There is no discussion of other chemical exposures potentially present in the workplace. Learning effects are potentially a problem in cross shift studies and this question is not addressed but may be assumed to be the same for both groups.

AUTHORS' CONCLUSIONS

Some disruption of neurobehavioural performance is related to styrene exposure, with the bulk of this effect being those of exposure to >50 ppm.

REVIEWER'S CONCLUSIONS

Presentation of the data does not allow us reach any clear decision about the level at which effects of styrene exposure began to appear. It does seem likely, however, that the effects seen were indeed acute effects as the morning scores were unrelated to duration of exposure. Little can be concluded from this study other than that, with adequate numbers and an experienced research team acute exposure may affect performance on some tests.

Matikainen E, Forsman-Gronholm L, Pfaffli P, Juntunen J.

Nervous system effects of occupational exposure to styrene: a clinical and neurophysiological study.

Environmental Research 1993; 61:84-92.

AIM

To use quantitative spectral and frequency analysis of EEG to estimate the effects of styrene on the central nervous system.

DESIGN

Cross sectional study using internal comparisons and values in a normative database.

SUBJECTS

100 male workers were identified from workplaces where hygiene studies had previously been performed. The number of workplaces is not stated. Workers were brought to the Institute of Occupational Health in Helskinki for testing. It is not clear how recently they had last been exposed to styrene.

OUTCOME

The study reports results of EEG evaluation and of symptoms. No information is given for the way in which symptom information was collected. EEG was recorded from 19 channels and submitted to both visual and quantitative analyses. The quantitative analysis from each individual was compared with a normative database to determine whether the results were normal, abnormal or not classifiable.

EXPOSURES

The average duration of styrene exposure was 12.6 years with a range of 0.5-32 years. Individual measures were made of styrene concentrations in the breathing zone and of end of shift urinary metabolites. In addition an exposure index involving the number of years of exposure, the working method, the level of daily exposure and the time weighted mandelic acid levels was used, essentially to estimate cumulative exposure to examine chronic effects. At the time of testing the average eight hour time weighted average styrene concentration was 32 ppm but many of the workers had been exposed to much higher levels earlier in their employment. Styrene oxide, acetone and dust were also measured but these values are not included in the analysis.

ANALYSIS

The exposure index was grouped into three categories corresponding to low, moderate and high. In some analyses (Table 6) all three levels were used, with some only the low and high categories were contrasted (Table 4) and in others a different classification was adopted (Table 3).

RESULTS

Headaches, memory disturbance, forgetfulness, sensory symptoms in the hands and feet and excess sweating were reported more commonly in those with higher exposure. Workers with higher exposure had a higher mean total EEG power than those with low exposure and this was most marked in the frontal and temporal regions. In comparison to the normative database the quantitative EEG was classified, in a neurometric analysis, as normal in 28 cases, abnormal in 30 cases and not classifiable in 31 cases. Abnormal cases had a somewhat higher level of urinary mandelic acid and environmental styrene concentration but levels of statistical significance are not given. Those in the highest exposure index group appeared less likely to have a normal EEG using this approach.

CONFOUNDING

Age and alcohol consumption were discussed as potential confounders and it was concluded that there was no difference on these factors in the three exposure groups. No formal adjustment was reported.

AUTHORS' CONCLUSIONS

The authors conclude, although the data is not shown in the paper, that the number of abnormal EEGs was not increased when visual inspection was used and there was no correlation between the number of abnormal EEGs and exposure. They conclude that quantitative analysis showed consistent, although with slight changes in the more highly exposed group, but that the changes found did not suggest increased cerebral disease in the workers.

REVIEWER'S CONCLUSIONS

This paper attempts to measure effects of chronic exposure. There are some problems however. The authors conclude that these effects are seen at relatively low exposure but many of the workers had been exposed for years and will have had a history of much higher exposure in the past. It is not clear how much time had been allowed from last exposure, and unless this was several days, the effects seen might be ones that were acute and reversible. The marginally positive result depends on comparison of these data to a normative database, but there is no evidence that the information collected for this study and for the database was collected in the same way. In particular, it required a technician to determine levels of wakefulness and the degree of artefact, and to choose those parts of the EEG that were felt to be non-artifactual. The presentation of data by different groupings of the exposure index (rather than by the three groups initially defined) suggests that any effect was very slight and could only be demonstrated by careful choice of the way in which data was presented.

The authors make only very modest claims for the relation between EEG and styrene exposure, and that is appropriate.

Mergler D, Huel G, Belanger S, Bowler RM, Truchon G, Drolet D.

Surveillance of early neurotoxic dysfunction.

Neurotoxicology 1996; 17:803-12.

AIM

The objective was to examine nervous system functioning prior to and following workplace interventions.

DESIGN

A cohort study in which both workplaces and individual workers form the unit of analysis. The study was designed to assess the effect of interventions to reduce exposures.

SUBJECTS

Three workplaces involving styrene exposure in the manufacture of reinforced plastics were studied twice, two years apart. 118 workers completed the neurobehavioural test battery and exposure assessment at time 0, and 75 of these were still employed at one of the three plants two years later. 57 of the 75 agreed to repeat testing although environmental measures and urine samples were obtained for all 75.

OUTCOMES

A large number of tests were carried out. These were selected from the neurobehavioural core test battery supplemented by three tests of color vision, contrast sensitivity and olfactory threshold selected from a battery of tests developed by the authors. The battery included the profile of mood states which gives scores on six dimensions and a symptom checklist which is not described.

EXPOSURES

Exposures were measured at time 0 and time 1 using passive dosimeters for a total of eight hours and an eight hour time weighted average was determined. End of shift urine samples were collected on the same day as the environmental exposure measurement and mandelic acid corrected for creatinine was determined. Workers included in both assessments had been employed for a mean of 6 years at time 0 (8 years at repeat testing). The median exposure at time 1 was 75 mg/m^3 and at time 2 76 mg/m^3 for these 57 workers. It is reported that at one plant (plant 3) the levels of styrene and mandelic acid were significantly reduced between baseline and follow-up but that there was no change at the other two plants. Median levels broken down by plant are not given in the paper.

ANALYSIS

The analysis focused on changes between baseline and follow-up for the entire population, for plant 3 (where there had been improvements) compared with the other two plants and for individual changes in relation to end

of shift mandelic acid concentrations. Paired t-tests were used to compare mood and Spearman's rho to compare trends, that is changes in mandelic acid concentrations compared with changes in test score.

RESULTS

Overall the 57 subjects improved their scores on the digit span and digit symbol tests and did less well on the contrast sensitivity test. No overall difference was found on any of the other behavioural or mood scales. Workers at the plant in which improvements had been made did better than the other two plants on a test of manual dexterity (Santa Ana), on the digit span and digit symbol tests, and were less likely to report anger and more likely to report vigor. When the 57 workers were grouped according to changes in their mandelic acid levels (increased, the same or decreased) over the two years a significant relation was found with color vision, simple reaction time, digit symbol forward and tension and fatigue on the mood scale. There was also some tendency toward an increase in number of reported symptoms for those who mandelic acid concentrations were higher on the second test.

CONFOUNDING

With each subject used as their own control in the analysis between mandelic acid and performance tests, confounding is of less importance. The authors suggest that for some tests changes in conditions of testing may have been important and these would be confounded with time of testing. If learning is important, that would be demonstrated by the increase in score over time 2 compared with time 1, while the population will, by definition, have aged two years.

AUTHORS' CONCLUSIONS

The authors conclude the most striking result of the study is an association between the differences in mandelic acid and outcomes. They note that on some tests this was due to deterioration in those whose mandelic acid levels had increased rather than improvement in those with decreased levels, but nevertheless suggest that on some functions, particularly color vision, effects appear to be reversible. They suggest that a protocol of this kind may be useful for ongoing surveillance of groups of exposed workers as part of a program of preventative intervention.

REVIEWER'S CONCLUSIONS

This is an important study in that it appears to show the reversibility of neurobehavioural effects associated with styrene exposure. As with many papers the information given is not complete and it would have been interesting to know more about those who had left the plant (were they those with the highest abnormal scores?) and to have known something about the baseline levels of mandelic acid in these three groups (increased, same and decreased). Though the illustration of this data in Figure 3 is very clear, it would have been useful to have had a more rigorous analysis with initial level of mandelic acid as a covariate. The large number of tests carried out (20 scores are reported) raises the usual problems of interpretation. Here, for example, improvement was seen on two tests and deterioration on one; it is not clear which of these may have been due to chance. The testing was done "at least 12 hours after last exposure" but probably with too short a delay to rule out acute effect of recent exposure.

Murata K, Araki S, Yokoyama K.

Assessment of the peripheral, central and autonomic nervous system function in styrene workers.

Am J Ind Med 1991; 20: 775-784.

Yokoyama K, Araki S, Murata K.

Effects of low level styrene exposure on psychological performance in FRP boat laminating workers.

Neurotoxicology 1992; 13:551-556.

These two papers report results from the same group of 12 styrene exposed workers, with Murata et al. giving details of neurophysiological measurement and Yokoyama et al. of behavioural testing. Although different comparison groups were used in the two papers, it is appropriate to include them in a single review.

AIM

The study aims to assess the effects of styrene exposure on peripheral, central and autonomic nervous function (Murata et al.) and on performance tests (particularly on a test of picture completion from the Wechsler adult intelligence scale) (Yokoyama et al.).

DESIGN

This was a cross sectional study using an external non-exposed comparison group.

SUBJECTS

The study involved all 12 workers from a single reinforced fibreglass factory manufacturing boats in Japan. One subject, with sinus arrhythmia, was excluded from the neurophysiological studies but included in the performance testing. For the neurophysiological measures a comparison group was chosen from men seen at a regional health clinic. They were healthy volunteers matched ±2 years to the age of the exposed men. Testing of this group was done over a period of two years following the workplace testing of exposed workers. For the behavioural testing a comparison group was taken of 11 male workers in a steel factory in the same area.

OUTCOMES

Peripheral nerve function was assessed using sensory and motor nerve conduction velocity, measured in the median nerve. Using a method developed by this group, the dispersion of conduction velocities was also calculated. This was done by determining, for exposed and control subjects separately, the nerve conduction velocity associated with successive proportions of active nerve fibres. In addition somatosensory evoked potentials were measured by stimulating the right median nerve at the wrist and recording latencies at four points, with the interpeak latencies representing conduction times in the spinal cord and brain (a measure of central nervous system functioning). Autonomic function was measured by the coefficient of variation of the R-R interval on ECG recordings. For the performance testing three measures were used, the picture completion and digit symbol tests from the WAIS and the long form of the Maudsley Personnel Inventory, giving scores on scales of extroversion and neuroticism and on a lie scale.

EXPOSURES

The mean duration of exposed workers was 5 years with a range of 0.5-9 years. Air sampling was carried out three months before and two months after the testing, using area sampling. The styrene concentration was estimated to be 26 ppm (range 1-77) before and 14 ppm (range 6-46) after testing. Urinary metabolites were measured at the end of the day on which testing (neurophysiological and neurobehavioral) had occurred. Mean concentrations of mandelic acid were 169 mg/g creatinine and phenylglyoxylic acid 277 mg/g creatinine. These were estimated to be equivalent to a mean exposure of 22 ppm of styrene in air.

ANALYSIS

Test results for cases were compared for those with control subjects using paired sample t-tests in the neurophysiological study and non-paired t-tests for the neurobehavioral study. Pearson correlations were calculated to examine whether there was a dose response between urinary metabolites and test score.

RESULTS

The distribution of nerve conduction velocities were interpreted as showing that the distribution of fibres was shifted towards the slower velocities in the styrene exposed group when compared with the controls. Sensory conduction velocity of the median nerve was slower in the exposed workers and there was less variation in R-R interval. No significant difference was found on motor nerve conduction velocity, on somatosensory evoked potential or on heart rate. It is reported, although no detail is given, that there was no relation between urinary metabolites and any of the neurophysiological test scores. On the behavioural measures of styrene workers there were no differences between the exposed and comparison groups on the digit symbol test or on the personality inventory but the exposed group had fewer correct responses on the picture completion test. No detail was given of the dose-response analysis but it was commented that no significant correlation was observed between scores on the picture completion test and urinary metabolites.

CONFOUNDING

In the neurophysiological study, in which workers were matched on age to non-exposed controls, differences between the groups on height, tobacco, alcohol and age were reported not to be significant and these factors were not included in the analysis. In the neurobehavioural testing part of the study the controls were somewhat older, less educated and drank less alcohol than the styrene workers but none of these differences was significant. However the values for age (with styrene workers being younger) and length of education (longer in styrene worker) would both have resulted in more favourable test results and it is of note that adjustment for these factors increased the estimated difference between the two

groups. Amongst other chemical exposures were acetone (mean 9 ppm) and methyl ethyl ketone (mean 1 ppm) but these were not included as confounders in the analysis.

AUTHORS' CONCLUSIONS

Murata et al. conclude that styrene affects the peripheral nerves, particularly the fast nerve fibres in the large myelinated nerves and that it causes significant dysfunction of the autonomic nervous system. Yokoyama et al. conclude that performance on the picture completion test was significantly and adversely affected but that effect (as reflected in MPI scores) was not.

REVIEWER'S CONCLUSIONS

These two papers have weaknesses in aspects of power, potential bias and interpretation. First the number of subjects was very small (11 in the neurological study) and did not have the power to reach conclusions of adverse effect. Measurement of controls in a different environment and, for the neurophysiological studies, over a very different period, gave the possibilities of systematic errors including those of instrumentation, reporting and observation. The interpretation of both studies is difficult, particularly in addressing the question of chronic or acute effects. It appears that the testing was done (of the exposed workers) on a mid-week working day when, particularly for the performance testing, acute exposures might be expected to affect performance. A lack of any difference in "effect" is not surprising as the test they used is one reflecting long-term personality traits rather than acute response to circumstances.

The attempt to determine whether fast or slow fibres are principally involved in peripheral effects of styrene was interesting but these reviewers have no experience with this testing and cannot assess its validity.

Pierce CH, Becker CE, Tozer TN, Owen DJ, So Y.

Modeling the acute neurotoxicity of styrene.

J Occup Environ Med 1998; 40: 230- 240.

AIM

To test the effects of four different exposure regimes on neuro-physiological and neuro-behavioural tests in normal, healthy volunteers. A secondary aim was to investigate the relationship between the exposure regimes and blood styrene concentration.

DESIGN

Experimental design, using an exposure chamber to compare the effects of four different exposure regimes. Neurological testing was performed prior to exposure, after 35 minutes of exposure (2 and 7 test) and at the end of each 100 minute period of exposure (P300 test).

SUBJECTS

Four healthy male subjects aged 26-30. None had any significant history of exposure to solvents, smoked cigarettes, took any medication, or drank more than 18g of alcohol per day.

OUTCOMES

Blood styrene was measured once before, six times during, and four times after each exposure regimen via an indwelling catheter placed in the ante-cubital vein prior to each experiment. The latency and amplitude of a P300 potential was measured using scalp electrodes following an auditory stimulus and used as a measure of neurophysiological function. A '2' and '7' recognition test, identifying '2's and '7's from a series of random digits, was used to test neurological function.

EXPOSURES

Subjects were exposed to styrene in a 13.8m³ stainless steel exposure chamber. Exposure regimens comprised:
Day 1: 100mins at 15ppm, 100mins at 32.5ppm, 100mins at 50ppm.
Day 2: 100mins at 50ppm, 100mins at 75ppm, 100mins at 99ppm.
Day 3: fluctuating exposure level between 10-150 ppm for 4 hours.
Day 4: constant exposure to 10ppm followed by highly variable auto-correlated period of exposure between 5-200ppm for 4 hours.

Three subjects (A, B and C) completed the exposure regimens for days 1 and 2. Two subjects (A and D) completed the exposure regimens for days 3 and 4.

Days 1 and 2 were consecutive days. There was an interval of several weeks between days 2 and 3, and days 3 and 4 were consecutive days.

ANALYSIS

A partial correlation coefficient using a t statistic was used to measure the association between exposure and the results of the 2 and 7 recognition test. A test of Kendall rank correlation was used to evaluate the relationship between observed toxicity (presumably the P300 test although this is not stated) and four measures of exposure: peak exposure; duration of exposure; product of average exposure level x duration (i.e. cumulative dose); and modelled peak brain styrene concentration.

RESULTS

No change in the P300 test amplitude or latency was observed. Scores for the 2 and 7 recognition test improved (presumably due to increased familiarity with the test) between days one and two but showed no other significant change.

CONFOUNDING

The before/after design reduced the opportunities for confounding.

AUTHORS' CONCLUSIONS

The authors conclude that exposure at the ACGIH TLV of 20ppm would protect workers from acute styrene toxicity, whereas the OSHA PEL of 100ppm would not protect exposed workers.

REVIEWER'S CONCLUSIONS

It is difficult to interpret these findings meaningfully in terms of workplace exposures. Exposure in an exposure chamber is unlike exposure in a work environment where exercise and environmental conditions may affect the absorbed dose. The experimental data for four subjects provides no evidence that short exposure to concentrations up to 150ppm has adverse effects on the nervous system.

Ska B, Vyskocil A, Tardif R, Carrier G, Thuot R, Murray K, Viau C.

Effects of peak concentrations on the neurotoxicity of styrene in volunteers.

Human and Experimental Toxicology 2003; 22: 407-415.

AIM

To identify acute neurotoxic effects associated with short term exposure to current occupational exposure limits in Quebec (213mg/m^3 (50ppm) 8 hour TWA and 426mg/m^3 (100ppm) 15min average)

DESIGN

Experimental design, using an exposure chamber to compare the effects of five different exposure regimes.

SUBJECTS

42 healthy men aged 20-50 years. None had previous exposure to styrene or other neurotoxic agents although 5 had had occasional exposure to paint solvents. All were self-reported 'social' consumers of alcohol.

OUTCOME

Three groups of tests were used to assess neurotoxic outcomes:

- Sensory: Colour vision measured using a Lanthony D-15 desaturated panel; a vision contrast test; and an olfactory threshold test.
- Neurobehavioural: simple reaction time; colour word stress test; symbol digit matching test; digit span memory test; and continuous tracking test.
- A questionnaire to assess mood and symptoms. Symptoms included local irritation and central nervous system effects.

EXPOSURES

Five exposure regimes each of 6 hours duration were used:

- Constant 106mg/m^3;
- Variable exposure, mean concentration 106 mg/m^3 but with four 15min peaks up to 213mg/m^3;
- Constant 213mg/m^3;
- Variable exposure, mean concentration 213 mg/m^3

(50ppm) but with peaks up to 426mg/m³ (100ppm);
■ Two stable low-level exposure to 5mg/m³. Used as a 'null exposure' for physiological effects but with a detectable odour.

The test exposures were carried out in a constant sequence.

ANALYSIS

Multivariate analysis of variance techniques were used to evaluate the effects of exposure on outcome measures incorporating three factors: moment of measurement; type of exposure; and mean styrene concentration.

RESULTS

Only results for subjects completing all exposures were included in analyses (24 of 42 initial subjects). No significant effect of exposure on sensory tests was noted. In the neurobehavioual tests subjects had a faster reaction time on the colour word stress test at the end of the day regardless of exposure, although accuracy remained unchanged; subjects had a faster response time on the symbol digit matching test after exposure compared with before, and as they progressed through sessions; and subjects remembered a higher number of digits on the digit span memory test as they progressed through sessions. No other significant effect was noted. No significant effect of exposure on mood or symptoms was reported.

CONFOUNDING

The before/after design reduced the opportunities for confounding. There is little comment on the subjects who did not complete testing. It is not stated why these individuals discontinued their participation and it is not known whether this might have biased the results towards the null. Period and order effects were not considered in the analyses.

AUTHORS' CONCLUSIONS

Short term exposures up to 213mg/m³ (50ppm) with peaks up to 426 mg/m³ (100ppm) did not induce neurotoxic effects. Caution should be exercised in extrapolating these results to occupational exposure situations.

REVIEWER'S CONCLUSION

This is a relatively large and well conducted experiment but its extrapolation to the workplace is problematical. Bio-available doses will have been lower for a given exposure in the exposure chamber as subjects were at rest. In addition, these exposures were relatively short term. Nonetheless, this study is reassuring about the acute effects of these levels of exposure in naïve individuals.

Stetkarova I, Urban P, Prochazka B, Lukas E.

Somatosensory evoked potentials in workers exposed to toluene and styrene.

Br J Ind Med 1993; 50:520-527

AIM

To detect possible sub-clinical impairment in the somatosensory pathway in workers exposed to styrene.

DESIGN

Cross sectional study with an external non-exposed comparison group and using normative reference data.

SUBJECTS

20 workers exposed to styrene (5 men and 15 women) in a factory making glass laminates. The comparison group was 13 male and 40 female volunteers without any known exposure to any neurotoxic agents. No information is given on how these were identified.

OUTCOME

Somatosensory evoked potentials were measured using electrical stimuli delivered to the right median nerve at the wrist and the right tibial nerve at the ankle. Peripheral, cervical and cortical sensory evoked potentials were measured after stimulation at the wrist and spinal and cortical SEPs after stimulation to the tibial nerve. Results of the sensory evoked potential measurements were considered abnormal if any latency conduction time or conduction velocity exceeded the 95% reference range. Abnormal SEPs were divided into three categories, peripheral, central or a combination of the two.

EXPOSURES

Exposed subjects had a mean exposure for 11 years with

85% exposed for more than 5 years with a maximum of 22 years. For the period from 1980-1989 the styrene concentration in air ranged from 140-570 mg/m³.

ANALYSIS

Mean values in the outcome variable in the exposed and comparison groups were examined using t-tests with multiple regression to adjust for sex, age and height.

RESULTS

Six women were determined to have abnormal sensory evoked potentials, two of the peripheral type, two central and two with impairment on all SEP pathways. In a table showing the relation of abnormal SEPs to exposure eight abnormal cases (including males) are included with a disproportionately high (not statistically significant) number in those with longer exposure duration. The 15 women exposed to styrene had significantly slower peripheral conduction velocity and reduced latencies compared with the comparison group.

CONFOUNDING

The methods section discusses use of multiple regression in the examination of latency but it is not clear whether these were used in the final analysis. Drinking habit was considered but the values in the exposed group were very similar to those in the comparison.

AUTHORS' CONCLUSIONS

The study shows evidence of functional impairment of all somatosensory pathways indicative of potential toxic polyneuropathy, myelopathy or encephalopathy due to chronic exposure to neurotoxic agents.

REVIEWER'S CONCLUSION

Although there are some problems in understanding some aspects of this study (for example the choice of comparison group or the decision to exclude male styrene workers from the analysis) it does not appear to be intrinsically flawed. The use of somatosensory evoked potentials is not open to manipulation by the subject. It would have been helpful however to have had a clearer description of how the proportion of abnormal potentials in the exposed group compared to those in the comparison group; no data on these differences in the comparison group are given.

Tsai S, Chen J.

Neurobehavioral effects of occupational exposure to low-level styrene.

Neurotoxicology and Teratology 1996; 18:463-469.

AIM

To evaluate possible adverse effects of low level styrene exposure on central and peripheral nervous systems in fibreglass reinforced plastics workers.

DESIGN

This was a cross sectional study with an internal non-exposed comparison group.

SUBJECTS

Workers in six manufacturing plants in Taiwan. 86 of 117 were found to be eligible, could perform the testing and were enrolled.

OUTCOMES

Symptom information was collected using the Swedish Q16 symptom questionnaire. Tests of vibration and thermal threshold were carried out, with vibration measured on the distal phalanx of the left index finger and the big toe, and the thermal test on the left forearm. A number of tests from the neurobehavioural evaluation system were adapted for the Chinese culture and language. They included the continuous performance test, associate learning, symbol digit test, pattern comparison, pattern memory, visual digit span, switching attention, and associate delayed recognition. In addition, information on mood scales was collected and a vocabulary test was completed.

EXPOSURES

The mean duration of employment was 8.3 years in those with direct exposure and 8.4 years in those with low or no exposure. Personal and area sampling was carried out to assess levels of styrene and other chemicals in the workplace. The styrene measures were used to divide the population into those with none or indirect exposure (mean styrene concentration 1.0 ppm) and those with direct exposure including layering, cementing and spray-

ing for whom the mean styrene concentration was 21.9 ppm. Acetone and methyl ethyl acetone were detected rarely and at very low levels.

ANALYSIS

Multiple regression was used to adjust for possible confounding and the Bonferroni Inequality used to take account of multiple comparisons.

RESULTS

No difference was found in either acute or chronic symptoms between the low and higher exposure groups. On behavioural testing the scores on the continuous performance test were significantly lower in those with more exposure, having taken account of the effects of age, sex, educational level and alcohol consumption. The vibration perception threshold was higher in both the hand and the foot for the exposed but no significant difference was seen on the thermal threshold.

AUTHORS' CONCLUSIONS

The authors conclude that acute symptoms are not found below 25 ppm. They note that chronic symptoms were not significantly related to exposure in this study but there was a tendency for those with high exposure to be more likely to complain of chronic symptoms. They note that having allowed for confounders the continuous performance test was significantly and adversely affected and feel this result agrees with a consensus that reaction time seems to be the most prominent effect of styrene exposure. They interpret the higher threshold for vibration as indicating that styrene affects the function of faster myelinated fibers. They conclude by saying that subtle but significant changes involving neurobehavioural performance and peripheral nervous function were detected in workers exposed to styrene at a mean dose of 22 ppm.

REVIEWER'S CONCLUSIONS

Although workers were tested away from the factory, the time away from exposure may have been for as little as 16 hours and it is not certain that the effects involved, for example on the continuous performance test, were due to chronic rather than acute effects of the substance. While this paper shows the generally weak design of comparing exposed with unexposed workers, where issues of bias and confounding are difficult to overcome, in this instance all were working in the fibreglass factories and the table showing confounding does indeed suggest that they were rather similar (except, perhaps, for years of education which is about 10 months longer in the less exposed). There is a comment that when the analyses were repeated using the vocabulary test instead of years of education, the results for the continuous performance test was marginally less significant (p=0.06). Vibration perception has been used rather little in styrene workers and has the disadvantage it may be affected by a low motivation to perform well. However it is of interest that the vibration threshold but not the thermal threshold is considerably different in exposed and non-exposed, suggesting that such motivational factors may not be very important.

Viaene M, Veulemans H, Masschelein R.

Experience with a vocabulary test for workers previously and still exposed to styrene.

Scand J Work Environ Hlth 1998; 24:308-311.

Viaene M, Pauwels W, Veulemans H, et al.

Neurobehavioral changes and persistence of complaints in workers exposed to styrene in a polyester boat building plant: influence of exposure characteristics and microsomal epoxide hydrolase phenotype.

Occup Environ Med 2001; 58:103-112.

These two papers present data from the same study and are considered together below.

AIM

The earlier paper seeks to explain, in terms of styrene exposure, an observed difference in vocabulary score between present and current workers. The later paper has three aims, to compare symptoms and behavioural test scores in workers who have been exposed to styrene and comparison workers who have not; to assess the relation between symptoms and test scores and industrial hygiene data in the exposed group; to examine whether there was a link between microsomal epoxide hydrolase (mEH) and test performance in workers exposed to styrene.

DESIGN

This was a mixture of a cohort and cross sectional designs. To enter the cohort workers had to have been employed in a single boat building plant sometime between 1984-1987. A comparison group was chosen from amongst people working in another plant, without exposure, at the time of follow-up. Although exposure measurements had been made in 1984-1987, all outcome data was collected at a single point, from 1992-1993. Blood for phenotyping was collected in 1994-1995.

SUBJECTS

185 workers who had been previously involved in exposure studies of styrene were traced and of these 117 participated. A comparison group of 64 non-exposed workers were recruited from a possible 111.

EXPOSURE

Detailed hygiene studies had been carried out in plant by the research group in 1984-1987. Individual records were kept for every worker on the tasks he carried out and this permitted calculation of the exact number of hours of exposure to styrene from 1982-1989 for formerly exposed workers (who were laid off at this point) and to 1993 for current workers. Currently exposed workers (n=27) had an average of 4700 hours of lamination compared with 3610 hours of lamination in the formerly exposed (n=90). The mean time weighted exposure for currently exposed workers was only 68 mg/m^3 and for formerly exposed 17 mg/m^3. Concentrations while laminating were 148 mg/m^3 and 157 mg/m^3 respectively.

OUTCOME

Behavioural testing included, as reported in the earlier paper, the Dutch version of the vocabulary test of the Neurobehavioural Examination System and, as reported in the later paper, performance tests from the same battery; namely, hand-eye coordination, simple reaction time, symbol digit substitution, associated learning, associated recall, digit span and color word vigilance. The subjects completed a 60 item questionnaire (the neurotoxicity symptom checklist) and were interviewed about their memory of complaints while they were at work with styrene and, if never exposed, in their present job.

ANALYSIS

In the earlier paper, vocabulary score was related to duration of exposure, having adjusted for years of education and age, in the group as a whole and in those previously exposed compared to those currently exposed. In the second paper, where the comparison group is included, comparisons were made between scores in those currently exposed, those formerly exposed and the comparison group. The statistical analysis included one-way analysis of variance, Fisher's exact test, multiple linear regression and multiple logistic regression.

RESULTS

The earlier paper showed a significant relation between vocabulary score and duration of exposure that appeared to be similar for those still employed and those formerly employed. No relation was found with level of exposure. The vocabulary score of the still exposed group was significantly lower than that of those who had been laid off three years earlier. In the later paper scores on the symbol digit substitution, digit span forward and hand-eye coordination (in former employees) appeared to be worse than those for the comparison group. The information given in the text does not appear to be identical to that in the tables but the text has been given priority. The exposed workers (including those currently exposed) remembered having more complaints at work than the comparison group did, and that diminished taste sensitivity persisted to the time at which the inquiry was carried out. Full details are not given of the results of the 60 item questionnaire but it appears that formerly exposed workers had worse scores than currently exposed. In a model including duration of exposure those with higher mEH (the faster metabolizers) did worse on the symbol digit substitution test, on the color word vigilance test and on the digit span test.

CONFOUNDING

Substantial differences were found in the educational level of currently exposed (the lowest), formerly exposed (intermediate) and comparison group (highest) educational level. This is a serious problem (see below). Other comparisons made included alcohol consumption, hours slept at night, smoking, and motivation. No statistical data are given but these appear to be very similar. No mention is made of any other chemicals that may have been present in the work environment.

AUTHORS' CONCLUSIONS

In the earlier paper, the authors conclude that the use of short vocabulary tests to estimate pre-morbid capacity (as "hold tests") may be limited as they are affected by exposure. In the second paper, the authors conclude that some subjective symptoms or complaints and neurobehavioural effects in workers were persistent even at TWA exposure concentrations thought to be safe in the 1980s. The results suggest that less than 10 years of exposure to a mean concentration of styrene of 155 mg/m^3 may result in persistent neurotoxic effects.

REVIEWER'S CONCLUSIONS

This study has many problems. First, the design is very weak. The collection of symptom complaints from a group of workers who had been laid off for three years, and for whom the method of parting may not have been ideal, raises serious questions of interpretation. As in almost all symptom studies, the use of an unexposed comparison group does not permit any firm conclusions. As such, the symptom data in these papers is not very useful. The data from behavioural testing is also difficult to interpret. First there is the problem of the earlier paper in which duration of exposure was seen to be associated with vocabulary score. As might be expected, vocabulary score is related to years of education in this study. Amongst the former workers those who agreed to take part had a higher educational level than those still employed, but as is apparent from the second paper, those who refused to participate had a much lower one. Years of education was also negatively correlated with duration of exposure (not significantly). In interpreting this entire study it is critical to decide whether the relation between duration of exposure and vocabulary score is due to inadequate adjustment for confounding or is a real effect. The authors conclude it is real, but it is somewhat strange that they make no mention in the second paper of the fact they have this vocabulary test data. It would have been interesting to know if adjusting for vocabulary score would have removed all the neurobehavioural differences described.

Viaene M, Pauwels W, Veulemans H, et al.

Neurobehavioral changes and persistence of complaints in workers exposed to styrene in a polyester boat building plant: influence of exposure characteristics and microsomal epoxide hydrolase phenotype.

Occup Environ Med 2001; 58:103-112.

See Viaene M, Veulemans H, Masschelein R.

Experience with a vocabulary test for workers previously and still exposed to styrene.

Scand J Work Environ Hlth 1998; 24:308-311.

Welp E, Kogevinas M, Andersen A, Bellander T, Biocca M, Coggon D, Esteve J, Gennaro V, Kolstad H, Lundberg I, Lynge E, Partanen T, Spence A, Boffetta P, Ferro G, Saracci R.

Exposure to styrene and mortality from nervous system diseases and mental disorders.

Am J Epidemiol 1996; 144:623-33.

AIM

To examine mortality from diseases of the nervous system, mental disorders and suicide in relation to styrene exposure.

DESIGN

Cohort mortality study.

SAMPLE

41,167 workers at 660 reinforced plastic plants were identified from 6 European countries. Those with no styrene exposure or with unknown date of birth, date of first employment or gender were excluded, leaving 35,443. Of these 44.8% were from Denmark, 20.9% from the UK, 15.2% from Italy, 8.5% from Sweden, 5.9% from Finland and 4.8% from Norway. Most of the workers were believed to be in laminating tasks.

EXPOSURE

An exposure matrix was constructed by country and calendar period using job title, type of product and method of production. The methods used were not clearly stated in the paper but it appears that a database was created using personal exposure measurement and urinary metabolites, with extensive data before 1970 only being available for Denmark. Occupational records were obtained from payroll data at each plant or, in Denmark, from the national pension scheme. Exposure levels were thought to have decreased from around 200 ppm before 1970 to around 40 ppm by 1990.

The time from first exposure, cumulative exposure, average exposure, and duration of exposure were calculated.

OUTCOMES

No description is given of the method of determining vital status or cause of death. Causes of death of particular interest were mental disorders, diseases of the nervous system and suicide. For internal comparisons a more detailed examination of cause of death was possible.

ANALYSIS

Standardized mortality ratios were calculated using national mortality rates by sex, age and calendar period. The follow-up for the mortality study started at the first exposure to styrene or the first date from which complete payroll information was available. The period of follow-up differed between countries with the longest being 1945-1990 in the UK and the shortest 1970-1990 in Denmark. A total of 446,784 person years with an average of 12.6 years of follow-up was available for the external analysis and 405,967 with an average of 12.4 years for the internal comparisons. Poisson regressions were used for the internal comparisons with the pattern of the dose response for duration and cumulative exposure being examined using more complex regression models.

RESULTS

The external comparison showed no excess from all causes (SMR=0.96, 95% CI 0.92-1.00) from mental disorders (SMR=1.01, 95% CI 0.62-1.54) or from disease of the nervous system (SMR=0.76, 95% CI 0.51-1.09). There was an excess of external causes of death (SMR=1.13, 95% CI 1.02-1.25) which was reflected in an increased rate of suicide (1.10) and other violent causes (1.17). For the internal comparisons 27 of the 30 deaths from diseases of the nervous system were analysed in relation to time since first exposure, duration, average exposure and cumulative exposure having adjusted for country, age, calendar year and sex. Trends were observed by each of these exposure variables, with significant trends being seen for duration of exposure and cumulative exposure. Consistent trends with exposure parameters were also seen for sub-groups of epilepsy (N=7) and for the 11 deaths from degenerative nervous system diseases, of which 7 may have been ALS. Both mental disorders and suicide were more frequently seen in those with exposure duration of less than one year.

CONFOUNDING

The internal comparisons adjusted for country, age, calendar year and sex but no information was available on other workplace or lifestyle confounders.

AUTHORS' CONCLUSION

Mortality from central nervous system diseases and especially those from epilepsy increased with exposure to styrene. They comment that selection out of employment by sick individuals will have attenuated the dose response relationships. They also point out that, because of the small numbers, the results must be treated with caution.

REVIEWER'S CONCLUSIONS

Although the numbers of cases overall, and for epilepsy in particular, are very small, the pattern of increased risk with duration and cumulative exposure is suggestive of some risk associated with styrene. However the estimates must be considered very unstable and this follow-up of a cohort is probably too soon, with less than 7% of deaths within the cohort to date, to truly evaluate whether styrene was related to degenerative disease of the nervous system. In any further follow-up the lack of information on confounding by lifestyle factors might be an important limitation.

Wong O, Trent LS.

Mortality from nonmalignant diseases of the respiratory, genitourinary and nervous systems among workers exposed to styrene in the reinforced plastics and composites industry in the United States.

Scand J Work Environ Hlth 1999; 25:317-25.

AIM

To determine mortality from diseases of the nervous system and other diseases in a US cohort of styrene exposed workers.

DESIGN

Cohort mortality study.

SUBJECTS

The cohort consisted of 15,826 styrene exposed workers in 30 plants with inclusion limited to those who had worked in areas with styrene exposure for at least six months between 1 January 1948 and 31 December 1977.

EXPOSURE

A job exposure matrix had been established by a team of occupational hygienists who visited each plant around 1980. This was based on job titles (reduced to a list of 19 job categories) and history of job functions, work practices, past industrial hygiene measurements, process changes, engineering control and personal protective equipment in use. Levels of styrene were measured in 1980. These were used to produce a cumulative exposure in ppm-years. In addition 6 process categories were created. Typical exposures were low, with the majority of jobs being estimated to have exposure of 10 ppm or less. Exposure information was truncated in 1977, at the end of the initial study.

OUTCOME

Vital status at 31 December 1989 was determined by all means available. This information was used to obtain death certificates from individual state health departments. Analysis against external referents used US national age-gender-year specific death rates to compute cause-specific standardized mortality ratios. Because race was not generally available, the entire cohort was assumed to be white. Internal analyses were performed using Cox's proportional hazards analyses.

RESULTS

In the SMR analysis no excess was found for diseases of the nervous system with only 14 (all male) deaths (2 from epilepsy); indeed the SMR 0.56 (95% CI 0.51-0.95) suggested a deficit of such disease. When these 14 cases were divided into time since first exposure there was an increase in SMR from 0.10 for those with less than 10 years from first exposure to 0.68 for those exposed 10-19 years and 1.13 for those exposed for more than 20 years. When a similar analysis was carried out by duration of exposure the highest SMR (1.03) for diseases of the nervous system was seen for deaths in those who had a duration of more than 10 years, the highest exposure category. For cumulative and average exposure, the highest SMR was for intermediate categories. When the deaths were broken down by industrial process no death from diseases of the nervous system was seen for those in the open mould process or mixed and closed mould processes, where exposure would be expected to be highest. A Cox regression with age and cumulative exposure as independent variables showed no relation to diseases of the nervous system. A further analysis including age, cumulative exposure, duration and average intensity also showed no excess risk for this outcome.

CONFOUNDING

The Cox regression adjusted for age and gender and the SMR for era. Information was not available for lifestyle factors that may have acted as effect modifiers (particularly alcohol). Computation of SMRs by major process categories might have allowed identification of other workplace exposures that were acting as confounders.

AUTHOR'S CONCLUSIONS

The authors conclude there was no increased mortality from diseases of the nervous system in general or from epilepsy in particular and that no pattern of mortality was found in relation to styrene exposure. They go on to comment that the levels of exposure in the US cohort were relatively low and that the number of deaths was small. They concluded that the power to detect a modest increase in risk was low.

REVIEWER'S CONCLUSIONS

With only 14 deaths the opportunities for analysis of this cohort was limited. However, it would have helped to have seen more detail of the internal analyses. There is some uncertainty about the interpretation of Table 7 and 8 as there may well have been considerable correlation between age (measured when?) and cumulative exposure and, in Table 8, cumulative exposure, duration and average intensity. It would have been helpful to have some tabulations of rates by grouped factors from the internal analysis. While this would have been informative, it is unlikely to detract from the overall conclusion of the paper that no excess is present.

Yokoyama K, Araki S, Murata K.

Effects of low level styrene exposure on psychological performance in FRP boat laminating workers.

Neurotoxicology 1992; 13:551-556.

See Murata K, Araki S, Yokoyama K.

Assessment of the peripheral, central and autonomic nervous system function in styrene workers.

Am J Ind Med 1991; 20: 775-784.

Yuasa J, Kishi R, Eguchi T, Harabuchi I, Arata Y, Katakura Y, Imai T, Matsumoto H, Yokoyama H, Miyake H.

Study of urinary mandelic acid concentration and peripheral nerve conduction among styrene workers.

Am J Ind Med 1996; 30:41-7.

AIM

To determine whether a low level of styrene exposure can affect the central nervous system and to clarify the parameter of electrophysiological response is most sensitive to early change in styrene exposed workers.

DESIGN

Cross sectional study with an external comparison group of unexposed subjects.

SUBJECTS

32 exposed subjects (27 men and 5 women) were identified in three Japanese factories employed in the manufacture of plastic materials. The comparison group existed of 23 men and 5 women who were clerical workers.

OUTCOME

Measures of conduction velocity were made in the ulnar and peroneal nerves and of sensory conduction in the ulnar and sural nerves. Latency, amplitude and duration of the action potential were also measured. Six symptoms of peripheral neuropathy were examined.

EXPOSURES

Subjects had been exposed for a mean of 11.6 years (range 1.3-27 years). They were exposed to styrene for 7 hours daily and wore gloves but not masks. End of shift urine samples were collected on the Monday evening after a shift at work. Mandelic acid concentrations were grouped into low and high, where "high" was more than 250 mg/l. The maximum airborne concentration was 117 ppm but levels were usually less than 10 ppm.

ANALYSIS

Paired t-tests and analysis of variance were used to compare the nerve conduction parameters between exposed and comparison groups. Pearson correlations were used to examine the relationship between urinary mandelic acid and nerve conduction. Multiple regression adjusted for confounding.

RESULTS

Motor distal latencies in the ulnar and peroneal nerves were longer for the group with the highest mandelic acid than for those with low exposure or in the comparison group. Motor nerve conduction velocity was significantly slowed in the group with the highest level of mandelic acid and some depression in sensory CV was seen also. There was in general no correlation between mandelic acid and the neurophysiological measures, but there was a weak relation with peroneal motor distal latency. In a

multiple regression analysis, having included estimates of relevant confounders (age, height, weight, body mass index, number of cigarettes smoked, alcohol consumption and skin temperature) urinary mandelic acid concentration was significantly related to ulnar and peroneal motor distal latencies. No relation was found between reported symptoms and exposure.

CONFOUNDING

Information was collected, and included in parts of the analysis, on height, weight, body mass index, skin temperature and alcohol intake. No mention is made of other chemicals at work that might have acted as confounders.

AUTHORS' CONCLUSIONS

A low concentration of styrene affected the peripheral nervous system. Motor distal latencies of the upper and lower extremities seem to be sensitive parameters for toxic neuropathy induced by styrene exposure.

REVIEW'S CONCLUSIONS

It is not clear that clerical workers are the most appropriate comparison group but collection of the information on some of the more obvious confounders might have minimized the importance of any unmeasured confounders. Although the authors make considerable play of the relatively low levels in which they observed effects (most workers currently exposed to 10 ppm for most of the time) much higher values may have been encountered in the past and this is not discussed. It should also be noted that no overall difference on any neurophysiological factor was seen. All the differences reported are within sub-group analysis of those of relatively high versus low levels of mandelic acid.

APPENDIX – LIST OF PAPERS

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2. Bergamaschi E, Smargiassi A, Mutti A, et al. Peripheral markers of catecholaminergic dysfunction and symptoms of neurotoxicity among styrene-exposed workers. Int Arch Occup Environ Health 1997; 69:209-214.

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The Effect of Styrene Exposure on Color Vision: A Review

Gregory W. Good, OD PhD, Jason J. Nichols, OD MS MPH[1]

ABSTRACT

Introduction

Styrene (CAS RN 100-42-5) is an organic, lipid soluble chemical that has been reported to have several health effects. In humans, styrene exposure in the workplace has been primarily associated with effects on the nervous system. As such, it is thought that color vision assessments may play an important role in determining the neurotoxic status of individuals exposed to styrene. The purpose of this report was to review the scientific literature in order to determine the relation between styrene exposure and color vision loss.

Methods

A primary literature search was conducted using MEDLINE and other scientific journal sources for the terms "styrene" and "color vision," and 23 articles were identified. However, not all 23 articles identified from the primary search were reviewed; three were in a non-English language, and four were review-type articles or peripherally related to the topic.[1-7] Two letters to the editor, 14 original research articles, and three case series/animal studies were included in the review and assessments. Articles were reviewed generally in terms of their overall study design and epidemiological methods, color vision test methods, styrene exposure assessment, sample size, control selection, recruitment procedures, statistical methods, internal and external generalizability, and impact of results on the workforce/workplace (if applicable).

Results

Most all studies reviewed were cross-sectional in nature, whereby individuals were sampled based on styrene exposure with the assumption that they would have altered color vision. Control groups were generally sampled from other manufacturing plants, or from other sources (i.e., convenience samples). Styrene exposures were assessed by air samples (personal or area) for ambient levels and through urinary concentrations of mandelic acid (MA) or phenylglyoxylic acid (PGA), which are the major urinary metabolites of styrene. Most studies used the Lanthony desaturated D-15 (dD-15) panel arrangement color test to assess color vision, given its sensitivity to very mild color vision alterations; the test was usually scored using the Color Confusion Index (CCI). Seven studies showed that, compared to controls, styrene-exposed workers had significantly

Styrene exposure, at high levels, is associated with color vision deficiency. However, the magnitude of these deficiencies is relatively minor, especially relative to congenital color vision defects and it is unlikely that the level of defect would have much functional significance.

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higher CCI values where the range of the CCI for the high exposure groups was 1.14 to 1.33, while the range of CCI values for the low exposure groups was 1.02 to 1.17. Studies tested for further dose-response type effects of styrene exposure by dichotomizing CCI values based on various styrene exposure levels, or through correlation and/or preferably, regression analyses in which styrene exposure was used to predict the CCI in the exposed sample. Six studies showed a relation between higher exposures (greater than 20-50 parts per million) and color vision deficiency, compared to control groups. Often, low exposure groups did not significantly differ from the control group, or population, age-specific normal values for the test. Two studies were able to show that styrene exposure levels were able to predict the CCI. Some preliminary evidence suggests that the mechanism of color vision loss associated with styrene is retinal, rather than in the optic nerve or higher processing levels.

Conclusions

Styrene exposure, at high levels, is associated with color vision deficiency. However, the magnitude of these deficiencies is relatively minor, especially relative to congenital color vision defects and it is unlikely that the level of defect would have much functional significance. The literature seems to support the notion that styrene levels above 50 ppm may lead to alterations in color vision, although the exact exposure dose is unclear. Workers performing tasks associated with high styrene exposure levels should use precaution and personal protective devices when possible (i.e., respirators). Questions that remain unanswered included the contributing exposure (i.e., local vs. systemic), the proximate toxicant (styrene or its metabolites), functional significance of the color vision defect, permanency of color vision loss associated with the exposure, the type of color vision (i.e., red-green vs. blue-yellow), and the exact mechanism of action of styrene exposure on color vision loss in the ocular system.

INTRODUCTION

Theories of Color Vision and Congenital Color Vision Deficiencies

Human color vision is possible due to the three component (trichromatic) color processing system in a normal visual system.[8] The perception of color is based on the differential responses across these three systems; i.e., the short-wavelength (blue), middle-wavelength (green), and long-wavelength (red) sensitive systems. About 92% of males and over 99% of females possess these normal retinal components.[9] Of the 8% of males and 0.4% of females who have inherited abnormal components, color discrimination can vary from near normal to severely inferior.

Many non-human land-animals possess dichromatic visual systems.[10] Only the short wavelength-sensitive and a single, medium wavelength-sensitive photopigments are present to allow color interpretation. Although some humans inherit a dichromatic color system, this is an abnormal condition. Normal human color vision is trichromatic, arising from the three types of retinal cone cells.

The genes for the middle (green) and long (red) wavelength-sensitive photopigments are carried on the X chromosome.[11] As the abnormal genes are recessive in nature, red-green color vision deficiencies are therefore inherited in a sex-linked recessive pattern. The two longer wavelength-sensitive pigments in human photoreceptors are extremely similar in chemical composition (vary at only 15 of the 364 amino acid sites). Additionally, the two genes responsible for their development are in juxtaposition near the distal end of the long arm of the X chromosome. The close proximity of the two genes and the nearly identical amino acid sequences suggest that these two photopigments evolved from a single gene. The presence of these two photopigments sensitive across the long wavelength end of the spectrum allows for greater discrimination of greens from yellows from reds, and thus is thought to provide for better ability to search for ripe fruit and vegetables.[12]

Red-green color vision deficiencies affect 8% of males and 0.4% of females. Approximately 2% of males (and 0.05% of women) are limited by a two color system (dichromatic). These individuals must judge all colors using only the short wavelength-sensitive and either the middle or the long wavelength-sensitive systems. Although these individuals are not totally "color-blind," their color is substantially reduced. Colors which are easily perceived as different by color vision normals can look identical and be confused by dichromats, and therefore, color naming errors are commonplace.

Six percent of males (and 0.35% of women) have three component systems, however, one of the two longer wavelength-sensitive components (either the middle or the long wavelength sensitive components) has an abnormal sensitivity. The abnormal red-green photopigments have sensitivities that are shifted relative to normal such that less difference between the middle and long wavelength sensitive photopigments is found. These *anomalous* trichromats can have widely varying color discrimination. Mild anomalous trichromats can perform most color discrimination tasks as well as those with normal color vision, while severe anomalous trichromats have very

reduced color discrimination and often can not be differentiated functionally from dichromats.

The gene for the short wavelength photopigment is present on chromosome 7.[11] Although a genetic abnormality in the short-wavelength photoreceptor is considered rare, its inheritance pattern is dominant. The prevalence of congenital tritanopia is thought to be 1 in 10,000.[11] Because of the rarity of the condition, testing for its presence is seldom accomplished in normal clinical practice.

Red-green color vision defects in females are relatively rare due to their sex-linked recessive inheritance pattern. An interesting note is that females that are heterozygous for color vision defects may show mildly abnormal discrimination abilities. This can occur with the inactivation of the dominant gene in a cell of the developing fetus. This process was first described by Lyon and has been termed "lyonization."[14] Thus, a single cell, and ultimately all of its daughter cells, may display a "recessive" characteristic in spite of a dominant allele being present. Lyonization occurs in many X-linked conditions of the eye.[16] Modern genetic testing has shown that this X-inactivation is related to methylation and unmethylation on the activated X-chromosome and inactivated chromosome, respectively.[17] The process is random, thus, portions of the retina will display a color vision defect, while others will not.[18]

Several investigators have described mildly reduced color discrimination for female carriers of color vision defects.[18-22] These deficiencies are relatively minor and have little effect upon everyday activities; however, when conducting color vision research or when assessing the effects of toxins and the mild acquired defects they may cause, it is imperative to be aware of this effect. Approximately 15% of females will be heterozygous for color vision deficiency. Therefore, care must be taken when choosing females for test and/or control subjects for color vision testing.

Color vision is possible for humans because of the three cone photopigments present in the normal retina. Very early in the visual pathway, however, opponent neural processing is found.[8] This color-opponent processing leads to the presence of 4 fundamental colors instead of 3; red, yellow, green, and blue. The red, green, and blue fundamental colors are typically believed to result from direct input from the long (red), middle (green), and short (blue) wavelength sensitive retinal photopigments. The input for the yellow signal results from the neural addition of the middle (green) and long (red) wavelength sensitive systems. In 1870, Ewald Hering recognized that colors had specific opponent colors which lead to various perceptual effects. This led Hering to propose his "opponent" color theory. In this theory, red is opposed to green, yellow is opposed to blue, and white is opposed to black. Modern day 'zone' theories of color vision describe trichromatic processing at the outer-retinal (receptor) level and opponent processing within the inner retina and along the visual pathway to within the visual cortex. Due to the paired color opponents and the differential mode of inheritance, color vision defects are often termed as either red-green or blue-yellow.

FIGURE 1

CIE-XYZ Color Diagram. The monochromatic spectral colors are represented as shown. White is at the "middle" of the diagram at x=0.33, y=0.33. Colors add in the diagram along straight lines according to the "center of gravity" principle. If equal amounts of color A and B are added, the resulting color, C, is on a straight line exactly halfway between the two. Colors are graphed within this diagram by measuring the relative amount of each of three primary colors (colors X, Y, and Z for this standard diagram) which must be mixed in the precise amounts to exactly match the color in question. The relative amount of primary X in the mixture is represented along the "x" axis, while that for primary Y is represented along the "y" axis.

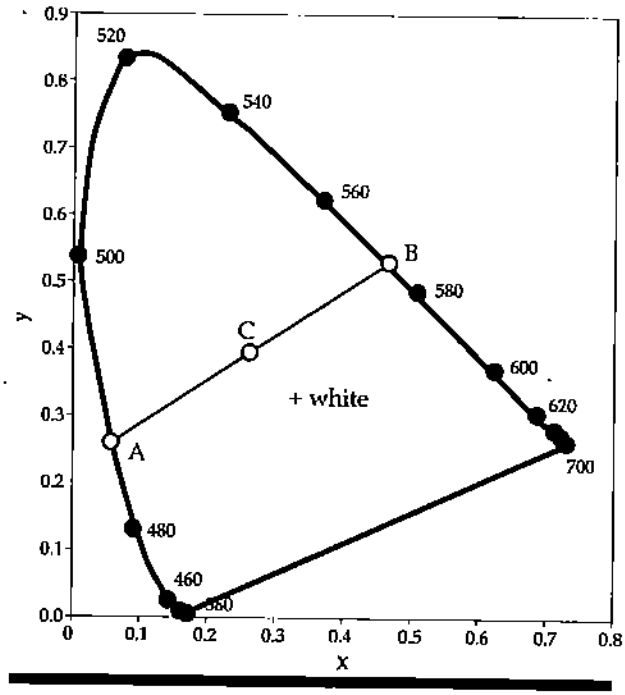
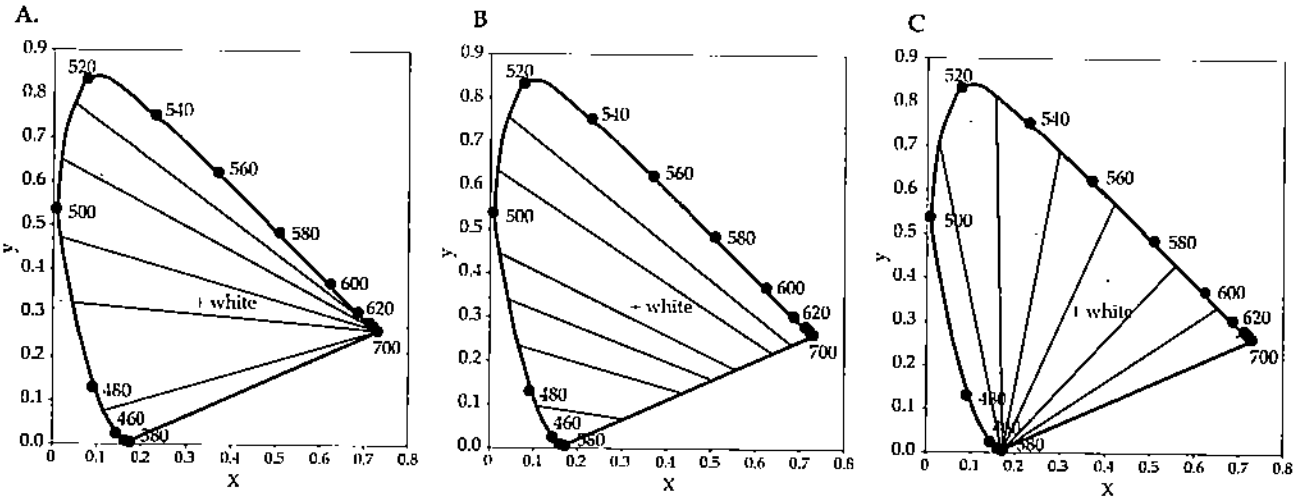


FIGURE 2

Color confusion Lines on CIE-XYZ Diagram. The color confusion lines for the three types of dichromats (A-protanope, B-deuteranope, C-tritanope) with the lines of color confusion radiating from the confusion points. Colors which graph along a confusion line appear identical to the respective type of dichromat.



Color Space and Color Discrimination Deficits

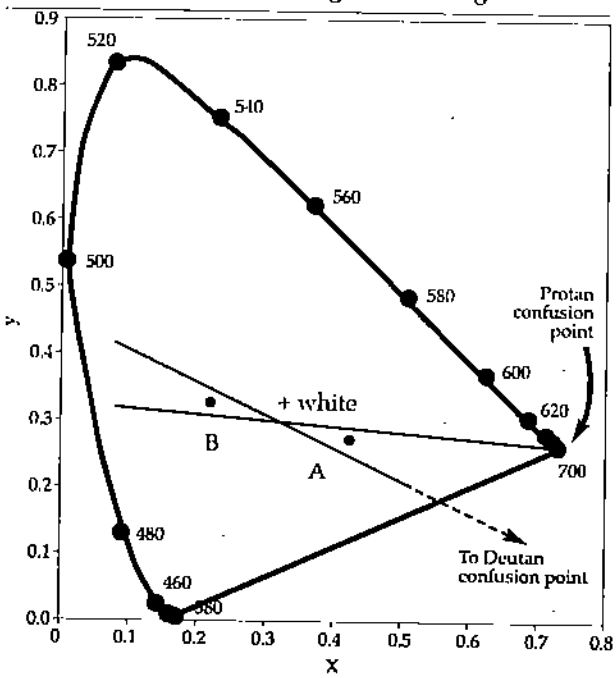
The "trichromatic" nature of human color vision was first expressed in a formal theory by Thomas Young around 1800.[8] The functional consequence of a three-receptor color system is that all colors can be exactly matched by mixing together only three appropriately chosen primary colors. Precisely mixing three colors to match all possible colors allows for color space to be mathematically represented. Figure 1 illustrates the CIE (Commission Internationale de l'Eclairage) XYZ color diagram.[5] The positions of specific colors are determined by the relative amounts of each of 3 primary colors (primary X, primary Y, and primary Z) which must be mixed to match a specific color exactly. The relative amount of the X primary is represented along the "x" axis, while that for the Y primary is represented along the "y" axis. The relative amount of the Z primary required in the mixture of primaries is not shown on the diagram. This value can be calculated, however, by knowing that within the diagram, the x, y and z coordinates always sum to 1. Using this diagram, the color resulting from a mixture of two colors (color A and B in Figure 1) is found along a straight line joining the two colors at a point determined by the amount of each of the two colors in the mixture (center of gravity principle).

Color vision defectives that have only a two-receptor visual system (dichromats) do not see the wide range of colors seen by color vision normals. The color diagram, therefore, provides excess information for these dichromats. Figure 2 illustrates "confusion lines" for the three types of dichromats. All colors along a single line will be confused, i.e., seen as identically matching, for the indicated type of defect. These lines of confusion are used when designing color vision tests. For example, color tests using pseudoisochromatic plates will use color "A" in Figure 3 for background dots on plate and use color "B" for the test numeral. For this example, red (protanopic) or green (deuteranopic) dichromatic subjects will not see the difference in color and will be unable to recognize the numeral. Color vision normals will be able to discriminate the different colors for the background and the test numeral and will see the "hidden" numeral. The background and numeral colors do not lie along the same confusion line for blue (tritanopic) dichromats. Thus, a tritanopic subject will also be able to see the figure.

Because the protanopic and deuteranopic color confusion lines are nearly parallel in most of the color diagram, it is difficult to use many color vision tests to discriminate between these types of defects. The tritanopic color confusion lines, however, are drastically different than the "red-

FIGURE 3

Color Plate Design using Color Confusion Lines. Color A may be used as the background dots in a Color Plate Test, while color B would be used for the "hidden" figure. A red-green color vision defective would not be able to see the "hidden" figure, as both colors lie close to confusion lines for protan and deutan type defectives. Color vision normals would be able to easily see a difference in color between the background and figure.



green" confusion lines. When analyzing the results of color vision testing, the "axis" of the color confusions is noted to help diagnose the type of defect that is present.

CONGENITAL VERSUS ACQUIRED COLOR VISION DEFECTS

Deficiencies in color discrimination are frequently caused by genetic abnormalities resulting in congenital color defects. As mentioned previously, these defects are most often confined to the long and middle wavelength sensitive photopigments (i.e., the red and green sensitive systems). These defects are combined to comprise inherited red-green defects. Abnormalities of the short wavelength sensitive system are also possible, but as previously stated, they are much rarer.

Individuals with inherited color abnormalities have stable color discrimination deficiencies which are relatively predictable and easy to diagnose.[10] The color discrimination losses in the two eyes are comparable; therefore, testing can be done with both eyes viewing. Besides the color discrimination deficits, these individuals have otherwise relatively normal visual functioning.

Individuals with acquired color deficits, however, have less predictable losses which typically progress in severity over time.[10] The two eyes can show very different effects; therefore, tests of color discrimination must be performed monocularly. Very often, acquired defects initially show discrimination loss more typical of blue-yellow defects. Additionally, as these defects result from "damage" along the visual system, changes in visual acuity or other visual measures may accompany the color discrimination loss. Although initial damage to the visual system may be confined to a single color-processing channel (for example the blue-yellow system), as the condition progresses, more diffuse vision losses result which are often difficult to categorize.

While many acquired color vision losses begin by affecting blue-yellow discrimination, some initially affect red-green discrimination. Köllner's rule[10] provides a general rule to predict the type of color vision loss to be initially present depending upon the location within the visual system. This rule states that blue-yellow defects are found with diseases affecting the outer retina, while red-green defects accompany inner retina and the conductive pathways to higher visual levels. Verriest (1963) more thoroughly investigated acquired losses and described Type I, Type II and Type III defects. These classes are described in Table 1.

EFFECTS OF DEFECTS ON EVERYDAY ACTIVITIES

The ability to perform everyday activities with mild or severe color vision defects is highly varied. The effects of color deficiencies on these activities depend upon the depth of the defect and the types of activities performed. Steward and Cole[23] surveyed 102 red-green color vision defectives and 102 color vision normals concerning various activities and the role color discrimination plays in ease of their accomplishments.[23] While a reporting by color vision normals of having a difficulty with color was rare, only 7% of the color defective patients reported no problems at all with color discriminations.

Overall, a greater percentage of dichromats reported having some difficulty with everyday activities than did anomalous trichromats; however, even some dichromats reported having few difficulties related to color discrimi-

TABLE 1

Verriest Classifications of Acquired Color Vision Defects

Name	Description	Examples of Causation
Type I (Red-Green)	Protan type of defect with shift of luminous efficiency from photopic to scotopic	1) Cone dystrophy 2) Chloroquine toxicity
Type II (Red-Green)	Deutan type defect with milder blue-yellow loss	1) Optic neuropathy 2) Ethambutol toxicity
Type III (Blue-Yellow)	Tritan type defect	1) Glaucoma 2) Diabetes 3) Nuclear cataracts 4) Aging 5) Macular degeneration

nation. Examples of tasks in which the most difficulties were reported are selecting colors of clothes and other objects (automobiles, furniture, carpets, etc.) and distinguishing colors of wires, threads, and other things with craft work and hobbies. Other tasks to which fewer problems were reported were assessing by color if meat is fully cooked, recognizing when fruit and vegetables are ripe, and recognizing skin conditions such as sunburn. None of the control subjects with normal color vision reported any difficulty with these particular tasks involving color.

In general, the more severe the defect as indicated by color vision testing, the greater the number of respondents reporting difficulties. It should be noted, however, that some individuals described as having severe deficiencies reported no difficulties and some having relatively minor deficiencies reported several areas of difficulties.[24] For example, 1 of 7 anomalous (deuteranomalous) trichromats that reported no difficulties judging color failed the relatively easy Farnsworth D-15 test, and 27 of 44 anomalous (deuteranomalous) trichromats that reported some difficulties passed the Farnsworth D-15 test. The reasons for these unexpected responses can be related to the different types of color discriminations these individuals require in their daily lives and individual differences in their desires for precision.

An additional note reported by Steward and Cole was that 5% of the dichromats (2 of 37) and 25% of the anomalous trichromats (16 of 65) were unaware of their color vision defect at the time of their participation in the study.[23] Therefore, when assessing subjects to determine color vision status (for inclusion or exclusion from a study, for example), it is imperative that clinical tests be performed to ensure diagnostic accuracy. Relying on patient reporting of color vision status is an inadequate method to determine color vision normalcy. This is an important consideration when studying acquired color vision deficiencies, as patient-reported history of color vision defects is poor.

Clinical Color Vision Tests

Clinical tests of color vision are designed to evaluate different aspects of color discrimination. For example, pseudoisochromatic plates are typically designed to determine if an individual has "normal" color vision. That is, does the individual possess the three, normal retinal receptors? Other tests are designed to determine color discrimination ability regardless of the normalcy status (arrangement tests). It is a basic tenet of color vision testing, that when trying to predict the ability to perform a vocational color discrimination task, it is best to use a test which simulates the vocational task as closely as possible. Therefore, when determining a subject's overall color vision aptitude, a battery of tests is typically administered. Several standardized color vision tests are described along with their vocational uses.

Pseudoisochromatic Plates (PIP). These tests are the most used tests in clinical practice. Subjects must report which figure made from dots or spots is seen against a similar background. The tests are typically designed to identify congenital red-green color defective subjects.[10] In this regard, they usually do an exceptional job. Nearly all color vision defectives fail the tests, while nearly all color vision normals pass. Few false positives or negatives are found. While, in general, mild defectives make relatively few errors and severe defectives make many, the number of errors made on the tests are not highly correlated with functional performance. PIP tests are easy to administer, however, and are frequently used as color vision screening instruments. Individuals that fail the PIP screening test are referred for further testing.

Farnsworth-Munsell (FM) 100 Hue Test. The FM 100-hue test was developed to measure the fine color discrimination in persons with normal color vision and to evaluate losses in those with defective color vision.[17] The test consists of 85 caps of color from around the color circle. The colors of the caps were chosen so that approximately equal perceptual steps of color are represented from one cap to the next. Subjects work with one-fourth of the caps at a time and must replace the caps into a tray in order according to color. The test can be very difficult for a person with defective color vision as the difference in color between adjacent caps is very small. The test is not frequently used for vocational evaluations due to substantial testing time that is required (may take 15 to 20 minutes per eye for administration).

Farnsworth Panel D-15. The Farnsworth D-15 test is an arrangement test using 16 color caps. One of the caps is affixed to the test tray.[10] The other 15 caps must be replaced into the tray according to color (similar to the FM 100-hue test). The difference in color between adjacent caps is much larger than for the FM 100-hue test. The test was designed for vocational evaluation to fail only those with moderate to severe color vision defects. Individuals fail the test when two or more errors (major errors) of replacement are shown which demonstrate gross color confusions from across the color circle. The test is simple to administer and typically takes less than five minutes for administration and grading. Approximately 50% of congenital color defectives fail the test.[25]

Lanthony Desaturated D-15. The Lanthony Desaturated D-15 (dD-15) is similar in design and administration to the Farnsworth D-15. The principle differences between the two are the boldness of the colored caps and the degree of color difference between adjacent caps. The dD-15 has colors which are lighter and less intense than the Farnsworth D-15 colors. It is much more difficult to complete and was designed specifically to evaluate subtle color discrimination changes as a result of acquired color vision defects. The test has been also used as an indicator of fine color discrimination. It is quickly administered and the results are relatively easy to evaluate. Approximately 20% of congenital color defectives pass this test.[26]

Anomaloscope. An anomaloscope is an instrument designed to diagnose the color vision status of subjects.[10] Two spectral colors are mixed to exactly match in brightness and color a third spectral color. The precise mixture ratio of the mixed colors and the range of acceptance of this ratio provide a diagnosis of the exact color vision status. It is the only clinical test which accurately differentiates normals from mild defectives. Although the test is very useful in clinical practice, the results do not always predict absolute functional ability. Therefore, individuals measuring abnormal on the anomaloscope typically are tested using an arrangement test to determine functional aptitude.

The Color Confusion Index (CCI)

In numerous studies that investigate the toxic effects of chemicals upon color vision, the dD-15 Panel Test is used to assess fine color discrimination. Small caps of different colors are replaced into a tray according to color. The caps vary gradually in hue from blue around a color circle to purple. The order with which the caps are replaced gives an indication of which colors may be confused.

The dD-15 is similar to the Farnsworth D-15, however, with the Lanthony test, the caps are lighter and possess less color; i.e., they are more white. Figure 4 shows the positions of the caps for the Farnsworth D-15 and dD-15 tests in CIE-LAB color space. The CIE-LAB color space is a transformation of the CIE-XYZ color space (Figure 1). Color discrimination across the CIE-LAB diagram is more uniform than for the XYZ diagram. Distances across the CIE-LAB diagram are used to represent measures of subjective color differences.[8]

FIGURE 4

Farnsworth D-15 and Lanthony Desaturated D-15 Tests graphed in CIE-LAB Color Space.

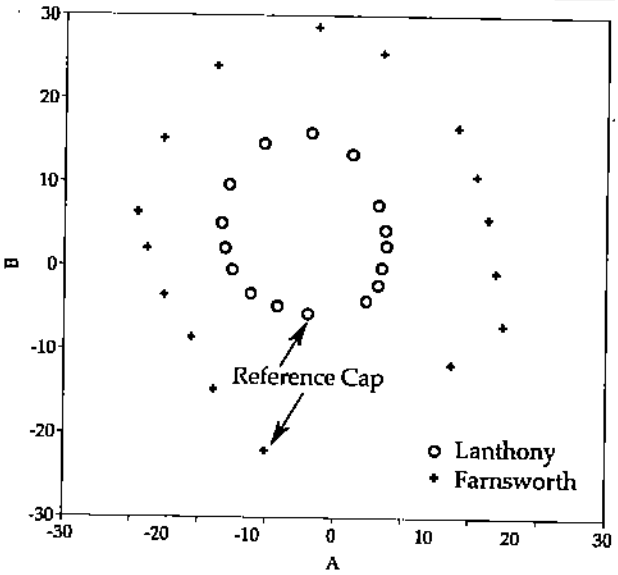


TABLE 2

Average age-specific Color Confusion Index scores for color vision normal subjects.

Age Range (Years)	Bowman et al, 1984[28] (n = 120)	Muttray et al., 1998[29] (n = 296)
10-19	1.083	1.041 (n = 3)
20-29	1.043	1.061
30-39	1.099	1.081
40-49	1.149	1.086
50-59	1.254	1.185
60-69	1.303	1.153 (n = 2)

Bowman[27] recommended a scoring method for the Farnsworth D-15 arrangement test which also can also be used for the dD-15.[27] The method calculates the total distance in CIE-LAB color space represented by moving from cap to cap for each specific replacement order. This total difference provides the Total Colour Difference Score (TCDS). For perfect replacement with the Farnsworth D-15 the TCDS is 116.9. The color chips for the dD-15 are graphed in CIE-LAB color space inside the Farnsworth color circle. Therefore, the circumference, i.e., the TCDS, is much less, being 56.4.

The color confusion index (CCI) is calculated by dividing a given TCDS by the distance representing perfect replacement.[28] Bowman and coworkers[28] and Muttray[28, 29] and coworkers have reported CCI values for the dD-15 for subjects of different ages with normal color vision.[28, 29] Color discrimination decreases with age due to both optical (transmission changes through media of eye) and neural reasons. These values are summarized in Table 2. In his paper, Bowman[28] provides the color space distance values when going from any given cap to any another, so that other investigators can use this scoring algorithm.[28] These values, however, are based upon the locations within color space for the Farnsworth D-15 test. Values for the dD-15 test were not listed, which has led to some confusion when analyzing the dD-15 using the CCI method. At least one investigator using the dD-15 test has apparently used the Farnsworth D-15 cap locations to calculate the TCDS while using the dD-15 test.[30] When investigators report dD-15 test results using the CCI instead of the TCDS, it is not possible to determine if the correct cap positions were used or whether the Farnsworth D-15 values were incorrectly substituted. Muttray and coworkers[29] provide color space distance values for both the Farnsworth and the Lanthony tests. (An analysis of the functional significance of incorrectly using the Farnsworth D-15 CIE-LAB positions for grading of the Lanthony dD-15 test is provided in Appendix A.)

The analysis of studies reporting to have used the dD-15 is further confused by the similarity in name of a scoring method termed the Color Confusion Score (CCS).[31] This method assigns a score equal to the percentage increase in distance traveled around the color circle based upon the specific color cap replacement. Castillo and coworkers[32] report using this method and reference it appropriately but report scores more indicative of the Color Confusion Index.[32] Although the dD-15 test is a desaturated version of the Farnsworth test, the color space locations are not equivalent, even in relative terms. Therefore, errors in analysis can occur if the distances used in calculating the Color Confusion Index are not from the appropriate test.

When assessing the fine color discrimination of a group exposed to a potentially toxic substance, it is important to eliminate congenital color vision defectives

TABLE 3

Color Confusion Index scores resulting from the examination of a sample of red-green color vision defectives using the dD-15. Red-green deficiency confirmed by anomaloscope.

Red-Green Anomalous Trichromats	Red-Green Dichromats
1.00	2.38
1.00	2.48
1.04	2.50
1.10	2.79
1.15	2.81
1.16	3.02
1.21	3.07
1.37	3.21
1.48	
1.74	
1.89	
2.30	
2.35	
2.41	

from both the test and control population groups. The effects of age, alcohol consumption, smoking and various other known toxins are small compared to the drastic discrimination deficits accompanying many congenital defects. CCI values for 22 known red-green defectives are shown in Table 3 for comparison.[19] Many of the studies investigating the effects of styrene on color vision report that potential subjects with congenital dyschromatopsia (color vision deficiency) have been eliminated from the subject pool. However, as Sheedy has noted, often these studies fail to explain how the diagnosis of dyschromatopsia has been made.[33] Steward and Cole report that approximately 18% of the 102 color vision defective subjects they studied were unaware of having a color vision defect prior to participation in that particular study.[23] This is important to remember when evaluating the effects of toxins on color vision. If a color vision defective is erroneously selected as a test or control subject, a significantly high CCI value may bias results. Many of these CCI's for color vision defectives shown in Table 3 are well outside the 95th percentile values of color vision normals reported by Muttray and coworkers.[29]

Calculating the Color Confusion Index for arrangement color vision tests has often been used to document fine color discrimination. The method does provide an objective measure of fine color discrimination. The method, however, provides only a measure of the total distance around the color circle relative to the specific cap replacement. The specific colors that are confused (i.e., the axis of the defects) is not mathematically provided and must be evaluated subjectively.

Vingrys and King-Smith[34] have described a method of evaluation for arrangement tests (FM 100 hue, Farnsworth D-15, and dD-15) which provides mathematical measures of both the color discrimination loss and the primary axis of confusion.[34] This method utilizes vector analysis of the improper replacements to calculate the magnitude and orientation of the principal color confusions. The value of the "C' index" representing a given color deficit is highly correlated with the CCI, and thus provides the same color discrimination information. For the sample values for color vision defectives in Table 3, the correlation coefficient between the C' index and the CCI is 0.976. Therefore, when using the Vingrys/King-Smith method, the same color discrimination information is provided as when using the Color Confusion Index. However, the Vingrys/King-Smith method provides additional information concerning the orientation of color confusions which is not provided by the CCI (i.e., red-green vs. blue-yellow). A computer program utilizing the Vingrys/King-Smith method can be obtained from P. Ewen King-Smith at The Ohio State University: king-smith.1@osu.edu.

Styrene

Background, Sources, and Exposures

Styrene (i.e., vinylbenzene, ethenylbenzene, styrole, CAS RN 100-42-5) is a colorless liquid that is often detected by its somewhat sweet odor. It is very lipid soluble but not very soluble in water, and a volatile liquid at room temperature. Due to its lipid solubility, it is found in higher concentrations in animal tissues such as brain, myelin, and adipose, and can impair neural membranes interfering with the propagation of nerve impulses or neurotransmitters.[35-37]

Styrene is one of the top 50 chemicals produced worldwide, although it occurs naturally as well (i.e., cinnamic acid containing plants). About 10 billion pounds are produced each year. The major uses of this chemical include plastics, latex paints, synthetic rubbers, and polyesters. Styrene can be detected in the atmosphere, generally at low levels, which is mainly due to industrial and auto emissions associated with its production and use. Thus, air is the primary exposure route for most individuals. One study conducted in Canada sampled 18 urban sites not related to styrene manufacture or processing, and showed that the range of ambient styrene exposures was 0.09 to 2.35 $\mu g/m^3$ (where 1 part per million (ppm) of styrene is equivalent to 4,260 $\mu g/m^3$).[38] Another recent study estimated that total daily exposure of individuals is between 18.2 to 55.2 μg/person based on a review of the literature.[39] The World Health Organization (WHO) reports that general ambient air levels are 0.3 $\mu g/m^3$ leading to an estimated daily intake of between 6 and 40 μg/person for nonsmokers in industrialized nations.[40] In general, typical ambient exposures are thus thought to be less than 1 part per billion. Although it is difficult to estimate these exposure levels due to the numerous factors involved, it is generally believed that daily ambient styrene exposure levels are very small. This is because styrene is very reactive in the presence of hydroxyl radicals and ozone found in air; thus, much of it is oxidized in the first two hours after release.

Polluted air and the close physical proximity to styrene-associated manufacturing plants have been shown to be associated with higher styrene levels, although ambient styrene levels in the monomer production and the polystyrene industry are much lower than the reinforced plastics industries.[41] This workforce includes a number of industries including styrene and polymer production, reinforced plastic/rubber produc-

tion, and other products containing styrene. Typical occupations include reinforced plastics workers involved in the manufacture of boats, automobile parts, pipes, protective coatings, and baths/showers. One study sampled the vicinity of seven reinforced plastics industries in the United States showing ambient styrene levels ranging from 0.29 to 2934 $\mu g/m^3$.[42] LeMasters and coworkers (1985) examined occupational styrene exposure values (n = 1,500) from 28 reinforced-plastic manufacturers in the United States showing that the open mould process (24 to 82 ppm) is associated with styrene levels that are two-to-three times greater than the closed mould process (11 to 26 ppm).[43] The WHO reports ranges between 20 and 30 $\mu g/m^3$ for in Europe, with an estimated intake of 400 to 600 Bg/person for individuals involved in this process.

Other sources of styrene and routes of exposure include automobile emissions, cigarette smoke, food, drinking water, and the elimination (thermal degradation) of styrene-containing materials. Cigarette smoke has been reported to contain between 18 and 48 μg/cigarette of styrene, although the WHO reports that active smoking may be associated with styrene exposures levels between 400 and 500 μg/day.[44,45] Small amounts of styrene may migrate from polystyrene packaging into the packaged food; small amounts of styrene may also be present in some foods that have not been packaged in styrene-derived materials. Although styrene has been measured in drinking water, it is thought to evaporate to air rather quickly.

Pharmacokinetics

Absorption of airborne styrene occurs primarily through the lungs, but also occurs through the dermis and other mucous membranes. Styrene is distributed readily through the body, and can accumulate in fatty tissue. Its elimination is linear at low concentrations of ambient styrene. The main metabolic end products of styrene include mandelic acid (MA; 57 to 80% of inhaled styrene) and phenylglyoxylic acid (PGA; 10% of inhaled styrene), which are both found in human urine. Wieczorek and Piotrowski showed that urinary excretion of these two metabolites is biphasic, with the first half-life occurring at about 2.5 hours and the second occurring at about 30 hours.[46] The relation between inhaled styrene and MA is linear up to 150 ppm (639 mg/m^3). Some prefer MA assessment over ambient styrene measures as it allows one to measure the dose of the agent received, although both are generally used together. In general, MA sample collected at the end of a workshift is indicative of acute exposures.

Health Effects in Humans

There has been concern about the association between styrene exposure and cancer; studies have shown a relation in mice and humans between styrene exposure and lung cancer.[47,48] However, the International Agency for Research in Cancer (IARC) has concluded that there is limited evidence for carcinogenicity in human.[49] Other health effects anecdotally reported include symptoms of irritation of mucosal tissues such as the throat, respiratory tract, and eyes, central nervous system depression, ototoxicity, pulmonary toxicity, asthma, and contact dermatitis.[5,50-52] Given styrene's lipid-soluble characteristic, it is often associated with several neurotoxic effects resulting from depression of the central nervous system (e.g., slower reaction times, vestibulomotor alterations, weakness, headache, fatigue, malaise, dizziness, and nausea). An additional effect on the nervous system that has been reported is impairment in color vision discrimination.[32,53-60]

Prior to 1997, the American Conference of Governmental Industrial Hygienists (ACGIH) established the Threshold Limit Value (TLV) for safe occupation exposure at 50 ppm based on the potential for neurotoxicity. In a previous literature review on the impact of styrene on color vision, Sheedy concluded that "High exposure levels are associated with color vision deficiency, whereas low exposure levels are not."[23] However, since that time, ACGIH has reduced the limit for safe occupational levels from 50 ppm to 20 ppm, which is presumably based on more recent 'neurotoxic' evidence. There have also been several new reports evaluating the impact of styrene on color vision discrimination, some of which have evaluated the association between lower levels of styrene exposure.[32,54,59-63] The purpose of this report is to further evaluate the issue of styrene exposure and color vision discrimination through a critical assessment of the literature.

METHODS

A MEDLINE search for "styrene" and "color vision" yielded 23 articles or letters to the editors published prior to June 2003. However, not all 23 articles identified from the primary search were reviewed; three were in a non-English language, and four were review-type articles or peripherally related to the topic.[17] Two letters to the editor, 14 original research articles, and three case series/animal studies were included in the review and assessments. Each of these 17 "studies" (non-letters to the editor) were critically reviewed based on the following principles, and are presented in a temporal sequence:

1. Overall study design and epidemiological methods
2. Color vision test methods
   a) Appropriate testing conditions
   b) Test scored appropriately
   c) Examiners masked in regards to exposure status (when applicable)
3. Styrene exposure assessment
   a) Ambient styrene measures (i.e., personal vs. area sampling)
   b) Biological monitoring (MA or PGA assessments)
4. Sample size and recruitment
   a) Inclusion of control group
   b) General comparison of the exposed and non-exposed groups
   c) Matching conducted
5. Statistical methods
   a) Assumptions met
   b) Sufficient power
   c) Dose-response analyses
   d) Consideration of interactions between variables and effect modifiers
6. External generalizability of results
7. Impact of results on the workforce/workplace (if applicable)

CRITIQUES

Pratt-Johnson (1964)[64]

The author presents a case of retrobulbar neuritis associated with exposure to styrene. The individual was a 48 year old male who had a sudden, painless loss of vision over the course of one week. His history revealed that he worked in the fiberglass industry and he frequently used styrene without protection. His medical history was unremarkable. His examination revealed a visual acuity of 20/400 in each eye, but no refractive error. The anterior and posterior segments of his eyes were unremarkable for any disease and his intraocular pressures were normal. However, visual field testing revealed centrocecal scotomas in each eye. Laboratory blood and urine analyses were unremarkable. The patient was diagnosed with bilateral toxic retrobulbar neuritis and was treated initially with daily intramuscular injections of vitamin B compound, nicotinic acid tablets, and Prednisolone. After 10 days, there was no improvement in the patient's vision or visual field. The vitamin B and nicotinic acid were continued for 6 months and one year after first presentation, the patient's vision and visual field had returned to normal.

Assessment

This case presentation is notable as it is one of the first references in the MEDLINE literature regarding the effect of styrene on the visual system. Given what is known at present about styrene exposure and vision, this patient must have been exposed to extremely high levels of styrene in order for this to occur. As we know, patients with retrobulbar optic neuritis experience very early color vision losses associated with parvocellular visual loss, although there is no indication of color vision testing in this patient case report.[65,66] Additionally, there is a good possibility that there is a strong nutritional deficiency component to this case.

Kohn (1978)[67]

In this study, 345 workers (338 men) who were exposed to styrene were questioned via case history and underwent a visual examination. The author reports that the exposure level for these workers averaged 5 ppm; however, the range of exposure levels was wide, and could have been up to "a few thousand parts per million momentarily during heating of commercial polystyrene." Ophthalmic findings included one individual with an afferent papillary defect, although this was accounted to ocular toxoplasmosis. There were "infrequent" reduced visual acuity findings (< 20/30), and causes associated with reduced acuity included macular scars and amblyopia. Conjunctival irritation was noted in 22% of workers by history and was more common in workers exposed to levels above 50 ppm. By history, several workers reported "styrene beads imbedded in their corneas." There were no cases of retrobulbar neuritis or central vein occlusions, which had been suggested in previous reports as an ocular complication associated with styrene. The author concludes, however, that exposure to styrene is correlated with conjunctival irritation.

Assessment

This report was important as it was one of the first 'studies' to evaluate the ocular effects of styrene. Although no reasonable study design was outlined in the publication and the methods are insufficient, it was one of the first manuscripts to address the impact of styrene on vision. There is no real quantification of exposure assessment. The biological mechanism for conjunctival irritation associated with styrene exposure is unclear. Unfortunately, there was no indication of an assessment of color vision in this patient sample.

Skoog & Nilsson (1981)[68]

The authors note that information regarding styrene exposure and central nervous system effects is scarce. The purpose of this research was to evaluate the effect of

styrene on the electroretinogram (ERG) and standing potential (SP) of the eye. The effect of toluene on these outcomes was also examined, but is not reported on in this review. Cynomolgus monkeys (two male, and three female) were anesthetized and the animals were coupled with the apparatus. Following baseline recordings, the animals were infused with styrene by intravenous injection (0.3 to 15 mg/kg body weight). Blood styrene levels were monitored through venous samples and gas chromatography. The results indicated that the c-wave amplitude nearly doubled about 10 minutes after injection with 15 mg/kg. In addition, the standing potential of the eye followed with similar changes after these styrene injections, especially with higher doses. The authors conclude that the c-wave and standing potentials evoked are approximately proportional to the injected styrene, and no apparent changes were seen in the a- or b-waves. The lowest dose of styrene in these monkeys evoking c-wave changes was 3 mg/kg which corresponded to about 4 ppm in the blood. The authors suggest that it is difficult to relate blood concentrations of styrene with ambient levels. Finally, they state that "It is not possible to explain the observed effects on the c-wave and the SP on the basis of the present study" although both potentials are generated primarily in the retinal pigment epithelium.

*Note: The ERG essentially consists of 4 main elements: the a, b, c, and d waves. The a-wave is seen first and is thought to reflect the summed effect of the photoreceptors within the retina. Under certain testing conditions, the effects of rods versus cones can be seen separately. The b-wave is next seen and is thought to be generated within the retinal Mueller cells. Although Mueller cells are support cells within the retina and don't directly participate in the transmission of the visual signal, the b-wave, however, is thought to reflect receptor (rod and cone) activity. The c-wave is the long-standing potential within the ERG and is present as long as the light stimulus is on. It is thought to be generated with the retinal pigment epithelium but again to reflect rod and cone activity. The d-wave signals the end of the light stimulus (off-effect) and also reflects post-receptor processing (similarly to the b-wave). The standing potential of the eye is that potential which is used when measuring the electro-oculogram (EOG). It is the potential difference between the front and the back of the eyes. It changes slowly over time and can be reflective of the overall integrity of the retina.

Assessment

This study examined the acute exposure of styrene on the visual system, thus, it is difficult to draw conclusions about the effect of styrene in this regard on altered retinal function and color vision impairment. Additionally, the route of administration of styrene in this study was different than that for workers exposed on the job. However, this is an interesting article in that it places potential damage from styrene to the visual system within the retina. Also, it is interesting to note that the ERG returned to normal after the styrene blood level dropped.

Gobba and Coworkers (1991)[53]

This study examined a cross-section of styrene exposed industrial workers for color-vision deficiencies; comparisons were made with a group of 'normal' subjects. The styrene exposed sample included workers from seven small fiber-glass plastics factories and there were 34 men and 41 women included in the sample. The 60 normal individuals (34 men and 26 women) were workers in a rock wool plant. There is no discussion of sampling methods used or sample size considerations. Exclusion criteria included the following: 1) congenital dyschromatopsia, 2) a medical history of hypertension, diabetes, or cerebrovascular disease, 3) use of medications associated with color vision loss, 4) alcohol use greater than 250 grams per week, and 5) poor visual acuity (worse than 20/32). Biological sampling (MA and styrene concentration in urine) was conducted at the end of the workday and environmental monitoring was conducted via personal passive sampling; both were conducted on a Thursday to allow for workweek accumulation. The primary study outcome was acquired dyschromatopsia, assessed by the Lanthony dD-15 panel at the beginning of the workday under a fluorescent daylight lamp (color temperature 5000K). Study subjects were tested monocularly with no time limit. The test was scored using the Color Confusion Index (CCI). Visual acuity was measured as well, although the chart type is not specified. A questionnaire was administered which examined work history, occupational and recreational exposure to solvents, and alcohol and drug use. Statistical methods included testing for data normality (Kolmogorov-Smirnov test), the Mann-Whitney U test and paired t-tests, Spearman's correlation coefficient, and multiple linear regression (dependent variable was the CCI and predictor variables included age, seniority, and exposure levels).

Although the authors stated that they tested their data for normality, there is no indication that such tests were done in the results. The mean (± SD) ambient styrene for the exposed sample was 69.02 ± 3.6 mg/m^3, while the mean urinary styrene was 49.5 ± 44.8 μg/L and the mean MA was 342.9 ± 425.3 mg/L . Correlation results indicated a significant relation between age and CCI in the

exposed ($r = 0.44$, $p < 0.01$) and control groups ($r = 0.37$, $p < 0.01$). As such, the authors stratified their data by age (Group 1 = ≤ 29 years, Group 2 = 30-39 years, and Group 3 = ≥ 40 years) and then compared the median CCI for the exposed and unexposed groups. The only difference was in Group 3, whereby the exposed had a significantly higher median CCI value than the control group (1.301 vs. 1.203, $p < 0.05$). Given the smaller sample sizes associated with this subgroup analysis, it is not known what the observed power was in any of the group comparisons. However, in another post-hoc analysis, the authors 'matched' subjects on age (± 2 years), and then statistically compared CCI values. The results showed that the mean CCI for the styrene exposed workers was significantly higher than in the control group (1.265 vs. 1.151, $p < 0.01$). Environmental sampling data were used to examine a dose-response effect, whereby subjects exposed to styrene levels above the 'threshold limit value' (i.e., 215 mg/m^3 = 50 ppm) showed significant increases in CCI values when compared to the low-exposure group ($p < 0.05$). There was also a significant relation between urinary styrene excretion and CCI in the exposed group ($r = 0.27$, $p = 0.02$). Although the results are not presented, the authors state that a similar correlation was found for ambient styrene and CCI, but not for MA and CCI. Finally, multiple regression results revealed that age, ambient styrene, and urinary styrene concentration were significant predictors of CCI, although self-reported styrene exposure duration was not a significant predictor. Twenty styrene-exposed workers underwent dD-15 testing on one month later, and although no data are presented, the authors state "...no tendency toward a restoration of color vision was observed." In general, styrene exposed workers showed blue-yellow shifts in color vision, although it is noted that "in a few styrene workers, red-green discrimination also was affected."

There are five general conclusions put forth by these authors which include:

1) Age is correlated with CCI values in both styrene-exposed workers and normal controls;

2) Blue-yellow discrimination is primarily affected in styrene-exposed workers.

3) CCI values are higher in styrene exposed workers than in age-matched controls;

4) CCI values are higher in a high styrene exposure group (> 50 ppm) compared to controls, but the low exposure group did not statistically differ from the normal controls; and

5) CCI values can be predicted in multivariate analyses by age, ambient styrene, or urinary styrene excretion, but not MA concentrations.

Assessment

Although the analyses supporting the conclusions were appropriate, there are several issues with the study methods and design that should be addressed. First, there is no discussion of the sampling methods used to identify styrene-exposed subjects within the seven fiberglass plants, nor the control group. It is important to know the total number of styrene-exposed workers in all plants, and a random sample could have been taken from this subject pool eliminating any potential selection bias. With the currently described method, it is possible that certain low-exposure subjects may have been excluded due to the tendency to select those with more severe disease or exposures. There is no mention of masking of the examiners performing the test procedures regarding the exposure status of the subjects. Another issue regarding the subjects is that females were included in the styrene-exposed group. This may pose a problem as up to 15% of women are heterozygous for color vision deficiencies and will show errors on the dD-15 panel. This could spuriously inflate the CCI values associated with the styrene-exposed group leading to significant differences from the control group. The styrene-exposed sample should have included only male subjects free of congenital color vision deficiencies. Muttray and coworkers also suggested in a corresponding letter to the editor that alcohol use should have been controlled for in the analyses even when weekly consumption values were less than 250 g/week.[29] This too could have increased the CCI values; however, alcohol consumption would have affected the CCI values in both the styrene-exposed and control groups.

Again, a concern in this study was the apparent lack of testing data for normality; inspection of the data shows a highly skewed range of data. This could have accounted for the significant differences found between age-matched groups. The authors did not perform the appropriate analysis to derive the fourth conclusion regarding a dose-response effect. Given that three groups were present in the subgroup analysis, the appropriate statistical analysis would have been analysis of variance (ANOVA) with post-hoc testing of groups as necessary. The ANOVA technique would have allowed the authors to control for the effects of age as well. It is unclear why excreted MA did not predict CCI values. It is unfortunate that the authors did not provide any sort of power calculations for their observed findings in this case as it appears as there is much variability in the styrene-exposed group in this outcome (i.e., the standard deviation is larger than the associated mean for MA concentration). Also, it is dissatisfying that data and analyses were not presented regarding the affect of short-term elimina-

tion of styrene exposure (i.e., the 4 week vacation) on color discrimination. This is an important outcome that needs resolution. Overall, this study provides some evidence for the impact of styrene on color vision.

Fallas and Coworkers (1992)[8]

This study examined a cross-section of 60 shipyard workers who worked in the construction of glass reinforced polyester materials. The sample was composed entirely of men who ranged in age from 20 to 56 years, and whose styrene exposures varied from one to 29 years (mean exposure duration = 6.5 years). The workers were placed in four subgroups based on their styrene exposure duration [Group 1 (n = 14) = less than 1 year, Group 2 (n = 18) = 1-5 years, Group 3 (n = 14) = 5-10 years, Group 4 (n = 14) = more than 10 years]. Styrene exposure was quantified for a period of three months by gas phase chromatography and mass spectrometry via 16 sampling units in the work area. In addition, urinary excretion of MA and phenylglyoxylic acid (PGA) were measured at the end-of-shift by high performance liquid chromatography. A 60 member male control group was assembled, and the controls were additionally matched to the styrene-exposed workers on age, intellectual level, and ethnicity. Members of the control group were unskilled workers, boilermakers, and warehousemen. Two styrene-exposed workers were excluded for hypertension and alcoholism, and three controls were excluded—"one for education and two for psychiatric history."

Color vision was assessed using the Farnsworth 100 hue test during working hours and in the daylight. The test was scored using the number of errors subjects made, and comparisons of the error scores were made in relation to age. It is unclear what statistical test was used for this. The chi-square test was used to compare the number of subjects in the red-green and blue-yellow range for the Farnsworth test results. Other outcomes included a psychometric examination developed by the World Health Organization (WHO), which included a social and medical history, occupation, drug usage, mood assessment, reaction time, and other neurobehavioral items.

The mean value for MA was 230 mg/g creatinine (range = 2 to 1460) and the mean value for PGA was 57.4 mg/g creatinine (range = 0.4 to 421.2). The mean Farnsworth 100 hue error score for the styrene-exposed workers was 259.9 ± 136.9, while the mean for the controls was 262.7 ± 114.0; these values did not differ statistically. There were 32 workers with red-green and/or blue-yellow ranges in the styrene-exposed group compared to 20 in the control group ($p < 0.05$). The authors conclude that exposure to styrene "may lead to impairment of colour vision in the form of the anomalies already reported for workers exposed to mixtures of solvents," and suggest that the 50 ppm exposure limit may be too high given this finding.

Assessment

There is one general conclusion put forth by these authors—that is, styrene exposure leads to impairment of color vision. They go on to speculate that the damage occurring in the visual system may be either retinal and/or optic nerve. However, this conclusion is less than substantiated by the results presented. First, there was no statistical difference in error scores when comparing the styrene-exposed workers and the control group. The authors did not state whether one orientation of defect class was more prevalent than the other nor how they determined whether a defect was predominantly red-green or blue-yellow. The analyses presented are of poor quality, and there is no mention of controlling for confounders (i.e., age). It is likely that the analysis of error scores was probably underpowered to find a difference between these two groups given the variability presented. Although age-based subgroups are proposed in the methods, there is no indication that statistical analysis of these groups was carried out in the results. Further, it is questionable why there was no attempt to evaluate any association between urinary MA concentration, PGA, and color vision test results (i.e., after controlling for age in the styrene-exposed workers, does MA or PGA predict the error score). There is lack of sufficient details regarding the sampling methods used in order to ascertain any potential selection bias associated with subject recruitment. There is no mention of masking of the examiners who performed the testing regarding subject exposure status. Finally, the styrene exposure sampling method was for the workshop rather than through personal monitors and there is no indication duration or frequency of exposure to the peak styrene levels (469 ppm).

Interestingly, the actual error scores presented are much higher than what might be expected, both in the styrene-exposed workers and the control group. The authors stated that they used "a specially devised automated procedure for analysis of the results" but do not sufficiently describe the method. Further, the actual testing methods used for color vision testing are not described sufficiently. The lighting used is not optimal, and there is no mention of whether testing and scoring was carried out monocularly or binocularly.

In a subsequent letter to the editor, Muttray (1993) criticized this study for several reasons. Muttray and coworkers concluded that the work by Fallas et al. does not provide evidence of an effect of styrene on color

vision.[51,52] The criticisms are listed below:

1) Improper illumination used for color vision testing.
2) Lack of control over subject's glasses and contact lens status (i.e., colored lenses).
3) Failure to exclude those with congenital color vision defects.
4) The term "range" was not defined.
5) Failure to screen vision or for ophthalmic conditions.
6) Skepticism of error score results given their high means compared with other studies.
7) Testing of subjects during the workshift with concurrent exposure to styrene. It is suggested that this would impact the ability to differentiate between acute and chronic effects of styrene in terms of color vision.

In response, Fallas and coworkers suggested the following:

1) They clarify the lighting used by stating that "The Farnsworth Munsell procedure guidelines indicate that "sunlight" is irrelevant and that "daylight" together with fluorescent lighting is more appropriate. We have therefore applied the Farnsworth Munsell procedure."
2) They claim that none of the subjects wore colored glasses or contact lenses.
3) They state that acquired and congenital color vision defects are difficult to differentiate, and state that "it is hard to understand why workers exposed to styrene should be more affected by congenital defects than those of a control population living in the same area and matched for age, sex, and ethnic origin."
4) The term "range" referred to circumferential errors associated with the Farnsworth-Munsell test.
5) Screening examination were conducted on an annual basis by an occupational physician.
6) They did not discuss the issue of above average mean error scores.
7) They state that they make no claims as to the chronic or acute effects of styrene on color vision and their study was not designed to address this issue.

Although some of the criticisms of Muttray and coworkers (1993) are somewhat artificial, the responses provided by Fallas and coworkers are less than reassuring.[54] It becomes more clear that this group is unfamiliar with many aspects of color vision, including the disease itself and testing methods. The response to the issue of lighting alone leads one to this conclusion, and it is still unclear exactly what was done in this regard. Further, the use of the term "range" also suggests the researcher's lack of familiarity with the testing method used in this study. The lack of details regarding the scoring method used for the test results does not allow one to determine if improper scoring is associated with the high mean error scores. However, as Sheedy suggests, this is likely the cause of the inflated mean.[11] He also suggests that the researchers probably included the base value of "2" for each color chip in the test.[11] Correcting for this factor would make their mean error scores in line with what might be expected based on the results of other studies. In summary, the data and analysis presented by Fallas and coworkers (1992) does not substantiate the claim that styrene exposure is associated with acquired color vision deficiencies.[51]

Gobba and Cavalleri (1993)[53]

This publication represents data presented previously (Gobba, 1991) in addition to some new data.[53] Two groups of subjects from 10 fiberglass reinforced plastics factories were included. Seventy-five styrene-exposed workers from seven of these plants (Gobba and coworkers, 1991) composed Group A and 40 workers from the remaining three plants composed Group B. The control group for Group A included 60 unmatched normals, while the control group for Group B styrene-exposed workers included 40 normals that were matched on gender, age (± 3 years), alcohol consumption (± 10 gm/day), and tobacco smoking (± 10 cigarettes/day). Further exclusion criteria included alcohol consumption greater than 50 gm per day, congenital color vision deficiencies, diabetes, hypertension, cerebrovascular disease, color vision altering medications, and visual acuity worse than 20/40.

Ambient styrene exposure was assessed via personal passive sampling on Thursdays. Urine collection was used for sampling styrene and MA concentrations at three time points: prior to the workshift, noon, and end-of-workshift. In addition, blood samples were taken at noon on Thursdays for styrene determination.

The primary study outcome was color vision discrimination, assessed by the dD-15 panel. Scoring was conducted using the method of Bowman (1984), in which the CCI is calculated. All color vision testing was conducted prior to beginning the workshift under a daylight fluorescent lamp (5000K color temperature).[28] Testing was conducted monocularly, although results presented represent the average of both eyes. Statistical tests used to compare group data included the paired t-test, Mann-Whitney U test, the Wilcoxon test, the chi-square test, and ANOVA. Correlations were determined using Spearman's method and multiple linear regression was used to determine predictors (i.e., age, seniority, exposure levels) of the CCI index.

As discussed in the assessment of Gobba and coworkers (1991), there was a significant correlation between age and CCI in the 75 styrene exposed workers and the control group.[53] However, because these groups were not

matched on age, the styrene-exposed workers were significantly older than the control group (37.6 years vs. 32.0 years respectively), which could artificially inflate the CCI associated with the styrene-exposed workers. The authors state that this is the reason for their assembly of a second group of styrene-exposed workers—in order to match for potential confounder. In this second group, the mean ambient styrene exposure was 68.2 mg/m³ while the mean urinary styrene was 41.4 μg/L. The average MA concentration for this new group is not presented. The mean (±SD) CCI for styrene-exposed workers in this new group was 1.206 ± 0.200, while the mean (±SD) CCI for the control group was 1.053 ± 0.070 ($p < 0.001$). The authors state that only one styrene-exposed worker could perform the test perfectly, while 16 controls were able to do so (chi-square test, $p < 0.01$). In order to assess a dose-response affect of styrene on color vision, subgroup analysis was performed on styrene-exposed workers whereby these workers were divided based on ambient styrene exposures above and below the threshold limit of 213 mg/m³ (the American Conference of Governmental Hygienists, 1992). The results indicated that subjects above the limit had higher mean CCI values compared to those below the threshold limit (1.29 vs. 1.11, $p < 0.05$) respectively. Finally, there was a significant correlation between urinary styrene concentrations and CCI ($r = 0.27$, $p = 0.02$), and environmental styrene levels and CCI (analysis results not presented), but not between MA and CCI (analysis results not presented). The authors state that "These results were confirmed by applying multiple regression analysis" but the results of the analysis are not presented. In a small group of styrene-exposed workers, the color vision testing was repeated about one month later with interrupted exposure to styrene and the results were found to be similar to previous testing.

From these results, the authors draw the following conclusions:

1) Styrene exposure can impair color vision.
2) Styrene and age appear to have a "synergistic" impact on color vision impairment.
3) There is a dose-response effect of styrene on color vision.
4) Short-term discontinuation of styrene exposure does not reduce the color vision impairment.
5) Blue-yellow color discrimination is primarily affected (data not presented).

Assessment

As mentioned above, some of this work has been previously presented. However, due to potential problems associated with the sample of the first group (i.e., no matching was conducted), a second sample was assembled, with age, gender, alcohol, and tobacco matched controls. Although this strengthens the study's validity, there is still lack of details regarding sampling methods used to assemble the styrene-exposed workers and controls. With the new sample, the conclusion that styrene exposure can impair color vision seems valid. However, the conclusion that age and styrene exposure have a synergistic effect on impairment of color vision is not supported. As far as one can tell, no interaction terms were examined in the multiple regression analyses. As such, it is not appropriate to suggest this relation between these two predictor variables on color vision impairment. The data as presented suggest that there is a dose-response affect of styrene on color vision when comparing high and low level styrene exposure groups. However, as in their previous work (Gobba and coworkers, 1991), it appears as if the low exposure CCI mean (1.11) probably does not statistically differ from the control group mean CCI (1.053). This finding in a second sample of patients may suggest that low exposures of styrene (< 50 ppm) do not impair color vision. In fact, the low exposure group CCI mean (1.11) is actually very similar to the previous *control* group CCI mean (1.151). The conclusion that short-term eliminations of styrene exposure does not seem to be supported by the data presented, as only a very small sample of patients underwent this test protocol. The authors conclude that most color vision discrimination alterations are blue-yellow in origin, although no data are presented to that end. Finally, it is unfortunate that there is no discussion of masking of the examiners who performed testing in terms of exposure status of the subjects. Overall, this article provides moderate evidence that styrene exposure alters color vision.

Campagna and Coworkers (1994)[70]

The authors state that this publication is part of a larger, prospective study examining the reversibility of neurotoxic effects of styrene. Three reinforced plastics manufacturing plants were used to sample workers exposed to styrene. Personal passive sampling was conducted and data were analyzed using gas chromatography. Urinary samples were taken after the work-shift and were analyzed for MA concentration, corrected for urinary creatinine. Workers completed a questionnaire assessing sociodemographic information, work environment and history, and medical outcomes including ophthalmic symptoms (eye irritation, tearing, and blurred vision). Color vision was assessed using the dD-15 panel tested monocularly, and near contrast sensitivity was assessed using the Vistech 6000 at five spatial frequencies. Color

TABLE 4

Descriptive results from Campagna and coworkers, 1994.[20]

Outcome	Mean Percent
Workers with Environmental Styrene Above ACGIH limit of 213 mg/m³	31.8%
Urinary MA Concentration Above 0.6 mmol/mmol creatinine	23.9%
Eye Irritation	37.5%
Blurred Vision	33.0%
Tearing	23.9%

vision and contrast sensitivity testing was conducted 12 hours after cessation of styrene exposure. Individuals were considered to have acquired color vision deficiency if the caps were misplaced by at least 2.

Study exclusion criteria included less than six months of exposure in the plant, "poor" near visual acuity, ocular injury, congenital dyschromatopsia, cataracts, diabetes, ophthalmotoxic medications, and incomplete exposure. One hundred fifty one individuals were eligible to participate in the study, 128 agreed to participate, and this publication represents the data from 88 individuals. No information is presented on statistical procedures used or model parameters.

The mean (±SD) age of the sample was 29 ± 8 years and the mean (±SD) time on the job was 5 ± 4 years. The average education level was 11 ± 1.7 years. The average alcohol consumption was 160 gm/week. Some descriptive results from this study can be found in Table 4.

Workers with styrene levels above 213 mg/m³ reported eye irritation more frequently than those below that level (chi-square, $p < 0.01$). Similarly workers with MA concentrations greater than or equal to 0.6 mmol/mmol creatinine reported eye irritation, blurred vision, and tearing more commonly than those below that level (chi-square, all $p < 0.05$). Of the 88 workers, 34.1% were found to have acquired dyschromatopsia, and workers with MA concentrations at or above 0.6 mmol/mmol creatinine were more likely to be those with acquired dyschromatopsia than those lower than this level (57% vs. 27% respectively, chi-square, $p < 0.05$). There was no relation between ambient styrene and acquired dyschromatopsia (33% vs. 36%, chi-square, p = not significant) or symptom reporting and acquired dyschromatopsia (data not reported). Regression models showed that age and alcohol consumption were associated with color vision loss; as such, models were adjusted for these factors. The final models showed that the adjusted CCI was predicted by log of the MA concentration ($F = 5.95$, $p < 0.05$) and the log of ambient styrene for those not wearing masks ($F = 9.39$, $p < 0.01$). There was no relation reported by styrene exposure and contrast sensitivity. The authors conclude that color vision losses occur with styrene exposure at higher levels, and the fact that no relation was observed between exposure and contrast sensitivity or symptoms indicates very early impairment.

Assessment

Although this is a somewhat short, technical publication, it does provide evidence of the fact that styrene exposure does impact color vision, especially at higher levels. The study's validity would have been strengthened by the inclusion of a control group of individuals not exposed to styrene. This is particularly important in this regard as examiners are not masked regarding exposure status in the current design. Additionally, there is no mention of the distribution of men and women in this patient sample. It would have been appropriate to exclude women from these analyses given their possible heterozygous genetic color vision status. As such, the inclusion of women in this sample could have spuriously inflated the CCI values reported. Overall, this article provides moderate evidence that styrene is associated with color vision deficiency.

Chia and Coworkers (1994)[19]

The purpose of this study was to evaluate the effect of styrene exposure on color discrimination, as well as to evaluate the effect of styrene exposure on neurobehavioral function. Included in the study sample were 21 workers (out of a population of 70 workers) from a fiber-reinforced plastic boat manufacturing facility. Subjects were only included in the sample if they had at least 5 years of work experience and if they did not have central or peripheral nervous system diseases. Medical examinations were used to screen for these illnesses. Workers generally wore gloves, but not masks, during the workshift. A control group of 21 workers was also assembled from the same factory (the carpentry division), and the authors state that "their workplace was inspected to ensure that there was no exposure to any neurotoxic chemicals." However, ambient sampling does not appear to have been completed in the carpentry area. Control group subjects were matched on age, years of education, and socioeconomic status to the styrene-exposed group subjects. However, it is also stated in the methods that the

styrene-exposed workers and the control group did not differ on smoking and drinking history as well—on average, neither group reported drinking more than 50 grams of alcohol per day. The workers were also asked to restrain from alcohol use for the three days prior to testing. It is stated that none of the workers used any long-term medications that might affect their test results.

Urine collection was completed at the end of the workshift on the day of color vision testing for determination of MA and PGA concentrations. Personal, ambient styrene sampling was not determined in this study. However, they estimated that the workers were exposed to less than 10ppm from previous studies conducted in the factory.

Testing was conducted on Monday mornings, and one trained worker who was masked to exposure status completed all testing. Color vision testing was conducted by the dD-15 panel under a daylight fluorescent lamp providing 1,000 lux. All subjects wore their habitual visual correction, and it is not stated if the test was conducted monocularly or binocularly. The authors state that scoring was conducted via the method of Bowman, although a total color difference score is reported. Statistical analysis of the data was conducted using log transformations. The chi-square test, Fisher's exact test, correlations, and ANOVA were used to examine the data. As an additional precaution, age, years of education, and alcohol use were controlled for in the ANOVA models.

The mean styrene exposure duration was a period of 18.8 years (range: five to 23 years), although 18 workers had more than 15 years of exposure. The mean MA concentration in the styrene-exposed group was 84.0 mg/g creatinine (median = 21.4 mg/g creatinine, range = 1.3 to 504.1 mg/g creatinine), and in the control group was 3.3 mg/g creatinine (median = 2.9 mg/g creatinine, range = 1.6 to 9.6 mg/g creatinine). The mean concentration of PGA in the styrene-exposed group was 66.0 mg/g creatinine (median = 30.1 mg/g creatinine, range = 0.3 to 297.4 mg/g creatinine), while in the controls, the mean concentration of PGA was 0.7 mg/g creatinine (median = 0.58 mg/g creatinine, range = 0.3 to 1.9 mg/g creatinine).

The total color difference score for the dD-15 panel in the styrene-exposed group was 164 (standard error = 0.04) and was 131.8 in the control group (standard error = 0.04); these values were reported to differ statistically ($p = 0.0006$). They report that there was not a significant correlation between the total color differences score and MA ($r = 0.33$, p = ns) or GA concentrations.

The authors conclude that styrene exposure can impair color vision. Secondarily, they conclude that most color discrimination errors in that group are "in the blue-yellow and red-green ranges." Finally, they suggest that even though these workers were exposed to low levels of styrene (less than an estimated 10 ppm), their long-term exposure to the chemical may be associated with their color vision deficiencies.

Assessment

Unfortunately, the conclusions made by the authors are obscured by several factors. First, the total color difference scores reported by the authors do not appear consistent with the scoring mechanism proposed by Bowman and coworkers (1982). The CCI value associated with the case group (≈ 1.40) appears to be quite large, and may reflect the inclusion of individuals with congenital color vision deficiencies (there was no mention of exclusion of congenital deficiencies and with a sample size that is so small, the inclusion of just one individual in this group could skew this mean). This significantly impacts the validity of this study. Another factor of concern is the lack of personal air sampling for styrene. We believe that no conclusions can be drawn regarding the effect of styrene exposure on color vision from the data and results associated with this study.

Campagna and Coworkers (1995)[9]

The purpose of this work was to examine the relation between "internal and external" styrene exposure factors with color vision discrimination and contrast sensitivity at near. A sampling frame of 151 workers was composed from three Canadian reinforced plastics factories, and from this, 128 volunteered to participate in the study. An additional 49 workers were excluded for the following reasons: "poor" near visual acuity (n = 11), additional solvent exposures (n = 1), medications affecting vision (n = 2), acute styrene exposures (< 6 months, n = 8), incomplete background data (n = 12), and "diabetes, color blindness, ocular disorders and injuries" (n = 13). In summary, a total of 81 final subjects were included (79 male, 2 female) with a mean age of 29 ± 8 years and an average of 5 ± 4 years of seniority on the job.

Styrene sampling was conducted via personal passive sampling monitors worn for about 4 hours during the workshift. End-of-shift urine was collected to determine MA concentration and were analyzed using HPLC (corrected for urinary creatinine). Outcomes assessed included sociodemographic information, work condition, work history, and general and ocular symptoms (i.e., eye irritation, tearing, and blurred vision). Near visual acuity was tested monocularly with habitual correction using the National Optical Visual Chart at 0.4 meters. Color vision was assessed monocularly using the dD-15 panel, and

subjects were classified with either a blue-yellow or red-green loss. Scores for subjects were used to calculate CCI values according the Bowman's method, with an average presented from both eyes. Subjects were further classified for color vision deficiencies if he or she misplaced a color cap by 2 in the following categories: type III blue-yellow loss, type II red-green with concurrent blue-yellow loss, and type I red-green loss. One cap misplaced was considered normal. The Vistech chart (VCTS 6000) was used to assess near contrast sensitivity at 0.4 meters monocularly. There is no statement about the use of habitual visual correction during this test. Five spatial frequencies were tested and the data are presented as a mean of both eyes.

Statistical analyses included the log transformation of some data due to non-normal distributions including alcohol consumption, seniority, ambient styrene, and MA concentrations. Paired t-tests and Spearman's correlation coefficients were used to examine the relation between variables. Multiple linear regression was used to examine predictors (i.e., age, seniority, alcohol use, mask use, and MA) of either the CCI or contrast sensitivity. Logistic regression was used to examine predictors (those aforementioned) of either one subjectively reported symptom or "qualitative dyschromatopsia."

The average self-reported alcohol intake was 160 ± 181 grams per week. The average MA concentration of the group was 0.36 ± 0.52 mmol/mmol creatinine and the average ambient styrene concentration was 205.78 ± 262.35 mg/m^3. For the group, there was a significant difference between the two eyes on the CCI (1.20 ± 0.22 vs. 1.09 ± 0.12, $p < 0.0001$) and contrast sensitivity, although the spatial frequency for this difference in contrast sensitivity between the eyes is not reported. The between-eye average CCI was 1.14 ± 0.16. Investigation of the data shows that the difference between eyes in contrast sensitivity may apply to all spatial frequencies. Linear regression models show that both age (95% confidence interval [CI] for $\beta = 0.003$ to 0.013) and urinary MA (95% CI= 0.052 to 0.263) predicted the CCI. Contrast sensitivity at 12 and 18 cycles/degree) were associated with age, while at 6 and 12 cycles/degree, it was associated with MA. In separate regression models, mask use and ambient styrene were used as predictors of the CCI and contrast sensitivity. In general, mask use was associated with reduced CCI values, while ambient styrene was associated with increased CCI values. However, when controlling for mask use in the day of exposure assessment, ambient styrene was no longer significantly associated with increased CCI values. In general, mask use and ambient styrene was not associated with contrast sensitivity loss.

Of the 81 styrene-exposed subjects, 25 (30.9%) were considered to have the "profile of acquired dyschromatopsia." Of these, 22 had blue-yellow defects, one had a red-green defect, and two had mixed defects. They also state that six other workers had defects with nonspecific axes. Logistic regression was used to predict acquired dyschromatopsia, and after controlling for age, alcohol consumption, and mask use, neither MA concentration nor ambient styrene were significant.

Eye irritation was reported by 35 subjects, followed by blurred vision (n = 31), and tearing (n = 23). Logistic regression was used to examine predictors associated with symptom reported. MA significantly predicted eye irritation (OR = 5.50, 95% CI = 1.28 to 23.67), tear (OR = 4.73, 95% CI = 1.07 to 20.87), and blurred vision (OR = 6.47, 95% CI = 1.43 to 29.28), after controlling for age and alcohol consumption. Ambient styrene predicted eye irritation (OR = 1.53, 95% CI = 1.09 to 2.14), but not tearing (OR = 1.36, 95% CI = 0.95 to 1.95) or blurred vision (OR = 1.37, 95% CI = 0.98 to 1.93).

The authors make several conclusions from this work including:

1) Color vision loss occurs during 8 hour exposures to styrene in workers who do not wear masks;
2) The effect occurs through respiratory absorption rather than through the mucous membranes of the eyes;
3) Styrene exposure induces blue-yellow defects;
4) The effect may be due to an alteration of the liposoluble fraction of the cones or through altered dopaminergic modulation in the horizontal cells;
5) MA concentration is associated with altered high spatial frequency contrast sensitivity caused by "changes in neurooptic transmission rather than ocular defects;" and
6) These workers reported visual symptoms "more frequently than others" caused by a local effect on the eye.

Assessment

This study was generally well-conducted and the analyses and results were carefully described. Unfortunately, the major criticism associated with this study however, is the lack of a control group in order for comparisons to be made. Although inferences can be made from the present study, a control group would have improved the validity of the results. That being said, the study does provide convincing evidence of color vision loss associated with both ambient styrene and MA. In terms of the contrast sensitivity, especially as the testing is down at nearpoint, the losses at the higher spatial frequencies could be due to refractive error and optical blurring. This fact greatly limits their findings relative to contrast sensitivity testing. Optical blurring would have little effect upon color vision testing.

Eguchi and Coworkers (1995)[7]

The purpose of this research was to examine the effect of low-dose styrene exposure (< 20 ppm) on color vision in a sample of Japanese workers. The original styrene-exposed sampling frame included 69 workers (five women) from six reinforced plastics factories, and the control group included 84 workers (15 women) from those same factories in addition to others. Patients were excluded if they had a congenital color vision defect, hypertension, poor vision (< 6/10), high alcohol use (> 250 g/week, were female, or used color vision-altering medications). Thus, the final sample included only 64 styrene-exposed men and 69 controls (all men).

Ambient styrene exposure was monitored by area sampling rather than personal passive sampling. Urine was collected at the end of the workshift on the day of color vision testing for MA concentration determination by HPLC. The dD-15 panel was used to assess color vision on Mondays at the beginning of the workday. The test was performed monocularly under a daylight fluorescent lamp providing 1,000 lux and habitual glasses were used. If subjects scored two or more errors, they were classified as red-green or blue-yellow as appropriate. Further, the total color difference score (TCDS) and CCI values were calculated. Pearson's correlation was used to evaluate the relation between CCI and age, while Spearman's correlation was used to evaluate the association between CCI and MA concentration. In addition, the Mann-Whitney U and Wilcoxon signed rank tests were used to make group comparisons. Multiple linear regression was used evaluate the relation between ambient styrene and CCI using a backward selection technique.

Ambient styrene was 18.5 ppm (range 6.6 to 36.4 ppm). In general, MA concentrations were low with a mean concentration of 0.22 g/l (range of 0 to 48 g/l). About 56.3% of workers had MA concentrations < 0.3 g/l, 34.4% had concentrations 0.3 to 1.0 g/l, and 9.4% had concentrations > 1.0 g/l. There was a significant correlation between TCDS and age in both styrene-exposed workers ($r = 0.39$, $p < 0.01$) and the control group ($r = 0.48$, $p < 0.01$). Following this analyses, post-hoc matching was conducted based on age (± 3 years). The average CCI in the styrene-exposed workers was 1.220 ± 0.235 while in the age-matched controls, the average CCI was 1.120 ± 0.128 (Wilcoxon signed rank test, $p < 0.01$). Styrene-exposed workers were subdivided based on MA concentrations (Group 1 < 0.42 g/l and Group 2 ≥ 0.42 g/l, where 0.42 g/l N 30 ppm), resulting in a reduction to 57 individuals in each group. The mean MA concentration in Group 1 ($n = 40$) was 0.20 g/l (≈ 8 ppm) and in Group 2 ($n = 17$) the mean MA concentration was 1.06 g/l (N 93 ppm). These two groups were then compared with the age-matched control group in terms of the average CCI and the results showed that only Group 2 differed statistically (Wilcoxon signed rank test, $p = 0.12$ and $p < 0.01$ for Groups 1 and 2 vs. controls respectively). However, for both of these groups, there was not a significant correlation between MA concentration and CCI values (Group 1 $r = -0.24$, $p = 0.12$ and Group 2 $r = 0.32$, $p = 0.18$).

The stepwise multiple regression analysis showed that age ($\beta - 0.007$, $p < 0.01$), smoking ($\beta = -0.006$, $p < 0.05$), and urinary MA ($v = 0.16$, $p < 0.01$) were significant predictors of the CCI in the styrene-exposed workers. Insignificant outcomes included volume of alcohol consumption, frequency of alcohol consumption, education, exposure duration, and the interaction of exposure duration and urinary MA. The adjusted R^2 for the model was 0.328. Finally, the authors state that most of the defects found in this sample were blue-yellow, although some showed "complex loss."

The authors conclude from the results that:

1) CCI values of styrene-exposed workers were significantly higher than controls;
2) Urinary MA is a predictor of color vision loss;
3) There is a dose-response function whereby those wither higher MA concentrations have more significant color vision losses; and
4) Duration of styrene exposure is not related to color vision loss.

Assessment

This study was conducted in a similar fashion to that of Gobba and coworkers (1991) including the design and analysis of the results (i.e., post-hoc data matching). It was appropriate to exclude women from the analyses as previously discussed, although the authors do not recognize the heterozygous deficits associated with females as the reason for their exclusion. The study does seem to provide evidence for the conclusions that CCI values of styrene-exposed workers are higher than controls, especially in higher styrene exposure levels measured through urinary MA concentrations. This was not the case for the low styrene exposure group, which did not statistically differ from the control group. A limitation of this study included area rather than personal ambient styrene sampling; this may be why the authors chose not to evaluate this variable as a predictor of color vision loss. An additional limitation of this study was that there is no mention of masking patient identifiers regarding their status as a styrene-exposed worker or a member of the control group. Overall, this study provides moderate evidence regarding the impact of styrene on color vision.

Mergler and Coworkers (1996)[51]

The purpose of this report was to examine the effect of a styrene-reduction intervention on neurotoxic effects associated with this chemical. The study design included a longitudinally-assessed cohort of styrene-exposed workers. A battery of neurofunctional tests was administered to will participants mainly composed of the Neurobehavioral Core Test Battery (NCTB) and the Field Assessment Using Sensory Tests (FAST) battery. The dD-15 and near contrast sensitivity (Vistech 6000) are the two components in the FAST battery. In addition, self-administered questionnaires including sociodemographic information, work and medical history, and drug, smoking, and alcohol use were administered. Styrene exposure measures were made by personal passive sampling and end-shift urinary MA concentrations were measured. A daily time-weighted average was used for each of these outcomes. Following baseline measures, styrene reduction recommendations were made to the plants which included the installation of local ventilation systems and personal protective device reinforcement. One plant heeded the recommendations, while the other two plants did not. Baseline outcomes were compared following two years of additional exposure in the three reinforced plastics plants using paired t-tests, Mann-Whitney U tests, or paired t-tests depending on the nature and distribution of the data.

At baseline, 118 workers completed the testing protocol, although only 75 were still employed 2 years later and 57 of these workers agreed to participate again (18 refused participation). No information regarding gender is provided in this study. At the 2-year follow-up, the 18 workers who refused to participate did not statistically differ from participants in terms age (29.0 vs. 32.3 years respectively) or years of education (10.7 vs. 10.7 years respectively). The participants were, however, more experienced than non-participants (6.0 years vs. 3.7 years, $p < 0.05$). At baseline, the participants had higher ambient styrene exposures (75 mg/m3 vs. 18 mg/m3, $p < 0.01$) and urinary MA concentrations (0.11 mmol/mmol creatinine vs. 0.05 mmol/mmol creatinine, $p < 0.01$), although they did not statistically differ in these two outcomes at their 2-year follow-up.

At the 2-year follow-up, there were significant reductions in ambient styrene ($p < 0.05$) and urinary MA concentration ($p < 0.001$) compared with baseline at Plant 3. This was not the case at the other two plants. Of the final 57 workers completing both phases of the study, there was no difference in their average CCI score when comparing baseline (mean ± SD = 1.18 ± 0.20) to the 2-year follow-up (1.19 ± 0.35). However, the authors found that contrast sensitivity (averaged over six spatial frequencies) was significantly reduced two years later (mean ± SD baseline 69.5 ± 20.8 to 62.1 ± 20.6, $p < 0.01$). When workers data was stratified by plant (i.e., plants 1 and 2 compared to plant 3), their mean differences in CCI scores (baseline – follow-up) were not significantly different from zero. The mean difference in CCI score for plants 1 and 2 was –0.12 ± 1.17 and for plant 3 it was 0.20 ± 0.78. Contrast sensitivity was reduced only in plant 3 (mean difference – 0.62 ± 1.05, $p < 0.01$).

Longitudinal changes associated with styrene exposure were examined by separating workers on the basis of their change in MA concentration (baseline – follow-up). The workers were grouped as follows: those with an increase ≥ 0.1 mmol/mmol creatinine ($n = 17$), those who remained within ± 0.1 mmol/mmol creatinine ($n = 22$), and those who had decreased by ≥ 0.1 mmol/mmol creatinine. The authors state that there was a strong correlation ($p < 0.001$) between increase in MA concentration over the two years and an increase in the CCI index, although they do not provide the actual correlation coefficient. Data for contrast sensitivity are not provided.

Based on these results, the authors make the following conclusion. Differences in neuro-outcomes were associated with changes in MA concentrations—"the strongest relation is with color vision which may be particularly sensitive on a short term basis to increases and decreases in exposure."

Assessment

Overall, the results supporting the relation between styrene exposure and color vision loss associated with this study are not substantial, although it is notable that this study represents the first full-scale longitudinal evaluation of exposure and color vision loss. For most neurofunctional outcomes, including color vision, there was no difference between baseline and the 2-year follow-up, although there were measured reductions in ambient styrene and MA levels (Plant 3). This is also somewhat true for Plants 1 and 2, who showed changes in styrene and MA levels, although workers showed no change in their CCI values. There is no mention of the sample's gender in the manuscript; thus, it is not possible to determine the percent of females. This might be problematic if CCI comparisons from this patient sample were made with normal individuals from other studies as the index may be slightly inflated. Finally, there was no control group used for statistical comparisons and changes in outcomes over time limiting the interpretation of the results.

Campagna and Coworkers (1996)[59]

The purpose of this work was to specifically evaluate the

potential dose-response effect associated with styrene exposure and color vision loss. Data that were used for these analyses included that collected and analyzed in two previous reports by Campagna and coworkers (1995) and by Gobba and coworkers (1991). For these analyses, subjects were excluded if they had medically confounding conditions (i.e., diabetes, hypertension, or cerebrovascular disease, cataracts), were taking ophthalmotoxic medications, and those with a history of poor near visual acuity, eye trauma, congenital dyschromatopsia, vocational exposure to other solvents, and those with incomplete data. Subjects were also excluded if they wore protective masks on the job, consumed alcohol more than 200 gm/week. No control subjects were included in these analyses, although data were collected on control subjects by Gobba and coworkers, 1991. The final sample thus included 118 individuals.

Individuals were assessed throughout the workshift for environmental styrene via passive dosimeters. Color vision discrimination was assessed using the dD-15 panel, and CCI values were used to quantify the data. The authors state that the test was administered in the same format and conditions across both study samples. Multiple linear regression was used to first examine the effects of age and alcohol use on color vision discrimination, which were both found to be associated with increased CCI values. As such, the CCI was then adjusted in further analyses by "adding the mean score to the residual." Spearman's correlation was then used to examine the relation between the adjusted CCI and styrene exposure. A maximum likelihood technique was then used to determine the threshold styrene exposure associated with color vision abnormalities.

There were no statistical differences in the patient samples from each of the two studies in terms of average age, alcohol consumption, work seniority, styrene exposure, or CCI values. The average adjusted CCI values were 1.14 ± 0.14 from the Canadian sample and 1.18 ± 0.16 from the Italian sample (p = not significant). The adjusted CCI showed a positive relation with styrene exposure (Spearman's $r = 0.246$, $p < 0.01$, $n = 118$). The maximum likelihood value was 3.8 ppm, where the Chi square value was 6.8. The upper bound for the 95% confidence interval associated with this styrene level was 25.7 ppm (lower limit could not be determined).

The authors conclude with the following points:

1) There is an association between styrene exposure and color vision discrimination loss across studies.

2) The threshold limit associated with color vision loss is about 4ppm, which is much lower than many current threshold limits for styrene exposure.

Assessment

There are two novel aspects associated with this manuscript. First, the data used for these analyses represented two distinctly separate patient samples (although they were shown to be very similar in terms of their respective demographics). In general, we would expect this to improve the generalizability of any results identified, as we have included a more diverse sample of patients and exposures. It does appear, however, that as this was somewhat of a post-hoc analysis, this may be problematic as the one cannot be entirely sure that the methods used were the same for both studies. The second novel aspect of this work is the specific dose-response analysis conducted from the overall data set. This method is based on a change point, which assumes no relation between exposure and the outcome of interest for low levels of exposure, but a relation for higher levels (i.e., where a dose-response may exist). However, the threshold of 4 ppm for color vision impairment is a bit surprising. Other studies have found no relation between styrene exposure and CCI for such low levels.[53, 57] The upper limit associated with this threshold value was 26 ppm, although the correlation coefficient associated with this limit was only 0.21. Typical ambient concentrations are around 1 part per billion (ppb) and 20 ppm in fiberglass-reinforced plastics plants today.

Given that this work represents the re-analysis of previously reviewed studies, many of the same previously identified problems still exist. These problems include the lack of masking examiners in terms of case (or control status) and the potential inclusion of females in the samples, who may give spuriously higher CCI values depending on their genetic status.

Gobba and Cavalleri (2000)[56]

The purpose of this report was to investigate changes in color vision associated with continued styrene exposure over a period of time. Although not relevant to this report, the investigators also examined the effects of percholorethylene (PCE) and metallic mercury on color vision in two separate samples of individuals. Two samples of styrene-exposed workers were examiner ($n_1 = 39$ and $n_2 = 30$). The authors indicate that styrene exposure was assessed by biological monitoring or personal passive sampling, although they are not specific. A questionnaire assessed work and medical history, exposure to other toxic solvents, alcohol and smoking habits, and ophthalmotoxic medication usage. Subjects were excluded if they used more than 50 g of alcohol per day, smoked greater than 30 cigarettes per day, and had visual acuity worese than 20/32. Controls were used for comparisons

in outcomes (with equal participants as the case samples) and were matched to the cases on gender, age, and alcohol and cigarette use.

Color vision was assessed using the dD-15 panel and expressed using the CCI. Each eye was tested individually, and testing was conducted in the morning before exposure under a daylight fluorescent lamp (1200 lux). The examiners were "unaware of exposure levels of workers." Statistical assessments were made after first testing data normality. The paired t-test was used for comparisons between groups on CCI data, and correlations were assessed using Spearman's correlation coefficient.

First, 41 styrene-exposed workers were assessed for color vision deficiencies "during the last weeks before summer holidays." The mean CCI from this group was 1.25 ± 0.22, which was significantly higher than controls (1.15 ± 0.14, $p < 0.01$). Color vision was then assessed the morning of the first day returning to work (30 days without work-related styrene exposure) in 39 of these same individuals. The mean CCI was 1.23 ± 0.19, which did not statistically differ from the baseline CCI value for these same workers (1.20 ± 0.21, $p > 0.05$). Styrene exposure levels were not reported for this analysis.

In a second group of 30 styrene-exposed workers (mean exposure = 13 ppm), the average baseline CCI was 1.24 ± 0.21 which was significantly higher than controls (1.14 ± 0.14, $p < 0.01$). This group was then assessed again 12 months later. Exposure levels were increased in 10 of the 30 workers, although this increase was not statistically significant (time 1 = 10.9 ppm vs. time 2 = 16.2 ppm, $p > 0.05$). The exposure levels were slightly reduced in 20 members of this sample, although this reduction was not statistically significant (time 1 = 14.1 ppm vs. time 2 = 10.2 ppm, $p > 0.05$). The authors suggest that the CCI values increased in the workers with increased styrene exposures, although the analysis did not show significance in this group (time 1 = 1.18 ± 0.16 vs. time 2 = 1.29 ± 0.21, $p = 0.08$). The workers with slightly reduced exposures showed no difference in CCI values over this 12 month interval (time 1 = 1.27 ± 0.18 vs. time 2 = 1.29 ± 0.16, $p > 0.05$). The authors conclude that the increased exposure level in the 10 workers was associated with increasing errors in color discrimination.

Assessment

Although the Methods section of this article is well organized and presented, the Results and Conclusions are less than satisfying. There is adequate discussion regarding controlling for potential confounders (i.e., age, alcohol use), although it does not appear that individuals with a congenital color vision deficiency were excluded from participation. The assessment of urinary MA concentrations would have also been a nice supplement to the data and comparisons. The authors do not discuss gender in their sample, which is problematic as the inclusion of females could have spuriously increased any CCI values reported. This may not be problematic if both the case and control groups have equal number of females, although we cannot be sure of this as the data was presented. Further, there is inadequate discussion regarding the control group, including it selection, demographics, and work history. Given the nature of the research, the selection of the control group could have just as much impact on the outcomes of interest as the selection of the cases. The statistical analyses as presented are appropriate given the desired comparisons, yet the actual results of the study do not correspond with the conclusions drawn by the authors. For the first group including 41 styrene-exposed workers, there was a significant difference as compared to the control group in terms of CCI values. However, there was no statistical difference in CCI values after 30 days without styrene exposure in the control group. Again, in the second group of 30 styrene exposed workers, there was a difference in CCI values when compared to a control group at baseline. However, there was not a statistical increase in styrene exposure over the twelve month period nor was there a statistical change in CCI values. No data were reported for the control group over this same time period. This draws into question the conclusion that any changes in color vision occur over time with changing levels of styrene exposure.

Kishi and Coworkers (2001)[60]

The purpose of this report was to supplement previous work conducted to assess a dose-response relation between styrene exposure and color vision loss. The authors specifically state the need for a larger sample size in order to achieve this aim relative to their previous study including 69 exposed workers.[57] In this study, 105 male subjects from seven reinforced plastics factories were included in the styrene-exposed group. A control group of 117 males was also assembled from the same and other factories; however, none of the controls reported any exposure to styrene or other solvents. A questionnaire was used to assess work history, solvent exposure, and alcohol and drug use. Subjects were excluded from participation for the following reasons: congenital dyschromatopsia, hypertension, ophthalmotoxic drug use, poor visual acuity (worse than 20/32), and alcohol use greater than 250 g/week. Collection of urine occurred at the end of the day of color vision testing for MA concentration determination and area sampling was used for ambient styrene levels.

Color vision was assessed using the dD-15 panel on Mondays or Tuesdays at the beginning of the work-shift under a daylight lamp (1000 lux). Testing was conducted monocularly with visual correction. The test was scored in two ways. First, individuals were categorized as abnormal (blue-yellow or red-green) with two or more errors in either eye. Second, a CCI score was calculated for each individual. Correlation coefficients (Pearson's and Spearman's) were used to assess the relation between CCI and age and CCI and MA concentration, respectively. The Mann-Whitney U test was used to make comparisons in outcomes between individuals. Multiple linear regression was used to examine the relation between styrene exposure and the CCI, while controlling for age. Finally, the CCI was adjusted for age and cigarette consumption and a threshold effect of styrene was determined using the maximum likelihood technique described by Campagna and coworkers(1996).

The average ambient styrene level was 21 ppm (range: 6.6 to 36.4 ppm) and the average MA concentration was 0.21 ± 0.44 g/L for styrene-exposed workers. The authors state that there were "no large difference for age, alcohol consumption, the number of cigarettes/day, and educational attainment among these workers," which appears to be true upon inspection of the data although no statistical comparisons were made. It does appear that the control group consumed more alcohol as compared to the styrene-exposed workers, although no statistical comparison was made. There was a significant linear relation between the CCI and age for both styrene-exposed workers ($r = 0.43$, $p < 0.001$) and controls ($r = 0.37$, $p < 0.001$). After matching for age (87 pairs for this comparison), it is reported that there was a difference in the mean CCI values when comparing the two groups (Mann Whitney U, $p < 0.01$) although the data are not presented nor is an indication of which group had larger CCI values.

The styrene-exposed group was categorized based on urinary MA concentrations: Group 1 (< 0.1 g/L N 8 ppm ambient styrene), Group 2 (0.1-0.2 g/L N 8 to 16 ppm ambient styrene), and Group 3 (> 0.2 g/L N > 16 ppm ambient styrene). The mean CCI values for these groups were 1.21 ± 0.26, 1.23 ± 0.20, and 1.27 ± 0.27, respectively, which did not statistically differ from one another (ANOVA). The CCI values from Group 2 differed from age-matched controls (1.12 ± 0.12, Wilcoxon signed rank test, $p < 0.05$), as did the CCI values from Group 3 compared with age-matched controls (1.13 ± 0.14, Wilcoxon signed rank test, $p < 0.01$).

The results from the multiple regression procedure showed that age ($p < 0.001$), urinary MA ($p < 0.001$), and smoking ($p < 0.05$) predicted the CCI in styrene-exposed workers. Exposure duration, alcohol use, and educational attainment were not significant predictors of the CCI in these workers. Using the maximum likelihood analysis, a threshold urinary MA concentration of 0.39 g/L (95% CI = 0.32 g/L to 0.48 g/L). This is equivalent to an ambient styrene level of about 30 ppm.

The authors conclude that for moderate and high styrene exposures, CCI values differ from age-matched normals indicating that there is a dose-response affect. Further, the lowest observed adverse effect level (LOAEL) was 0.39 g/L and most individuals had a blue-yellow defect associated with styrene exposure (no data presented).

Assessment

The Introduction and Methods sections associated with this article are clear and concise. Both the case and control samples seem to be appropriately selected, with very little differences between them other than the exposure of interest. No females were included in the analyses, which is appropriate. However, the authors state in the Introduction that the purpose of this report was to evaluate a dose-response affect of styrene on color vision with a 'larger sample of patients'; yet only 105 styrene-exposed workers were included. It is unclear as to how this sample size was determined to be large enough for the analyses required. The statistical analyses appear appropriate as they were conducted. The article suggests that color discrimination is altered in styrene-exposed individuals compared with age-matched controls, although no data are presented in this regard. In terms of dose-response evaluations, it appears that the analyses were underpowered to detect a difference between low, moderate, and high exposure groups. However, an additional analysis comparing these groups to age-matched controls shows that the CCI is significantly higher in the moderate and high-exposure groups than the control group. Thus, there is some evidence of a dose-response affect. The maximum likelihood threshold analysis shows a LOAEL of about 30 ppm, which is much larger than that of Campagna and coworkers.[59] The value found in the current work of about 30 ppm seems much more reasonable given the findings of this and other studies that higher levels of styrene exposure seem to impact color vision rather than low levels. It is unclear why no data were presented regarding the quantitative analysis of color vision (number of blue-yellow and red-green defects in the sample). In summary, this work does provide evidence that higher styrene exposures do impact color discrimination greater than low levels, which may not impact color vision.

TABLE 5

CCI data from Triebig and coworkers (2001).[62]

Test Period		CCI Monday Prior Morning		CCI Thursday After the Workshift		After 4 weeks Vacation	
Baseline	Cases	1.24 ± 0.25	p = 0.11	1.29 ± 0.27	p= 0.05	1.11 ± 0.11	—
	Controls	1.10 ± 0.11		1.10 ± 0.09		Not Assessed	
10 Months Later	Cases	1.11 ± 0.11	p = 0.61	1.16 ± 0.14	p= 0.18	1.05 ± 0.06	p= 0.56
	Controls	1.10 ± 0.16		1.08 ± 0.11		1.04 ± 0.06	

Triebig and Coworkers (2001)[62]

The purpose of this report was to examine how styrene exposure affects color vision and to examine changes in color vision over time after styrene exposure was stopped. A sample of thirty male laminators (out of 50 styrene-exposed workers) was included in the study; similarly, 27 males from the same company served as a referent group. The exclusion criteria for participation were as follows: congenital dyschromatopsia, diabetes, hypertension, visual acuity worse than 20/32, alcohol use greater than 30 g/day, ophthalmotoxic drug use, ophthalmic or systemic diseases altering color vision, and a MA and PGA sum of greater than 50 mg/g creatinine (control group). Given these restrictions, eight cases and 16 controls were eliminated from participation, leaving the sample with 22 cases and 11 controls. The article states that there were no significant differences between styrene-exposed workers and controls in terms of age, alcohol use, cigarette use, and seniority.

The examination included the following procedures: questionnaire, physical examination, Ichikawa color plate test, complete eye examination (including visual acuity, intraocular pressure, fundoscopy, and perimetry), and the dD-15 panel. The dD-15 was conducted under a daylight lamp (1000 lux) binocularly ("due to time constraints"). The test was scored using the CCI, and comparisons to age-dependent referent values were made to a "assess whether the CCI is normal or abnormal." Styrene exposure was assessed by urinary MA and PGA assessments, which were collected at the end of the workshift on Thursdays. An intervention was conducted in which a ventilation system was installed to reduce styrene levels and subjects were re-tested ten months later. The sum of MA and PGA were used as an assessment of total styrene exposure. Statistical analyses included the use of the U-test, Fisher's exact test, the Wilcoxon signed rank test, Spearman's correlation coefficients, and linear regression.

Following the ventilation intervention, there was a significant reduction in MA plus PGA levels in styrene-exposed workers (baseline = 472 and 10 months = 273, p = 0.02). This was not the case in the control group who showed no statistical change in styrene levels (baseline = 15 and 10 months = 24, p = 0.25). CCI data and analyses are presented in Table 5.

Further comparisons were made using these data. For both cases and controls, there was no statistical difference when comparing the CCI values from Monday morning and Thursday following the work-shift at baseline or at the assessment following the intervention 10 months later. However, there was a significant reduction in the CCI values taken Monday morning in the styrene-exposed workers following a 4 week vacation period. The mean baseline CCI was 1.24 compared to 1.11 after vacation (p = 0.01) while the mean CCI ten months later following the intervention in these same workers was 1.11 compared to 1.05 following the 4 week vacation period (p = 0.01). A categorical analysis is done whereby individuals were classified on the basis of an abnormal CCI result, although it is unclear how the authors define this term. The analysis suggests that eight styrene-exposed workers compared with one control worker had an abnormal CCI value on Thursday at baseline (p = 0.05); no other comparisons between styrene-exposed workers and controls with abnormal CCI values at any test period showed significance. They also state that "regression analyses revealed significant and positive correlations between the amount of internal styrene during the first examination phase and the score of CCI, but not for PGA alone." However, the regression equation and slope of the line between MA prediction of CCI score is actually insignificant (p = 0.07). Spearman's correlation coefficients are

then reported for the relation between MA an CCI ($r = 0.61$, $p = 0.01$) and between MA plus PGA and CCI ($r = 0.59$, $p = 0.01$); the correlation coefficient between PGA alone and CCI was not significant ($r = 0.28$, $p = 0.28$).

Finally, another attempt was made to examine a possible dose-response affect of styrene on color vision by categorizing styrene-exposed workers on the basis of a normal or abnormal CCI and then evaluating MA plus PGA levels for each of these groups. No specific details are given regarding the CCI cut-point used. The result presented is such that "MA plus PGA of approximately 500 to 600 mg/g creatinine was associated with a normal CCI score."

The authors make several conclusions based on the results of the study including:

1) Styrene-exposed workers generally have worse color discrimination than normal controls.

2) MA plus PGA of approximately 500 to 600 mg/g creatinine (≈ 20 ppm ambient styrene) is associated with the upper, age-normal values for the CCI.

3) Styrene-induced color vision deficiencies are reversible, both with complete elimination of styrene for a period of 4 weeks, after an "exposure-free weekend," and after an intervention to reduce styrene exposures in those workers.

4) There was too much overlap in blue-yellow and red-green errors in the styrene-exposed workers to come to any conclusions regarding discriminatory defects.

Assessment

In general, this article is well presented, and the methods and analyses seem to support the conclusions brought forth by the authors for the most part. Color vision testing was conducted binocularly rather than monocularly, which was done due to "time constraints." Unfortunately, this rationale is less than satisfactory as the test takes in general between one and three minutes to conduct. Urinary MA and PGA were sampled on controls in this research, which improves the internal validity of the study. Most other studies seem to use a self-reported assessment from control subjects that they have not been exposed to styrene. Although subjects were excluded due to heavy alcohol consumption, it would have been nice for the investigators to actually examine the affect of low-to-moderate alcohol use in the styrene-exposed workers and referent group, controlling for this as needed. It is stated in the Methods section that "results were considered significant if < 0.05;" however, none of the regression procedures evaluating MA, PGA, or MA plus PGA as predictors of CCI showed significance at this level, although the authors suggest significant positive relations from these analyses. The authors do show significant Spearman correlations between CCI and MA and CCI and MA plus PGA, although this sort of analysis is less satisfactory. The significance of these nonparametric correlation analyses suggest that the variables of interest did not meet the assumptions required for the linear regression.

From the data and analyses presented, there is no reason to doubt that styrene-exposed workers have worse color discrimination than normal, non-exposed individuals. We also believe that although the sample sizes in both the styrene-exposed and control group in this study were small, there is no methodological reason to doubt that the discrimination losses are reversible both after short-term elimination and long-term reduction in exposure. This differs from the result of Gobba that showed no improvement in color discrimination after a four week vacation period.[53] In that study, 20 styrene-exposed workers underwent dD-15 panel testing after one month of reduced exposure. Although no data are presented, the authors stated "...no tendency toward a restoration of color vision was observed."[53] Triebig and coworkers (2001) explain this difference by suggesting that the stricter alcohol consumption exclusion criteria employed in the current study may have lead to the finding. Mergler and coworkers (1996) also conducted a similar study, reducing ambient styrene levels and MA concentrations in 29 workers in one reinforced plastics plant did not statistically improve their color discrimination.[61] It is unclear why these discrepancies exist, and future studies need to address the issue. It is unclear how repeated applications of the dD15 panel influence these sorts of outcomes, especially when the test is administered binocularly. Overall, this study provides moderate evidence of the relation between styrene exposure and color vision function.

Castillo and Coworkers (2001)[52]

The purpose of this study was to examine potential changes in visual function associated with long-term styrene exposure in a cohort of workers.[58,61] The investigators hypothesized that cumulative styrene exposure reduces near contrast sensitivity, and current styrene exposure is associated with color vision alterations. All workers examined in this study participated in both previous assessments and remained employed at the plant. The plant from which individuals were sampled was a shower and bathtub manufacturers. There were 32 workers still employed who participated in the previous follow-up assessments, and 24 decided to participate (6 more were eliminated due to poor near visual acuity). There was an intervention in the plant after the first data collection period, in which additional ventilation was installed and respirator use was highly encouraged. Ambient

styrene was measured with personal passive devices and urinary MA was measured at the end-of-shift at the end of the work-week (corrected for urinary creatinine).

Data were corrected for respirator use, and a cumulative exposure index (CEI) was determined for each worker using styrene exposure data. Visual function assessments were conducted Saturday mornings, and included near visual acuity (monocular, habitual correction) and color vision (dD-15, monocular). It was reported that the Color Confusion Score (CCS) was used to quantify color vision test results. All data were log-transformed, and ANOVA and two-sample t-tests were used to compare year-specific data for individuals with each job classification. Job groups were also compared between years using Friedman's ANOVA (nonparametric). Data were combined when no differences existed.

Average ambient styrene levels ranged up to 600 mg/m³ at the first data collection period, 430 mg/m³ at the second follow-up period, and 300 mg/m³ at the current follow-up period. Median MA levels ranged from 0.25 mmol/mmol creatinine at the first follow-up period, 0.1 mmol/mmol creatinine at the second follow-up period, and 0.075 mmol/mmol creatinine at the current follow-up period, which represented a significant decrease over time ($p = 0.015$). For the 18 workers in this analysis, the range of CEI values was 198 mg/m³ to 6,022 mg/m³. Workers were divided into three exposure groups based on the CEI: < 1,000 mg/m³ ($n = 6$), between 1,000 and 2,500 mg/m³ ($n = 6$), and greater than 2,500 mg/m³ ($n = 6$); there was no difference in alcohol intake between these groups ($p = 0.42$).

The authors examined the influence of age, education, and alcohol as confounders for reduced CCS or contrast sensitivity finding that age was the only factor associated with these outcomes. Thus, scores associated with each outcome were age-adjusted. The authors made paired comparisons using Wilcoxon signed rank test showing significant increase in CCS scores between 1990 and 1992 (median values of 1.07 vs. 1.045 respectively, $p < 0.05$), but not between 1992 and 1999 (median values of 1.045 vs. 1.16 respectively, p = not reported). There was no relation between the current CCS outcome (1999) and ambient styrene exposure, MS, or cumulative exposure. There was a significant difference between years at 3.0 CPD (Friedman's ANOVA, $p < 0.05$), but not at 1.5, 6.0, 12.0, or 18.0 CPD. Group individuals by the CEI, there was a significant difference between groups at 3.0, 6.0, and 12.0 CPD ($p < 0.05$). The authors conclude that long-term, cumulative styrene exposure is not related to color vision loss; rather, it is associated with contrast sensitivity loss. Although not addressed in analyses, the group also suggests that as MA levels were low (< 0.25 mmol/mmol creatinine) suggesting "These low levels of actual exposure probably account for the absence of a dose-dependent relation between color vision loss and exposure."

Assessment

This work represents a third follow-up period to the previous two assessments of a group of workers in the reinforced plastics industry. As with the other studies, the results here are not all that impressive. The authors found a significant increase in the CCS score between the first two assessment periods, but not between the 1992 and 1999 assessments. It is somewhat confusing that the authors chose to analyze color vision in terms of the CCS, rather than the traditional CCI, which they used in their previous reports. The CCS differs from the CCI in that it assigns a score equivalent to the percentage increase in distance traveled around the color circle based upon the specific color cap replacement. Thus, it makes it difficult to compare across studies, and it is unclear why the authors chose to analyze data in this manner.

Another cause of concern is the analysis of longitudinal data in this study. The statistical models may not be appropriate, as repeated measures ANOVA was not used. Repeated measures ANOVA is generally a more powerful design, as it is based on within-subjects variance, providing a more precise estimate of experimental error. If repeated measures ANOVA was used here, it may have shown a significant difference between the three color vision assessment periods in terms of the CCS. It would have also allowed the investigators to control for age, although age adjustments appear to have been made. Additionally, when using vision testing at nearpoint within a longitudinal study, it is extremely important to control refractive status. The normal reduction in uncorrected near vision seen with age can greatly influence visual acuity and contrast sensitivity testing.

Gong and Coworkers (2002)[43]

The purpose of this report was threefold: 1) to determine the relation between current styrene exposure concentration and color vision, 2) to determine previous/cumulative styrene exposure and color vision, and 3) to determine the maximum level of previous styrene exposure and color vision. Patients were excluded from the control group if they had congenital color vision loss, eye disease (not specified), hypertension, diabetes, cerebrovascular disease, prior head trauma, ophthalmotoxic drug use, poor habitually corrected visual acuity, or alcohol consumption greater than 250 gm/week. Thus, a total of 57 styrene-exposed workers (all males) from a reinforced

plastic factory and a control group of 69 males were included in analyses. A history was conducted to examine work/lifestyle characteristics, previous solvent exposures, alcohol, cigarette, and drug use, and anamnesis.

From 1991 to present, workers were required to obtain physicals 2 or 3 times per year, and end-shift MA concentrations were measured during this physical. This allowed for the determination of a Cumulative Exposure Index (CEI), which was calculated based on the number of previous health-related physicals. Current exposure was also assessed by personal passive sampling monitors and end-shift MA concentration measures on Mondays (subjects were asked to abstain from drinking alcohol on these days). Urinary MA was corrected for urinary creatinine concentration. Color vision was assessed monocularly using the dD-15 test under a 1,200 lux fluorescent daylight lamp. Although the same examiner performed color vision testing to both groups, they were masked in regards to their exposure status. The CCI was used to quantify dD-15 test results. The Wilcoxon signed rank test, Wilcoxon rank sum test, Shirley-Williams test (a nonparametric test for comparing several dose levels with a zero dose control), and general linear model (GLM) were used to analyze the data.

Urinary MA concentrations were highest early on (≈40-60 ppm between 1991 and 1993), and decreased to about 25 ppm toward the end of the follow-up in 1998. The average styrene exposure period was 76.6 months and the average CEI was 77.1 months. The average current ambient styrene level in exposed workers was 49.90 ± 35.90 ppm, while the average MA level was 260 ± 350 mg/g creatinine, and the average level of PGA was 0.11 mg/g creatinine.

There were significant differences between styrene-exposed workers and controls in terms of age (29.3 vs. 38.3 years, $p < 0.01$), educational status (12.3 vs. 14.0 years, $p < 0.01$), cigarette smoking (11.9 vs. 8.0 cigarettes/day, $p < 0.05$), but not alcohol intake (89.9 vs. 77.6 gm/week, p = ns). As such, subjects were matched in terms of age (within ± 3 years), and 43 such pairs were obtained. Even after matching on age, there were still significant difference between the groups in terms of education and smoking status. Thus, these were controlled for in the general linear models.

The median CCI values for the age-matched groups were 1.31 (exposed) and 1.13 (unexposed), which significantly differed from one another ($p < 0.01$). No significant relations were found between the CCI and age, alcohol intake, cigarette use, education, MA plus PGA, or the CEI. In order to examine a possible dose-response effect, the exposed group was divided using a urinary MA plus PGA cut-point of 240 mg/g creatinine (10 ppm) and matched on age prior to analyses. The average (±SD) CCI value for the high dose groups was 1.14 ± 0.24, for the low dose group was 1.09 ± 0.13, and for the control group was 1.02 ± 0.04 (Shirley-Williams test, high vs. control $p < 0.01$, low vs. control $p < 0.01$). Another dose-response analysis was conducted in which subjects were categorized based on their maximal previous styrene dose. Those with over 0.85 g/g creatinine (> 50 ppm) were considered the high dose group, and the mean age- and alcohol-adjusted CCI value for this group was 1.00, compared to 0.95 for the low dose group ($p < 0.01$).

The authors conclude that styrene does lead to alterations in color vision discrimination, and given the somewhat low exposure levels in this study, the authors suggest that this is a factor in color vision loss. The authors also imply that MA plus PGA is a predictor of CCI loss, as it approached significance in the GLM. Finally, the authors suggest that doses as low as 10 ppm could lead to alterations in color vision compared to normals, and that one-time doses of greater than 50 ppm styrene could lead to impairments in color vision that do not resolve with time. In other words, the reversibility of color vision impairments due to styrene may be related to the maximum exposure dose rather than the cumulative dose.

Assessment

This article is well-written and the study appears to have been conducted in somewhat of a rigorous fashion, although there are several issues that cloud the conclusions suggested by the authors. The analyses suggesting that CCI values for the MA plus PGA group with exposures less than 240 gm/gm creatinine (the low exposure group) compared to the control group are not convincing. The median CCI value for the low exposure group was 1.03 (95% CI = 1.00 to 1.42, n = 29) while the median CCI value for the control group was 1.00 (95% CI = 1.00 to 1.15, n = 29). Similarly, the mean (± SD) values for these groups were 1.09 ± 0.13 and 1.02 ± 0.04, respectively. These mean CCI values for even the high exposure group are much lower than those found for the control group in other studies and other 'normal' individuals (Tables 12 and 14). The authors used the Shirley-Williams test to make comparisons of the mean CCI values between groups, suggesting that there were significant differences between both the high and low exposure groups compared to the control group. They base their conclusions that 10 ppm may lead to alterations in color vision on this analysis, although such current assessments may not reflect previous high-level exposures that may have induced permanent color vision defects. The regression

analyses showed for the right, left, and average of both eyes that MA plus PGA was not a predictor of color vision loss (although for the right eye, the p-value was 0.058 for this predictor, and the authors imply that this is significant). There is no real discussion about the discrepancy between analyses by the authors, and until such issues are resolved, one must cautiously interpret the results from this study. Additionally, we believe that the control group may suffer from somewhat of a selection bias; in particular, the inclusion of medical students in addition to employees of other manufacturing processes is not appropriate. The selection of a 'control' group in this type of study is of equal or greater importance when conducting a study of this nature than selection of the 'case' group. The control group must be representative of the sample from which the cases were derived, having the same characteristics as the cases except for the factor(s) of interest.[71-73] It is highly unlikely that the styrene-exposed workers were similar to medical students in many regards. Finally, Viaene has also questioned the result of Gong and coworkers, and has suggested that their calculation of a Cumulative Exposure Index is naïve, and "blurs the relation between CEI and CCI."[74]

DISCUSSION

The Effect of Styrene on Color Vision: General Issues and Threshold Levels

Determining a dose-response relation is a fundamental concept in toxicology. As such, it is an important assessment that needs to be conducted when evaluating the biological impact of chemicals such as styrene. Knowledge of a dose-response effect provides evidence of causality in terms of its observed effects and determines the lowest dose (i.e., the threshold dose) in which a toxic effect is observed. In this case, toxicity would be an alteration in color vision, outside of which is normal for an age-specific range. Consequently, one way to determine a threshold dose is to determine the point at which the body's normal ability to detoxify a chemical has been surpassed. The No Observed Adverse Effect Level (NOAEL) and Low Observed Adverse Effect Level (LOAEL) are important parameters in such analyses as they are associated with risk assessment. A dose-response curve would be the 'gold standard' assessment of the impact of styrene on color vision; however, an animal model in which various doses and a measured effect on color vision would be difficult due to the small, subjectively perceived differences in color vision that may occur. Therefore, the assessment of styrene's impact on color vision is generally performed using epidemiological study methods. These methods might include observational studies (i.e., cross-sectional, case-control, and longitudinal cohort studies), as it is probably not feasible to randomize individuals to a chemical such as styrene. Most of the studies of styrene and color vision are cross-sectional/case-control in nature, although sampling is generally conducted based on styrene exposure status with the expectation that these people will be 'diseased.' When using this somewhat of a meshed epidemiological design to study styrene's impact on color vision, a dose-response effect can be assessed in one of two ways. First, styrene-exposed individuals can be compared to 'normal' controls in terms of their color vision discrimination. Second, the 'dose' of styrene within the exposed group could be evaluated in relation to change in color vision. In doing so, the investigator may select cut-points for styrene exposure levels and examine color vision discrimination across the exposed groups or apply a regression procedure to describe and predict the relation between the variables (clearly, the correlation coefficient does not perform these functions).

Most studies evaluated in this literature review used a control group to compare color vision discrimination against the exposed group, and most found a small, but significant difference between the two groups (Table 12).[51, 54, 56, 57, 60, 62, 63] Seven studies showed that, compared to controls, styrene-exposed workers had significantly higher CCI values where the range of the CCI for the high exposure groups was 1.14 to 1.33, while the range of CCI values for the low exposure groups was 1.02 to 1.17. Studies tested for further dose-response type effects of styrene exposure by dichotomizing CCI values based on various styrene exposure levels, or through correlation and/or preferably, regression analyses in which styrene exposure was used to predict the CCI in the exposed sample. Six studies showed a relation between higher exposures (greater than 20-50 parts per million) and color vision deficiency, compared to control groups. Often, low exposure groups did not significantly differ from the control group, or population, age-specific normal values for the test. Two studies were able to show that styrene exposure levels were able to predict the CCI. Although this was the case, there was lack of sufficient detail or issues with the design in several studies which precludes their inclusion as evidence that styrene and acquired color vision deficiency are associated. Evidence for the association between styrene and color vision loss comes from the following studies: Gobba (1993), Campagna (1995), Eguchi (1995), Gobba (2000), Kishi (2001), and Triebig (2001). In particular, most of these studies support the notion that higher styrene exposure levels are associated with color vision discrimination loss.

Two specific studies associated with this literature review revealed surprisingly small apparent threshold doses associated with color vision loss.[59, 61] The study of Campagna and coworkers (1996) suggested that a dose as low as 4 ppm could induce color vision alterations that could be clinically detected, while Gong and coworkers (2002) suggested that a dose as low as 10 ppm was associated with such alterations. Although these levels are significantly higher than styrene levels found in the atmosphere, most studies have shown that styrene exposure induces color vision alterations in 'high' dose groups (i.e., greater than 20 to 50 ppm) compared with low dose groups.[53, 57, 60, 62] Because it is difficult to determine dose-response curves in human subjects, and thus, the threshold toxic dose associated with color vision deficiency, suggestions based on analyses like those of Gong and coworkers might be somewhat inappropriate. The analysis of Campagna and coworkers (1996) essentially fits two lines through the high and low exposure data, and determines the intersection point. Other studies that evaluated the low dose group relative to a control group generally show no difference in color vision discrimination. For these and other reasons discussed in the assessments associated with the review of these studies, we believe that these low limits of styrene exposure are unreasonable.

The question of the actual threshold associated with a change in color vision still remains. Although a cumulative review of the relevant literature suggests that a threshold dose of above 20 ppm appears to be associated with color vision loss, some might suggest that 50 ppm is a more reasonable estimate. However, one must use caution in suggest these values from a literature review as they are somewhat arbitrarily defined. A better approach might be to perform a meta-analysis/meta-regression to the data abstracted from the higher quality studies to determine a threshold value.

The application of cross-sectional/case-control research methods in studying occupational styrene exposure and color vision is problematic for several reasons, one being the potential measurement bias associated with the 'dose' of styrene received by workers. The dose of a substance is generally considered the amount of a substance given at any one time. In the case of occupational styrene exposures and color vision, there are several factors that need to be considered relating to the dose including the average level, peak level, and the duration of the exposure (e.g., work history). Because styrene is found in the air associated with various manufacturing processes, it is important to consider both the exposure dose (ambient styrene) and the absorbed dose (MA and/or PGA); truly, these assessments must be made not only in the present, but also in the past in order to determine the impact of styrene on color vision. Cross-sectional research designs generally address a specific dose (i.e., the current dose) in terms of the relation between magnitude of exposure and the outcome, although some investigators have tried to examine cumulative exposures (years or months) of exposure without much success. What cannot be addressed by cross-sectional methodology is the impact of previous peak doses of styrene on altered color vision. This seems to be an important part of the equation, as a very high peak dose might permanently alter color vision function in an individual, even though their current exposure dose is quite low. Thus, well-controlled, longitudinal designs clearly should be conducted in this regard.

Cross-sectional research designs also inhibit the investigator from establishing a sequence of events in terms of styrene dosing and color vision loss.[75] Although most studies reviewed indicate that they excluded patients with congenital color vision losses, there is generally no mention of the screening procedure used for such exclusion, and the reader is left to presume that this screening is by self-report. Research has shown that up to 18% of color vision defectives may be unaware of their color vision defect.[23] If appropriate screening for congenital defects was not conducted, this may have significant implications on CCI results.

Prospective study designs, which can assess changes in outcomes over time and factors that might explain that change, are considered the most rigorous of observational studies. The main advantages of using a prospective design to study this issue are that it allows one to ascertain incidence of disease, multiple risk factors associated with disease (including their interaction), and the ability to make more accurate measures of potential risk factors and confounders. For example, the interaction between smoking and styrene exposure could be synergistic, and these factors have not been thoroughly addressed in previous studies. Well-conducted, prospective studies are needed to help unravel the 'cause-and-effect' relation between styrene and color vision discrimination, which cannot be determined in these cross-sectional or case-control studies due to the unknown temporal relation between disease and exposures.

Other Substances and Color Vision Deficiency

Numerous substances have been identified through the years which have detrimental effects upon color discrimination. Often substances affect blue-yellow discrimination due partially to the especially vulnerable blue cones in the retina.[76] Because blue-yellow discrimination deteri-

orates "normally" with age, it is often difficult to document the toxicity of these agents separately from the aging processes. In addition, many ocular and systemic disease conditions have color vision deficits associated with them.

Aging. The measures of numerous visual functions (e.g., visual acuity, flicker fusion frequency, color discrimination) show similar age related changes. Sensitivity increases through the teens to early twenties, remains fairly stable for perhaps 2 decades, and then gradually deteriorates. Verriest and coworkers, Bowman and coworkers, and Muttray and coworkers have documented this relation with color vision using arrangement tests that measure fine color discrimination (see Tables 3 and 4).[78,79] The decrease in color discrimination with the elderly has been shown to be due primarily to the yellowing of the crystalline lens and causes discrimination losses principally in the blue portion of the spectrum.[22,77] The effect is exaggerated at low to medium light levels as senile miosis substantially reduces light levels on the retina.[78]

Ocular Disease Conditions. Type III color vision defects are shown for both glaucoma and diabetic retinopathy. Glaucoma color vision loss begins with a tritan-type defect that progresses in severe cases to include a red-green loss.[80] While the use of color vision tests has not proven very beneficial in glaucoma diagnosis, several studies have shown that visual field testing with a blue stimulus on a yellow background (Short Wavelength Automatic Perimetry - SWAP) may help document beginning visual loss. In diabetic retinopathy color discrimination loss can be great, again showing predominantly a tritan-type loss. As the condition progresses, red-green defects are found such that red-green pseudoisochromatic plates are also failed. The overall discrimination loss can be so great that an averaging technique is required with the FM-100 hue test to identify the blue-yellow confusion axis.[79]

A Type II color vision defect is typically shown for several conditions that affect the optic nerve. This is true for an optic neuritis due to demyelination (multiple sclerosis), inflammatory disease, or vascular disease.[80] A red-green color defect is also seen with Leber's hereditary optic atrophy. This condition is caused from abnormal mitochondria function and resulting degeneration of the optic nerve fibers.

Therapeutic Drugs. Numerous drugs have been reported to cause at least small changes in color perception. Because of this color vision testing is often used as an indicator of drug toxicity. Ethambutol is a drug used to treat tuberculosis. With prolonged use (or large dose short-term use) the optic nerve is affected creating a Type II red-green color vision defect. Digoxin, a cardiac glycoside, also can produce an optic neuritis and an associated Type II color vision defect. The color vision effects from both drugs are reversible after withdrawal from both agents. Quinoline derivative (e.g., chloroquine, quinine) are used prophylactically against malaria or for treatment of arthritis. The drug stimulate pigment deposition within the retina and initially produce a Type III tritan-type defect. Viagra is a drug used to treat erectile dysfunction. Transient effects of blue-tint to vision and mildly reduced color discrimination have been reported. Long-term oral contraceptives use has been reported to cause Type III tritan defects. An interesting side note is that with even short term use, oral contraceptives and caffeine in combination have produced reduced color discrimination.

Lifestyle. The use of tobacco has long been known to have toxic effects upon vision. Tobacco amblyopia was first described in 1868.[80] It is an optic neuropathy with demonstrates a central visual field loss and a Type II red-green color defect. The condition is often associated with nutritional deficiencies and responds favorably to vitamin therapy. More recently, tobacco use has been associated with cataracts and age-related macular degeneration (ARMD).[81-84] This is significant in that both these conditions have associated Type III tritan defects similarly to styrene toxicity. Concerning the ARMD, Hammond and coworkers has shown that cigarette smoking is associated with reduced macular yellow pigment which serves as protective role to potential hazardous effects of short wavelength light on the retina.[85] Muttray and coworkers showed an association between cigarette pack years and color discrimination loss which they attribute to subclinical macular degeneration changes.[29] Erb and coworkers noted an association between heavy smoking (20 or more cigarettes/day) and color vision discrimination loss; however, they were not able to determine the orientation of color vision loss.[86]

Chronic use of alcohol has also been associated with ocular changes with resulting color vision loss. In alcohol addiction malnutrition often plays a major role. Although the color vision findings are somewhat varied, it appears that Type II red-green defects are seen with extreme alcohol abuse. Mergler and coworkers studied the effects of alcohol consumption on fine color discrimination (dD-15).[87] While determination of the relative effects of the age and alcohol consumption could not be made from the published results, it appears that heavy alcohol use (> 750 g alcohol/week) significantly reduced color discrimination.[33] Adding to the confusion is that heavy alcohol use is associated with late ARMD, while moderate use has been reported to show mild protective effects to both ARMD,

cataract, and diabetic retinopathy. Of special note is a recent report that shows a possible interaction with alcohol and solvents (toluene, xylene, tricholorethylene, tetrachloroethylene). Consumption of more than 250 g alcohol/week in conjunction with solvent exposure showed significant detrimental color discrimination effects.[88]

Occupational Exposures. Color discrimination losses have been shown to occur after exposure to several chemicals used in industry. Iregren and coworkers (2000) provide a comprehensive review of the color vision effects for several of these toxins.[6]

Cavalleri and coworkers studied workers exposed to mercury vapor using the dD-15 test.[89] Workers with urinary mercury levels greater than 50 ug/g creatinine had significantly worse CCI (mean CCI = 1.285) than controls (mean CCI = 1.101). Urinary mercury level was correlated with CCI (r=0.448) for exposed subjects. Subjects were matched to controls by age, sex, alcohol, and nicotine. They reported the color confusions were mainly along the blue-yellow axis (Type III tritan defects). Cavalleri and Gobba (1998) reported that this effect was reversible after the occupational exposure was reduced.[90] One year after conditions had improved, color discrimination improved such that comparison to a control group showed no difference.

The effects of toluene on color vision have been reported in several recent studies. Zavalic and coworkers used the dD-15 test (CCI) to measure color discrimination in two groups of workers exposed to toluene and a control group.[91] One group had a high exposure (132 ppm), while the other had a relatively low exposure (32 ppm). The CCI scores were "corrected" for both age and alcohol consumption. The results of the study showed that the actual CCI value for the higher exposure group and the age and alcohol corrected CCI values for both exposure groups were significantly higher for both the high and low exposure groups than for the controls. The authors did not report on the predominant "axis" (i.e., red-green or blue-yellow) of confusion or even if one existed for their subjects. Results from this study are shown in Table 6. The authors conclude that toluene can impair color vision even with concentrations below 50 ppm.

Scientific concerns for this study involve the different gender make-up of the three comparison groups and how congenital color vision defectives were eliminated from the subject pool. The lower exposure group was predominantly female (93%). It is estimated that 15% of females are heterozygous for congenital red-green color vision defects and will have reduced color discrimination.[19] This would artificially raise the CCI for this group relative to the control group. Additionally, it is not stated how congenital color vision defectives were identified to be eliminated from participation. Slightly fewer subjects were eliminated from the study than is expected from general population estimates. These concerns create doubt for the significance of the toxic effects on color vision for the lower exposure test group.

TABLE 6

Toluene exposure and the CCI (Zavalic and coworkers, 1998).[91]

Exposure Group	CCI	p value*
High (mean 132 ppm, n = 32)	1.29	< 0.001
Low (mean 32 ppm, n = 41)	1.17	0.205
Controls (n = 83)	1.15	—

*p-values determined by comparison of exposed group with the control group.

Cavalleri and coworkers also studied toluene effects on color vision as compared against controls and showed significant differences between groups in both the CCI and the Total Confusion Index.[92] It was reported that the type of color confusions was primarily blue-yellow. The overall results are summarized in Table 7. Estimation of toluene exposure was done by measurement of urinary excretion of unmodified toluene. The mean toluene exposure was calculated to be 42 ppm. Subjects with high alcohol intake, smoked over 30 cigarettes/day, had poor visual acuity, or had a congenital color vision defect were eliminated from the subject pool. In spite of these limitations, the test and control subjects did have a significant difference in alcohol consumption, although as subjects with high alcohol intake were eliminated from the subject pool, the authors downplayed this difference. Additionally, no mention was made of the gender of sub-

TABLE 7

Toluene and CCI and TOTCI (Cavalerri and coworkers, 2000).[92]

Exposure Group	CCI	TOTCHI	p-value
Exposed (n = 33)	1.29	1.49	< 0.01
Controls (n = 16)	1.10	1.16	< 0.001

ject participants nor was it explained how congenital color vision defectives were identified.

Functional Significance of Color Vision Deficiency

In order to determine the functional significance of styrene induced color vision defects, a typical value for this effect must be specified. Most of the studies in this area used the dD-15 test and analyzed results using the CCI. For researchers that published mean values, the mean CCI for the styrene exposed subject populations never exceeded 1.30, even for the high exposure groups. The highest mean values were reported by Gobba (1991, CCI = 1.26), Gobba (2000, CCI = 1.29), Kishi (2001, 1.28), and Triebig (2001, 1.29). Chia and coworkers did report a mean equivalent CCI of 1.40 for the styrene exposed population; however, it was apparent that incorrect methods were used to calculate the TCDS.[30, 31] Therefore, for this analysis, those results will not be included.

Table 8 provides CCI scores for several sample replacements on the Lanthony Desaturated D-15 test. It appears that a CCI score of 1.30 can be achieved by making at least 1) three one-place replacement errors, 2) one two-place replacement error with a one place error, or 3) one major error across the diagram. As noted previously, the CCI scoring calculates the distance in CIE-LAB color space that is traversed from the reference cap to the final cap in the tray after replacement by the subject. CIE-LAB color space was developed based upon perceptual differences in color. The distance from one cap to the next is proportional to color perceptions by color vision normals. As reported by Sheedy, the total distance traversed with perfect replacement for the Farnsworth D-15 is 2.07 times that for the dD-15 test.[33] As each test contains 16 total caps, the average "distance" between caps in the two tests would also vary by this same ratio.

TABLE 8

Sample CCI score for specific cap replacement sequences Lanthony Desaturated D-15.

dD-15 Cap Replacement Order	CCI
Ref-1-3-2-4-5-6-7-8-9-10-11-12-13-14-15	1.12
Ref-1-4-3-2-5-6-7-8-9-10-11-12-13-14-15	1.21
Ref-1-4-3-2-5-6-7-8-9-10-11-13-12-14-15	1.30
Ref-1-15-2-3-4-5-6-7-8-9-10-11-12-13-14	1.30
Ref-1-3-2-4-5-7-6-8-9-10-11-12-13-15-14	1.36
Ref-1-15-14-2-3-13-12-4-5-11-10-6-7-9-8	2.35

For the dD-15, if an individual that makes a single major misplacement with return (e.g., from cap 1 to cap 15 to cap 2) with perfect replacement following, the CCI would be approximately 1.3 (see Table 8). As only one major error was made, it is expected that the distance across the color circle would be near the subject's just-noticeable-difference (jnd) in color. That is, if the subject's jnd were much larger than the diameter of the color circle, it would be expected that several major crossings would be made, for example, from caps 1 to 15 to 2 to 14 to 3 etc, resulting in an extremely large CCI. Therefore, along this axis (from cap 1 to 15) the subject's jnd may be expected to be approximately the diameter of the color circle for the dD-15 in CIE-LAB color space. This individual would not be expected to make a crossing error on the Farnsworth D-15 as its color circle is 2.07 times that of the dD-15. An individual with a 1.30 CCI on the dD-15 would not, therefore, be expected to make crossing errors on the Farnsworth D-15 test. Color vision defectives which pass the Farnsworth D-15 are considered mild and would not be expected to have difficulty with color identifications in an industrial setting.

For the CCI values for the 22 known color vision defectives listed in Table 3, none of the individuals with a CCI less than 1.30 failed the Farnsworth D-15. All of these individuals were anomalous trichromats and would be considered very mild defectives. Additionally, all these individuals demonstrated good color discrimination by having anomaloscope matching ranges of 10 units or less.

As previously noted, Steward and Cole have documented by survey the difficulties encountered by 102 color vision defectives.[23] For the anomalous trichromat results (n = 65) mentioned in the study, only 2 items were mentioned where over 30% of the participants reported to have difficulties. These items were 1) selecting colors of clothes, cars, carpets, etc. and 2) confusing traffic signal traffic lights with street lights. The color discrimination situations mentioned by mild anomalous trichromats of which difficulties are found are those where the individuals cannot see the mild amount of color in a desaturated stimulus. For example, a green traffic signal may appear white, or the green in a grey-green car may not be seen. The significance of the confusion with the green traffic signal may not be great in most circumstances as positional cues are present to aid in traffic signal color identification.

In summary, the level of color discrimination demonstrated by the styrene exposed individuals showing statistically significant color defects are mild compared to congenital color vision defectives. The performance of these individuals in recognizing color or in making color

decisions in everyday activities would not be expected to be subnormal in a functional sense.

Recovery from Color Vision Deficiency

There are several types of toxicity that may be associated with styrene toxicity as it related to color vision deficits. Acute toxicity generally occurs immediately following an exposure to a toxin (i.e., within hours or days). An acute exposure is one that is dosed within a relatively short period of time (i.e., one or several doses within a 24 hours period). Subchronic toxic effects result from repeated exposures to a toxin over a period of one to several months, and this is the most common exposure route for environmental toxins. Finally, chronic toxicities result from repeated exposures to a toxin over a period of several months-to-years resulting in cumulative damage to a tissue or organ structure. Although damage from chronic exposures may be sub-clinical for an initial period of time, the damage may become so severe as toxicity progresses that the tissue or organ no longer functions normally. It is likely that styrene exposures are somewhat chronic in this regard, although it is not known if the toxicity to the ophthalmic system (color vision alterations in this case) is permanent or temporary. In other words, the permanency of color vision impairment after styrene exposure is still in question.

Gobba and Cavalleri (1993) were the first to evaluate the issue of color vision recovery following styrene exposure in a group of workers.[55] In their study, a group of 39 styrene-exposed workers were examined with the dD-15, showing a mean CCI of 1.23 ± 0.19. One month later with no styrene exposure (they were on summer holiday), the workers were examined again on the first of returning to work, and their mean CCI was 1.20 ± 0.21. The authors report that these values did not differ.

In another study, Mergler and coworker (1996) also evaluated the impact of styrene exposure reduction on color vision deficits.[61] Baseline measures of styrene and color vision were established, and local ventilation systems were established in order to reduce styrene exposures. Reassessment of these outcomes was measured again on the same baseline workers two years later. The results from this study can be found in Table 9.

The authors then state that there was a significant reduction in ambient styrene ($p < 0.05$) and end-shift MA ($p < 0.001$) after two years of follow-up for workers in plant 3, but not plants 1 or 2 (data are not presented). They then report mean differences in CCI values (Baseline – 2 year follow-up) for plants 1 and 2, and plant 3 separately. The mean difference for plants 1 & 2 was –0.12 ± 1.17 (p-value: not significantly different from zero) and for plant 3 was 0.20 ± 0.78 (p-value: not significantly different from zero). Finally, the authors stratified mean differences in CCI values using the criteria based on change in MA between baseline and the 2 year follow-up as follows: increase by 0.1 mmol/mmol creatinine, remained within ± 0.1 mmol/mmol creatinine, and decreased by 0.1 mmol/mmol creatinine. Spearman's correlation coefficient was used to examine the relation between the respective group and the mean differences. No data are presented other than schematically, but the authors report a significant correlation ($p < 0.001$). Thus, the authors conclude that "the strongest relation [associated with styrene reduction] is with color vision." This is somewhat of a stretch, and the author certainly had to statistically hunt to find relation between styrene reduction and color vision improvement (i.e., there were no statistical differences in the mean differences between visits for the CCI). Correlation coefficients are particularly sensitive to sample size; thus, reporting only a p-value is inappropriate, as it does not allow the reader to examine the actual magnitude of the correlation.

A study by Gobba and Cavalleri (2000) more recently addressed this issue.[56] After an initial assessment, styrene-exposed workers were re-examined 12 months later. Results from this study are found in Table 10.

TABLE 9

Overall results from Mergler and coworkers (1996).[61]

Outcome	Baseline	Time 2*	p-value
Ambient styrene (all 3 plants)	75 mg/m3	76 mg/m3	Not Provided
End-Shift MA (all 3 plants)	0.11 mmol/mmol creatinine	0.13 mmol/mmol creatinine	Not Provided
CCI (n = 57)	1.18 ± 0.20	1.19 ± 0.35	ns

*Time 2 is a follow-up assessment performed two years after baseline following installation of ventilation systems.

TABLE 10

Results from Gobba and Cavalleri (2000).[56]

Outcome		Baseline	Time 2*	p-value
Group 1	Ambient Styrene	10.9 ppm	16.2 ppm	> 0.05
	CCI	1.18 ± 0.16	1.29 ± 0.21	0.08
Group 2	Ambient Styrene	14.4 ppm	10.2 ppm	> 0.05
	CCI	1.27 ± 0.18	1.29 ± 0.26	> 0.05

*Time 2 is a follow-up assessment performed one year after baseline.

The authors suggest, based on these analyses, that "These results suggest a progression in color perception impairment if exposure is increased, even if the difference in CCI values was not significant." We disagree. The results of Gobba and Cavalleri (2000) are inconclusive regarding the reduction or improvement of color vision in relation to small changes in ambient styrene. This may be because, again, this is cross-sectional research, and exposure and disease status are measured at the same time. We have no idea about the peak styrene levels occurring in the workplace throughout the 12 month follow-up period. Thus, it is impossible to draw any conclusions from this study.

A follow-up study to Mergler and coworkers 1996 report (based on data from 1990 and 1992) was conducted by Castillo and coworkers (2001).[32] In this study, a third follow-up assessment of color vision and styrene exposure was conducted in 1999, and reported in 2001. In this assessment, only 18 of the original participants were included and the authors analyzed color vision testing using the CCS rather than the CCI for some unknown reason. This is confusing as CCS outcomes are reported as 100 times the percent above the distance traveled in color space for perfect replacement; it appears as though CCI values were reported by the authors. This leads one to question the result and conclusions. Scores for the three assessment period (1990, 1992, and 1999) are reported figuratively, and are now age-adjusted. Median values obtained from this figure are as follows (year data obtained is in parenthesis): 1.07 (1990), 1.045 (1992), and 1.16 (1999). The authors report a significant improvement between 1990 and 1992 ($p < 0.05$), but no difference between 1992 and 1999 ($p > 0.05$). Again, the results of this study are completely in question, and no conclusions should be drawn.

Finally, Triebig and coworkers (2001) examined the effect of altered styrene exposure on color vision discrimination.[62] In this study, baselines assessments of exposure and color vision were made, and these same workers were then again examined after a four week vacation. In a second phase, the workers were again reassessed 10 months later following the installation of ventilation sys-

TABLE 11

CCI results from Triebig and coworkers (2001).[62]

Phase	Test Day: Monday Morning	Thursday After Work	After 4 Week Vacation	p-value A vs. B	p-value B vs. C
Baseline (MA + PGA = 472 mg/g creatinine)	1.24 ± 0.25	1.29 ± 0.27	1.11 ± 0.11	0.28	0.01
10 Months Later (MA + PGA = 273 mg/g creatinine, p = 0.02 compared to baseline)	1.11 ± 0.11	1.16 ± 0.14	1.05 ± 0.06	0.06	0.01
p-value: Baseline vs. 10 Month Follow-Up	0.01	0.01	0.01		

tems. A control group was initially included, but not retested after in the follow-up periods. The results from this study can be seen in Table 11.

This is the first study to provide substantial evidence that color vision alterations are associated with reductions in styrene. This indicates that in some regard, these color vision alterations may not be complete. The authors indicate that the styrene-exposed workers included in this study had at least 6 months of experience, although they do not report an estimated cumulative work history associated with styrene exposure in these workers. The authors suggest that the finding here may be different from other studies because stricter alcohol consumption exclusion criteria were employed. Regardless, there seems to be no methodological or reason otherwise to doubt there finding.

The evidence regarding the recovery of color vision following reductions in exposure is too inconclusive at this point to make any firm recommendations regarding the issue. As discussed, several studies have found negative or weak results, although this may have been due to methodological or other issues. The study by Triebig and coworkers (2001) does provide evidence for an effect, and other studies need to replicate this finding prior to conclusions being drawn.

Physiological/Anatomical Basis for Color Vision Deficiency

In his review of the effects of styrene exposure on color discrimination, Sheedy makes a compelling case that the site of initial damage for styrene's detrimental effects upon color discrimination to be within the retina.[33] He bases his argument on styrene's predominantly blue-yellow nature of the color discrimination loss and that components of the electroretinogram appear to be affected. The studies which have been performed since his review have strengthened his argument.

Köllner observed early in the 20th century that tritan (blue-yellow) type defects were common with conditions that affect the outer portions of the retina, while red-green type defects were common with those affecting the visual pathway (inner retina and optic nerve). While several exceptions to this rule have been documented, his observations have generally withstood the test of time.[93] The reason for this is the relative vulnerabilities of the blue visual mechanism in the retina and the red-green chromatic processing of the parvocellular system within the optic pathway.[94]

In this regard, studies investigating the initial effects on color vision of styrene exposure on larger number of subjects have generally described blue-yellow type defects. Sheedy reported that Campagna and coworkers, Eguchi and coworkers, Gobba and coworkers (1991), and Gobba and Cavalleri all found predominantly blue-yellow type defects.[33, 53, 55, 57, 95] He also reported that Mergler and coworkers reported a high incidence of mixed dyschromatopsia (both red-green and blue-yellow) but predominantly for the highly exposed workers.[57] This is somewhat expected as typically acquired color vision losses begin with confusion along a given axis but as the condition progresses the orientation of losses become less defined. More recently Triebig and coworkers reported color discrimination losses with styrene without a predominant red-green or blue-yellow orientation.[62] It should be noted, however, that these reports of orientation of color confusion are subjective in nature as the CCI evaluation method of color discrimination provides no objective measure of predominant color system loss.

The electroretinogram (ERG) has been used to evaluation electrical activity in the retina of individuals exposed to styrene. The ERG is a non-invasive method to measure the relative strength of the visual signal as it progresses through the retina. Several studies using the electroretinogram have shown abnormal retinal activity after styrene exposure. Skoog and Nilsson (1981) studied the acute effects of toluene and styrene on the ERG on anaesthetized monkeys.[96] Both agents affect the c-wave of the ERG and the standing potential of the eye. The c-wave is believed to be generated by potential changes within the pigment epithelium of the eye. The pigment epithelium is intimately involved in metabolic support of the retinal photoreceptors. As this study investigated the acute effects of styrene and the route of administration was intravenous, the generalizability of the results to chronic exposure to humans in industry are limited.

Mirzoev and Sultanov (1989) studied the ERGs of workers exposed to high levels of styrene.[95] Their results showed reduced a-wave and b-wave activity and long latency. The a-wave is believed to indicate photoreceptor activity. This is a significant finding to show styrene is toxic to the outer retina. As Sheedy notes, however, the study failed to control for alcohol and/or tobacco effects.[33]

The most compelling evidence to date to implicate changes in the retina to cause styrene induced color vision loss is a recent study on changes in amacrine retinal cells in rats after styrene exposure.[96] Ten rats were exposed to an atmosphere of 300 ppm styrene for 6 hours/day, 5 days/week for 12 weeks. Ten control rats were exposed to fresh air. Exposed rats showed amacrine cell loss and a related depletion of dopamine. Dopamine in inherently involved in the lateral processing of information across the retina (through horizontal cells) and has been shown to play a role in color perception.[97, 98]

In summary, evidence continues to mount to implicate the retina as the site of damage causing the color discrimination losses with chronic styrene exposure. While amacrine cell loss has been implicated by the Vettori and coworkers on rats, detrimental effects upon the pigment epithelium, photoreceptors, and other retinal structures can not be ruled out.[56]

SUMMARY

The impact of styrene on color vision has been assessed by numerous investigators over the past 10-12 years. The studies since the report by Sheedy have supported the evidence that exposure to styrene can cause detrimental effects upon fine color discrimination. Overall, this result is best supported by the work of Gobba and coworkers (1993), Campagna and coworkers (1995), Eguchi and coworkers (1995), Kishi and coworkers (2001), and Triebig and coworkers (2001).[35, 57, 58, 60, 62] Although there is no single, high quality study which confirms a causative association between styrene and acquired color vision deficits, taken together, these studies provide moderate evidence of the association. However, no study evaluated was perfect, and flaws were inherent in all of them. Although studies of this type of occupational exposure and biological effect are not easily conducted, some methodological, statistical, or other issue precluded the remaining studies from being included among those supporting the relation in our opinion.

The new data provide little evidence supporting a threshold effect at exposure levels below 50 ppm. We believe that higher doses (i.e., greater than 50 ppm) of styrene are associated with alterations in color vision compared with normal controls. In general, several studies showed a significant difference between the high exposure group relative to the control group or a significant linear relation between ambient styrene or MA and the CCI (Table 14). As suggested by others, we believe that the small amount of evidence accumulated to date suggests that styrene impacts color vision by altering retinal function—specifically, the amacrine cells. More recent studies have supported previous claims that the detrimental effects on color vision are at least partially caused by changes within the retina and that the detrimental color vision effects of styrene may be temporary.

However, the exact dose and mechanism of color vision loss associated with styrene is still in question, as is the proximate toxicant (styrene or a metabolite), and the dose metric (peak vs. metric). Other questions also remain. It remains uncertain as to the degree of restorability of the slight loss in color vision, and if there are more susceptible subgroups (e.g., elderly, smokers). Also, it is not known what the impact of an acquired color vision deficit of this magnitude would have on an individual functional status. Future research is needed to address these and other questions raised in this report.

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APPENDIX I

Color Confusion Index

In numerous studies which investigate the toxic effects of chemicals upon color vision, the Lanthony Desaturated D-15 Panel Test is used to assess fine color discrimination. Small caps of different colors are replaced into a tray according to color. The colors vary in hue from blue around the color circle to purple. The order with which the caps are replaced gives an indication of which colors may be confused.

Bowman (1982) recommended a scoring method for arrangement tests by calculating the total distance in color space represented by moving from cap to cap for each specific replacement order. Color confusion index (CCI) is than calculated by dividing this total by the distance representing perfect replacement. In his paper Bowman provides color space distance values when going from any given cap to any another. These values, however, are based upon the locations within color space for the Farnsworth D-15 test. Values for the Lanthony Desaturated D-15 test were not listed.

The Lanthony Desaturated D-15 is similar to the Farnsworth D-15, however, with the Lanthony test, the caps are lighter and possess less color (i.e., more white). Figure 1 shows the Farnsworth D-15 and Lanthony Desaturated D-15 positions in CIE-LAB color space. Although the Lanthony test is a desaturated version of the Farnsworth test, the color space locations are not exactly equivalent even in relative terms. Therefore, errors in analysis can occur if the distances used in calculating the color confusion index are not from the appropriate test.

In order to determine the degree of error which could be introduced, color confusion indices were calculated for sample Lanthony Desaturated D-15 results using the appropriate locations and using the Farnsworth D-15 equivalents. Results for 25 subjects with varying degrees of color discrimination were analyzed. The CCIs using the two methods are presented in Table 1. In general, using the Farnsworth color space positions for each cap instead of the Lanthony color space positions overestimates CCI when compared to the actual value and the degree of overestimation increases with CCI. However, for results showing few errors (i.e., CCI ≈1), the Farnsworth analysis can overestimate or underestimate the actual value. Figure 2 shows the Farnsworth calculated CCI graphed versus the actual values. Figure 3 shows the ratio of the two values graphed versus the actual value. For low CCIs, there is considerable variability of estimation shown. Overestimated and underestimated

TABLE 12

CCI results from studies assessing impact of styrene on color vision.

	Study Characteristics			Matched OR Adjusted CCI Values		
Study	Control Group	Average or Median Ambient Exposure	Average or Median MA Level	Case	Controls	p-value
Gobba, 1991	Y	16.2 ppm	0.34 g/L	1.26 ± 0.22	1.15 ± 0.14	< 0.01
Gobba, 1993	Y	≈ 34 ppm[a]	≈ 0.48 g/L[a]	1.21 ± 0.20	1.053 ± 0.07	< 0.001
Chia, 1994	Y	Not Measured	84.0 mg/g creatinine	1.40	1.13	0.0006
Campagna, 1995	N	48.3 ppm	0.36 mmol/ mmol creatinine	1.14 ± 0.16	Not Conducted	
Eguchi, 1995	Y	18.5 ppm	0.22 g/l	1.22 ± 0.24	1.12 ± 0.13	< 0.01
Mergler, 1996	Y	18 ppm[b]	0.12 mmol/ mmol creatinine	1.18 ± 0.20	Not Conducted	
Campagna, 1996	N	≈ 16 ppm[a]	Not Measured	1.16[c]	Not Conducted	
Gobba, 2000 (2 case groups, time periods)	Y	13 ppm		1.24 ± 0.21	1.14 ± 0.14	< 0.01
	N	T_1: 11ppm[f] T_2: 16 ppm[a] n = 10 T_1: 14 ppm[f] T_2: 10 ppm[f] n = 20	Not Measured	T_1: 1.18 T_2: 1.29 T_1: 1.27 T_2: 1.29	NA	0.08 > 0.05
Kishi, 2001	Y	Not Measured	0.21 g/L	1.28 ± 0.28	1.18 ± 0.18	< 0.01
Triebig, 2001 (Baseline)	Y	Not Measured	MA + PGA: 0.472 g/g creatinine	Mon: 1.24 Thur: 1.29	Mon: 1.10 Thur: 1.10	Mon: 0.11 Thurs: 0.05
Triebig, 2001 (10 months later after intervention)	Y	Not Measured	MA + PGA: 0.273 g/g creatinine	Mon: 1.11 Thurs: 1.16	Mon: 1.10 Thurs: 1.08	Mon: 0.61 Thurs: 0.18
Gong, 2002	Y	Not Measured	MA: 0.26 g/g creatinine PGA: 0.11 g/g creatinine	1.04[b]	1.00[b]	< 0.01

[a]Mean value reported., [b]Median value reported., [c]Not reported., [d]Represents the average of measures taken from Monday and Thursday; preshift MA measures., [e]Represents the average of the two study sample (Italian and Canadian)., [f]T_1 and T_2 represent baseline and 12 month outcome assessments.

TABLE 13

Results from studies using alternative methods of assessing color vision tests or scoring algorithms.

	Study Characteristics			Matched OR Adjusted Color Vision Scores		
Study	Control Group	Average or Median Ambient Exposure	Average or Median MA Level	Cases	Controls	p-value
Fallas, 1992	Y	24.3 ppm	MA: 0.23 g/g creatinine PGA: .057 g/g creatinine	259.9 ± 136.9 Farnsworth 100 Hue Error Score	262.7 ± 114	NS
Castillo, 2001	N	Overall Not Reported	0.08 mmol/mmol creatinine	1.17 (Color Confusion Score?)	Not Conducted	

TABLE 14

Dose-stratified CCI comparisons and regression analyses evaluating the dose-response effect of styrene on color vision.

Study	High Dose Group	Low Dose / Intervention Group	Control Group	p-value	Criteria
Gobba, 1991	1.27[b]	1.09[b]	Not Compared	< 0.05	215 mg/m³
Gobba, 1993	1.29	1.11	Not Compared	< 0.05	213 mg/m³
Chia, 1994	"No Significant Linear Correlations were present between TCDS and MA or PGA"				
Campagna, 1995	"Significant positive relations were found between the internal and external styrene exposure measurements and color vision loss adjusted for age, alcohol consumption, and seniority…"				
Eguchi, 1995	1.33 ± 0.29	1.17 ± 1.19	1.12 ± 0.13	H vs. C: < 0.01 L vs. C: = 0.12 H vs. L: < 0.05	MA < 0.42 g/l
Mergler, 1996	1.18 ± 0.20	1.19 ± 0.35	Not Compared	> 0.05	Intervention group included original workers after installation of ventilation.
Campagna, 1996	"Color Confusion Index (adjusted for alcohol consumption and age) exhibited positive relationships with environmental styrene exposure (Spearman r = 0.25, p < 0.01)"				
Gobba, 2000	"…the exposure was unmodified or slightly decreased in 20 subjects, and D-15 outcomes remained unchanged, while styrene levels had increased and color vision loss progressed in the other 10."				
Kishi, 2001	H: 1.27 ± 0.27 C: 1.13 ± 0.14 p < 0.01	L: 1.21 ± 0.26 C: 1.17 ± 0.19 p > 0.05			L: < 0.10 g/L M: 0.1-0.2 g/L H: > 0.2 g/L
Triebig, 2001	1.24	1.11*	NA	0.01	*4 week vacation—original cases
Gong, 2002	1.14 ± 0.24[a]	1.09 ± 0.13[a]	1.02 ± 0.04[a]	All < 0.01	MA + PGA < 0.24 g/g creatinine

[a]Mean value reported., [b]Median value reported.

values are shown depending upon the specific errors of replacement which were made. For this sample, once the actual CCI reached 1.25, the CCI using the Farnsworth values always overestimated the actual value. For the few subjects in this study with CCI below 1.25, the mean difference in the two calculations was only 0.0089 +/- 0.039. For CCI values at 1.25 and above, the mean difference was 0.12 +/- 0.090.

From this investigation it appears that the error introduced by using the Farnsworth test color space positions instead of those for the Lanthony test is small; however, the error appears to increase with CCI above CCI = 1.25. How these small errors could affect a study's results are difficult to determine without having the actual data to analyze. However, it is logical to assume that if the exposed group had higher CCI's, the incorrect calculation could magnify (slightly) a small difference. Also, for CCI's just above 1 (and below 1.25), the incorrect calculation could add variability and help "hide" a small but significant difference between control and exposed groups.

TABLE 1

Lanthony Based Analysis "Correct"	Farnsworth Based Analysis "Incorrect"
1.04	1.05
1.04	1.05
1.10	1.07
1.12	1.10
1.14	1.18
1.15	1.19
1.16	1.15
1.21	1.29
1.22	1.18
1.25	1.32
1.37	1.43
1.48	1.58
1.74	1.76
1.89	1.95
2.30	2.37
2.35	2.51
2.38	2.58
2.41	2.45
2.48	2.57
2.50	2.56
2.79	2.91
2.81	2.87
3.02	3.30
3.07	3.27
3.21	3.54

FIGURE 1

Farnsworth D-15 and Lanthony Desaturated D-15 Tests graphed in CIE-LAB Color Space.

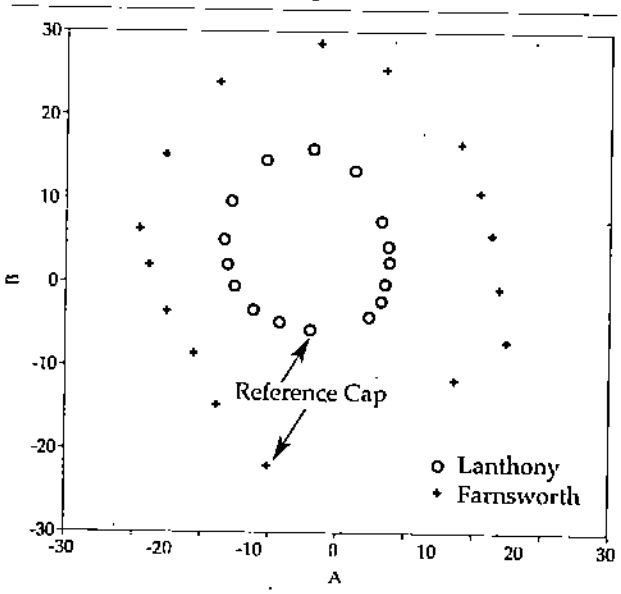


FIGURE 2

Farnsworth Calculated vs. Lanthony Calculated CCI.

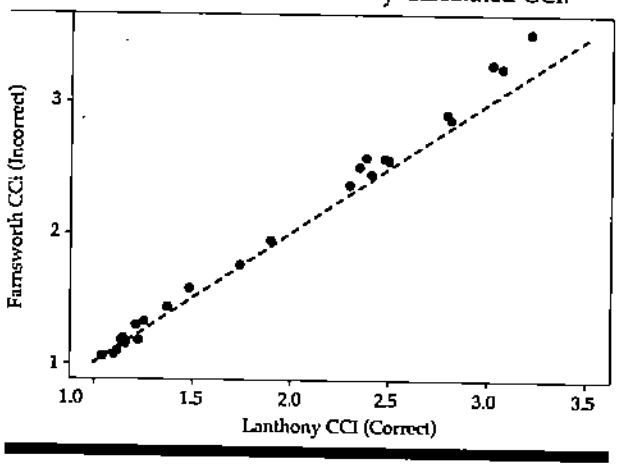
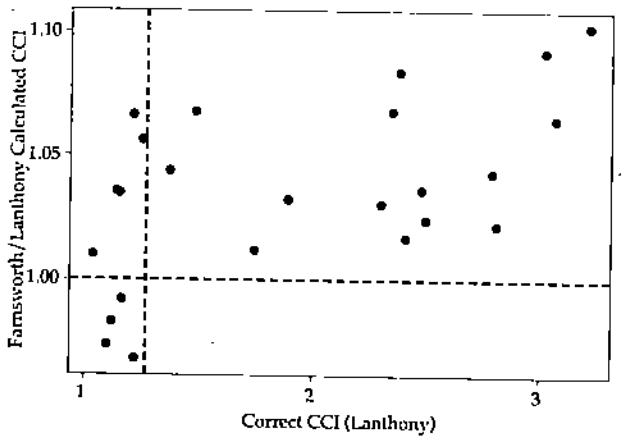


FIGURE 3

Ratio of Farnsworth Calculated vs. Lanthony Calculated CCI graphed versus the correct (i.e., Lanthony Calculated) CCI.



Quality of Life and Color Vision: The Significance of Acquired Dyschromatopsias

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EXECUTIVE SUMMARY

The effect of styrene exposure on color discrimination has been assessed by numerous investigators over the past 10-12 years. There is moderate consensus that high doses of styrene (i.e., greater than 50 ppm) are associated with acquired color vision deficits. These acquired deficits, however, are mild when compared to the color discrimination difficulties exhibited by congenital color vision defectives. Quality of Life instruments have been recently developed to help determine not only the functional deficits that accompany disease but also the individual's general well-being. As such, numerous instruments are available which are used to augment the more classic measures of individual health. Presently, however, there is no color vision specific quality of life instrument; and, of the instruments that are presently used, few questions relate at all to color discrimination ability. Studies have been done, however, in which individuals with congenital color vision defectives are questioned concerning the effects of their defects on life activities. It is only those individuals with relatively severe deficits, however, that report any substantial detrimental effects in everyday life. In summary, there is lack of information and studies that have assessed the significance of acquired color vision deficiencies on quality of life and everyday tasks. As the color discrimination losses accompanying styrene exposure are relatively mild, we believe that a study that assessed the significance of styrene-induced color vision deficiency might show little-to-no impairments in quality of life and functional ability. At present, there is not an appropriate color vision-specific quality of life survey instrument that could be used for this type study.

As the color discrimination losses accompanying styrene exposure are relatively mild, we believe that a study that assessed the significance of styrene-induced color vision deficiency might show little-to-no impairments in quality of life and functional ability.

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INTRODUCTION

The effect of styrene exposure on color discrimination has been assessed by numerous investigators over the past 10-12 years. There is moderate consensus that high doses of styrene (i.e., greater than 50 ppm) are associated with acquired color vision deficits.[1-5] Recent studies have supported previous claims that the detrimental effects on color discrimination are at least partially caused by changes within the retina, and that the detrimental color vision effects of styrene may be temporary.[6] The threshold dose and mechanism of color vision loss associated with styrene, however, is still in question, as is the degree of restorability of that loss. Although there are many specific questions that remain to be answered regarding the relation between styrene and

color vision loss, the actual significance and handicap of the associated dyschromatopsia on an individual's function in society and quality of life has not been considered. The purpose of this report is to examine the scientific literature in order to provide a better understanding of the potential significance that this and other acquired and/or congenital color vision deficiencies may have on an individual's quality of life.

HEALTH, DISEASE, AND QUALITY OF LIFE

Traditional concepts of health-care generally reflect a "disease-based" model. This is somewhat a medical approach whereby pathology is reflected by signs and/or symptoms of disease. Often, ill-health is reflected by a symptom or a change in 'usual' function noted by the patient. There are several traditional measures of health which include population-based outcomes (e.g., mortality and morbidity rates), individual indicators (e.g., laboratory/diagnostic tests, patient/clinician judgments, behavioral data), and service utilization rates. Today's concept of health and disease, however, has changed such that symptom response or survival rates are no longer enough to describe the health status of an individual or population. With the average life expectancy in the United States at 76.2 years, society is faced with chronic health conditions than ever before.[7] These include conditions such as cancer, heart disease, stroke, and diabetes; previously, these conditions were associated with significantly shorter periods of survival.

In 1948, the World Health Organization (WHO) defined health to be "a state of complete physical, mental and social well-being, and not merely the absence of disease."[8] The formation of this organization and its all encompassing definition of health shaped the way views on health and disease changed through next 50 years. Although the definition guides us in our views of 'health,' in reality, it is somewhat complex and abstract. For instance, how do you measure such a construct? Typically, scientific measures occur through the application of a standard scale to some variable of interest. In the case of 'health,' what do we use as the standard scale and what variables do we measure? This is not an easy task, and researchers and practitioners have come to rely on the use of health indicators to make such assessments. These health indicators represent elements of the concept of overall health, and there has been an evolution in their development since 1948. As previously discussed, health indicators originated as concepts such as survival and disease, but moved to concepts such as freedom from disease, ability to perform daily activities, and more recently, positive themes such as happiness, social and emotional well-being, and cognitive function. Because many of these outcomes are subjective in nature, surveys and self-reported instruments are required in order to make assessments. The main advantage to using these outcomes when defining health is that they provide insight into matters of human concern such as pain and suffering, depression, cognition, functional ability, emotion, and motivation. Because they are subjective, however, there is concern that they are too reflective of response bias.

The term 'quality of life' means many things to many people. Research in this area spans numerous topics including things like geography, urban planning, advertising, social science (e.g., sociology and psychology), and medical science. It is somewhat of an ill-defined term, as there is no universally accepted definition. Grant (1990) suggested that quality of life is 'a personal statement of the positivity or negativity of attributes that characterize one's life.'[9] For instance, general quality of life may encompass dimensions such as adequacy of housing, income, and perceptions of one's immediate environment. Those involved in health-care delivery, research, or utilization are typically concerned with 'health-related quality of life.' In some sense, however, health itself is a dimension of quality of life, rather than a overall concept. Again, there is no clear definition of health-related quality of life. One somewhat simplistic way to think about health-related quality of life is that it includes an assessment of two things: 1) What the person can do (e.g., functional status), and 2) How the patient feels (e.g., well-being). Clancy and Eisenberg (1998) provided a more sophisticated and detailed framework outlining four concepts associated with health-related quality of life.[10] These concepts include health perceptions (an individual's rating of health or symptoms), functional measures (an individual's ability to carry out daily activities), preference-based measures (choosing between two treatments for disease), and patient satisfaction (with medical care or outcome of incident). The theoretical structure upon which these elements are based is the previously described WHO definition of health. Finally, Ware (1987) suggests five inherent concepts including physical health, mental health, social functioning, role functioning, and general well-being.[11]

There are two basic types of instruments that are developed for use in quality of life assessments: generic and disease-specific.[12] Generic instruments generally provide assessments that are irrespective of health or disease status. An example of a generic, health-related quality of life instrument is the Social Function-36 (SF-36) instrument that was developed by the Rand Corporation for use in the

Medical Outcomes Study.[13] Although this instrument has been used in ophthalmic research, vision researchers mainly use the SF-36 only to show concurrent validity with a new ophthalmic instrument. The second type of quality of life instrument that can be used for assessment is the disease-specific instrument. This type of instrument targets 'domains' that are thought to be related to a specific disease (or similar diseases). An example of this type of instrument is the Glaucoma Symptom Scale.[14]

Quality of life (QoL) instruments should be reliable, valid, sensitive, and responsive if they are to be clinically useful.[15] Although these psychometric properties are interrelated, they are examined in somewhat different ways. For instance, patients whose disease status has not changed should, theoretically, make similar responses on disease-specific QoL instruments each time they are assessed. In order to distinguish responsiveness (the ability to detect change in status over time) and sensitivity (the ability to detect differences among different patient groups) from measurement error, it is important to assess instrument variability when administered to clinically normal patients on repeated occasions.[16] Validity assessment consists of the accumulation of evidence over time and across various studies to demonstrate that the scales are rational and respond as predicted. Once psychometric properties are established, an instrument may be ready for use in epidemiological studies or clinical trials.[17, 18]

QUALITY OF LIFE AND OPHTHALMIC DISEASE

Quality of life (QoL) assessments have become important outcomes in ophthalmic research over the last 10 years. These instruments complement and enhance our understanding of a patient's visual status, supplementing the traditional clinical tests, such as visual acuity, used for patient assessment. They also supplement our understanding of how diseases such as cataracts, glaucoma, macular degeneration, and diabetic retinopathy lead to functional impairments and disability. Examples of ophthalmic QoL instruments include the National Eye Institute Visual Function Questionnaire (NEI-VFQ), the Visual Function-14 (VF-14), the Glaucoma Symptom Scale, and the Graves' Ophthalmopathy Quality of Life Survey.[14, 19-21] Recent interest in refractive surgery has also led to the development of two refractive error-specific QoL surveys: the National Eye Institute Refractive Error Quality of Life instrument (NEI-RQL-42) and the Refractive Status and Vision Profile (RSVP) Survey.[22, 23] Of the aforementioned instruments, only the NEI-VFQ makes any sort of assessment of the significance of color vision on quality of life.

COLOR VISION DEFICIENCIES

There are two generally accepted types of color vision deficiency: congenital and acquired. Congenital defects are usually predictable, stable, symmetric between eyes, and, thus, they are easy to diagnose.[24] Patients with a congenital color vision defect usually have otherwise normal visual function. Congenital color defects are often subdivided based on the main axis of color confusion (i.e., red-green versus blue-yellow confusions) and on the mechanism of the defect (i.e., a missing cone type within the retina [dichromacy] or an abnormal photopigment sensitivity within a single cone type [anomalous trichromacy]). The genetic basis and clinical implications of congenital color vision deficiencies are generally well understood, and when proper test procedures are used, the diagnosis of congenital defects is unequivocal.

Damage to the retina or optic nerve from things such as trauma, inflammation, vascular disease, metabolic disorders, or chemical exposures may lead to an acquired color vision deficit. Individuals with acquired dyschromatopsias have less predictable losses that may progress in severity over time. Often, the two eyes are differentially affected, and there may be an accompanying loss in visual function (e.g., contrast sensitivity). Additionally, in the beginning stages of acquired color vision loss, it is often very difficult to determine if color discrimination has been affected at all.

There are many classification schemes for individuals with acquired color vision defects. Probably the most often used is that from Verriest.[25] The classification scheme is presented in Table 1. As previously stated, recent research has indicated that color discrimination loss from styrene exposure may be due to changes within the retina leading initially to alteration in color vision of the blue-yellow type.[6, 26] This would correspond to a Type III defect, similar to other toxic retinopathies and diseases like cystoid macular edema, central serous retinopathy, and diabetic retinopathy. While retinal damage from disease certainly affects both blue-yellow and red-green color processing, the few short wavelength sensitive cones (i.e., blue cones) present within the retina relative to the large number of green and red cones makes blue-yellow color processing especially sensitive to retinal insult. Also, while an initial acquired color vision loss often demonstrates itself as predominantly a blue-yellow confusion, red versus green insensitivity is often found, especially as the condition worsens. Although confusions along the blue-yellow axis are most typically described with styrene exposure, studies have reported confusions of the red-green type.[5, 21]

TABLE 1

Verriest Classifications of Acquired Color Vision Defects

Name	Description	Examples of Causation
Type I (Red-Green)	Protan type of defect with shift of luminous efficiency from photopic to scotopic	1) Cone dystrophy 2) Chloroquine toxicity
Type II (Red-Green)	Deutan type defect with milder blue-yellow loss	1) Optic neuropathy 2) Ethambutol toxicity
Type III (Blue-Yellow)	Tritan type defect	1) Glaucoma 2) Diabetes 3) Nuclear cataracts 4) Aging 5) Macular degeneration

Diabetes is a metabolic disorder exhibiting elevated blood glucose due to an insufficiency of or insensitivity to insulin. There is a resultant breakdown of regulatory mechanisms, some of which lead to alterations in the visual system. In particular, research has shown that there are changes in the short wavelength system (blue-cones) leading to alterations in visual function prior to clinically detectable retinal vascular abnormalities.[27] A report from the Early Treatment Diabetic Retinopathy Study showed that 50% of its sample (n = 2,701) had color vision scores worse than 95% of the normal population.[28] These are patients with confirmed retinopathy, indicating that they are more severe diabetics than the general diabetic population. The color vision defect that was most frequent was of the blue-yellow (tritan) type.

Glaucoma is an ophthalmic disease whereby the optic nerve undergoes changes resulting from high intraocular pressures leading to alterations in the optic nerve fiber bundle. This disease also has an effect on color vision.[29–31] Traditionally it was thought that glaucoma leads to a blue-yellow alteration in color vision; however, more recent research has shown that red-green alterations may be just as common or likely.[35] It was originally suggested that short wavelength cones may more susceptible to damage from elevated intraocular pressure.

COLOR VISION AND EVERYDAY FUNCTION

It has been known for well over a century that people who have color vision deficiencies may have occupational impairments. Some of the first observations of this were in transportation—specifically, the railroad and maritime industries. The thought is that there is an increased risk for an accident or crash with the misinterpretation of a signal light. These industries adopted color vision standards in the mid-to-late 1800's. The airline industry instituted similar color vision standards in the early 1900's. There seems to be no broad agreement regarding color vision standards for personal automobile use throughout the world. In the United States, however, individuals must be able to discriminate between a green, amber, and red traffic signal in order to obtain a Commercial Driver's License. Research has shown that a color vision deficiency alone is not a danger to safe driving.[36]

There seems to be even less of a consensus for color vision standards in other occupations and trades where color vision discrimination may be important. This may be because many of these trades and industries are self-regulated, and are not equipped to handle such a health standard. Nevertheless, color vision discrimination may be quite important in many occupations, as color is one of the most effective means of conveying information.[37] However, individuals with color vision deficits often engage adaptive behaviors to compensate for their deficits in color perception and discrimination.

Reports on the significance of color vision on functional ability are scarce. The best assessment of this issue is that of Steward and Cole.[38] In that study, the investigators surveyed 102 individuals with congenital color vision deficits and 102 normal controls. A questionnaire was administered which was composed of five sections: color vision status/perception, everyday difficulties with color, occupational difficulties with color, difficulty with color signal (i.e., driving), and reaction to color vision diagnosis. The results of this study were interesting. Two dichromats (5.4% of the dichromats) and 16 anomalous trichromats (24.6% of the anomalous trichromats) were unaware prior to the study that they had a color vision deficit. The majority of dichromats (71%) and a small minority of anomalous trichromats (27%) reported that they became aware of their deficit through difficulties with colors ($p < 0.002$). Of the 18 individuals not aware of their color

vision deficit prior to this study, the general response of the individuals was described as "denial" or "coping."

The results from questions about difficulties with everyday activities, driving, and their occupations were also interesting. In terms of everyday activities, the color vision defectives reported difficulties with selecting colors of cloths, accessories, paints, carpet, furniture, wallpaper, and cosmetics. They also reported significant difficulty with crafts, hobbies, flower identification, determining when fruits/vegetables are ripe, and in sporting activities. Color vision defectives also reported difficulty with traffic signals and sometime confusing traffic signals with street lights. Finally, between 29 and 43% of color vision defectives reported that their altered color vision impacted their career choice, and some reported difficulties with their current, everyday work.

The study of Steward and Cole generally showed that dichromats report more difficulty than anomalous trichromats with most tasks, and this is expected given the nature of these defects. They state, "Most anomalous trichromats reported their color vision defect to be of nuisance value rather than a major handicap in their everyday life." What is not known from this research is how an individual with an *acquired* color vision deficit might respond to these sorts of questions about functional, everyday impairments associated with his or her color vision loss. We might speculate that those with an acquired color vision deficit might respond similarly to a mild anomalous trichromat, who may have a deficit similar in magnitude to that shown by workers exposed to styrene.

OPHTHALMIC DISEASES AND QUALITY OF LIFE

To our knowledge, there have been no studies that have truly assessed the significance of color vision deficits on quality of life. The National Eye Institute Visual Function Questionnaire (NEI-VFQ) has a color vision 'scale' that is made up of only one question (Question #12: Because of eyesight, how much difficulty do you have picking out and matching your own clothes?). Surprisingly, there is no color vision-specific quality of life or functional questionnaire that has been standardized in the way in which these sorts of instruments generally are. One study of 147 glaucoma patients showed no correlation between visual field loss and the color vision question on the NEI-VFQ; similarly in this same study, there was no difference in the mean score on this question when comparing normals to glaucoma patients on this question.[39] A possible explanation is that glaucoma is a disease which mainly affects older individuals and blue-yellow color discrimination losses are seen normally with aging. Another report of diabetic individuals to whom the NEI-VFQ instrument was administered reported that the scores for color vision were among the highest (best) reported, and because deficits were uncommon, the scale was not used in predictive analyses (i.e., to answer the question "is retinopathy severity associated with quality of life-related color vision loss?").[40] This scale is surely limited, and probably not representative of the ways in which color vision may affect an individual's function and health status. This issue most definitely needs further study.

CONCLUSIONS

There is lack of information and studies that have assessed the significance of acquired color vision deficiencies on quality of life and everyday tasks. Cole and Steward showed that congenital color vision deficiencies are associated with mild-to-moderate impairments in everyday tasks and occupations; however, styrene-associated color vision changes are relatively mild compared to congenital color vision deficits. The significance of a chemically-induced color vision alteration on quality of life is uncertain, but likely less significant than that of congenital deficits. We believe that a study that assessed the significance of styrene-induced color vision deficiency on individuals might show little-to-no impairments in quality of life and functional ability. At present, there is not an appropriate color vision-specific quality of life survey instrument that could be used for this type study.

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Title

Consequences of low level styrene exposure?
Commentary to meta-analyses of Benignus et al. on neurobehavioral effects

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Abbreviations

MA	Mandelic acid
Ms	milliseconds
PGA	Phenylglyoxylic acid
ppm	parts per million
ACGIH	American Conference of Governmental Industrial Hygienists
CCI	Colour Confusion Index

Abstract

Benignus *et al.* (2005) presented an analysis on neurobehavioral effects of long-term styrene exposure based on simple and choice reaction time, and colour discrimination. Significant effects were declared for choice reactions and colour discrimination. In their discussion the authors stress the significance of the results for accident rates and color discrimination demands, arguing the costs of regulation for styrene should be seen against the benefit of reducing styrene-caused accident rates. Such far-reaching conclusions required a deeper analysis of the meta-analyses. The authors' interpolation of urinary metabolite levels to projected airborne styrene concentrations did not use standard procedures, did not explain how multiple values in individual studies were incorporated, and, although acknowledging it, did not account for a general trend for greatly reduced exposures over the time period covered by the studies included in the analysis. It is concluded that the analysis of neurobehavioral endpoints presented is not representative of the studies available. However, the tasks in two of the four studies included for choice reaction time do not fit the usual definition of choice reaction tasks. The seemingly arbitrary use of a linear model limits the ability to identify a threshold of effect and is not supported by individual studies. The perspectives offered in the discussion are analysed with the result that neither the considerations on accident rates nor those on improvements of colour vision after reduced styrene exposures can be accepted as contributions justifying a new assessment of the neurotoxicity of styrene.

Introduction

Previous assessments of the neurobehavioral effects of styrene have focused on assaying the qualities of individual studies and attempting to find the best study for hazard assessment (Fouremen, 1995; Rebert and Hall, 1994). Benignus *et al.* (2005) presented a modern approach in a creative way to assess this issue: i.e., a meta analysis of several studies. Quantitative meta analyses allow the use of more data to evaluate dose-response relationships, provided that the data are sufficiently robust and appropriate assumptions based on experimental evidence is provided. However, the information lost in making assumptions that allow pooling of data may outweigh the benefits of pooling the data. For occupational styrene exposure, the authors evaluated relations between cumulative styrene exposures (or their metabolites) and simple (SRT) and choice reaction times (CRT) and with color discrimination. They reported statistically significant relations for CRT and the Colour Confusion Index (CCI), but not for SRT. The presentation of data suggests that styrene exposure even in low concentrations causes remarkable functional impairments. The extent of functional impairments has been declared as a 6.5% increase in choice reaction time after 8 exposure years of 20 ppm (the ACGIH recommended limit) as well as an increase of colour confusion of 2.2% (measured by CCI with age norms) after the same exposure history which corresponds to 1.7 additional years of age in men. In their discussion the authors stress the significance of the results for national accident rates and color discrimination demands of real life situations, arguing the costs of regulation for styrene should be seen against the benefit of reducing styrene-caused accident rates. Such far-reaching conclusions require deeper analysis of the authors' meta-analysis, based on 4 studies of CRT and 5 studies of colour discrimination.

Issues of concern include: (1) the absence of inclusion / exclusion criteria for the selected studies and the representativeness of the studies chosen for the intended objectives, (2) the exposure data used and the assumption of a linear model of dose-response (3) the assumed link from inhaled styrene to prolonged reactions, and from these reactions to higher accident rates.

Representativeness of studies included

The inclusion criteria for the articles used in the meta-analyses were not outlined explicitly, but the authors implicitly mentioned: (a) exposure data - airborne ppm; urinary styrene, mandelic acid or creatinine; (b) exposure duration; (c) mean or individual data for the dependent variables; and (d) "long-term exposure" in the title (thereby excluding experimental studies). Using these criteria, several additional studies could also be included into the model calculations. Five additional studies for SRT meet these criteria (Kjellberg *et al.* 1979, Cherry *et al.* 1980, Fallas *et al.* 1992, Edling *et al.* 1993, and Viaene *et al.* 2001) with two possible others (Mergler *et al.* 1996, Edling & Ekberg 1985). One additional study concerning CRT (Mackay & Kelman 1986) meets these criteria. For CCI one additional study definitely should be included (Triebig *et al.* 2001), with two possible others (Sin-Eng Chia *et al.* 1994, Mergler *et al.* 1996). We believe that the Benignus *et al.* article failed to reflect the available literature and thus their conclusions are questionable. Table 1 presents some information on the relevant references that were not included.

(Table 1 about here)

In addition, some data probably were used twice in the meta-analysis concerning color discrimination. Table 4 of Benignus *et al.* lists five papers from which data were obtained, including both Campagna *et al.* 1996 and Gobba *et al.*, 1991. The Campagna 1996 paper states

that their analysis was based on data published by Campagna *et al.* 1995 (67 participants) and Gobba *et al.* 1991 (51 participants) giving a total sample of 118. Thus, the 51 participants from the Gobba *et al.* 1991 study (15% of total participants in the Benignus *et al.* analysis) appear to have been included twice in the Benignus *et al.* analysis. This, plus the fact that several relevant studies were not included by Benignus, indicates the color discrimination analysis is suspect.

Additionally, the criteria concerning the tests accepted as "choice reaction" tests in table 2, "...in which the subject must first select between options before deciding whether or in what way to respond" (Benignus *et al.* 2005, p. 532) is a non-standard and wide definition for choice reaction tests. The authors included results from Tsai and Chen (1996) in the analysis of CRT:

1) The tests performed by Tsai and Chen (Continuous Performance, Pattern Comparison, Pattern Memory, and Switching Attention) are tests for cognitive and executive functions and do not fit into the usual psychological test classifications for CRT (Lezak, 1995). The authors do not explain how they incorporated the means of the four tests into the meta analysis of CRT.

2) A second study in Table 2 (Jégaden *et al.* 1993) used a test requiring inhibition of reactions in 50% of the trials - this being compatible with "go/no-go" tasks rather than choice reaction tasks (Nieuwenhuis *et al.* 2003).

Only the studies of Mutti *et al.* (1984) and Triebig *et al.* (1989) used typical choice reaction tasks and thus we conclude that the majority of studies pooled for Table 2 do not reflect CRT.

Benignus *et al.* expressed dependent variables as a percentage of baseline, stemming from either control groups or low exposed groups serving as "implicit controls." However, this may be misleading. In the study of Gong *et al.* (2002) different control groups and "implicit controls" are available and it remains unknown how they were used in the analysis by Benignus *et al.* The authors refer to a "weight" statement of Prog REG. Probably the very high variance $R^2 = 0.91$ for CRT in Table 6 can be explained as a result of the weighting procedure of means.

In this context the authors cited Paramei *et al.* 2004 who standardized, by pooled standard deviations, their differences between exposed and controls in order to get effect sizes. "This transform conflates the measure of magnitude of an effect with its stability, so a transformed score may be larger or smaller depending on the variance of the group." (Benignus *et al.* 2005, p 533). From our point of view the procedure of Paramei *et al.* is traceable, because an implicit "quality control" of the studies has been installed which resulted in missing of homogeneity between the CCI-related studies in the Paramei *et al.* article. To do meta-analyses by using only percentages of baselines is possible statistically but it forgoes an important opportunity of accounting for variability in control groups.

Exposure data and the models used

The studies included by Benignus *et al.* had differing exposure characteristics; the treatment of exposures and analysis of the subsequent data indicate that exposures were underestimated.

Benignus *et al.* say that those studies were included which provided exposure data from the end of the shift urinary mandelic acid (MA) (exception Mutti *et al.*, 1984, back adjusted). In the case of Jégaden et al. (1993) it is unclear what data were used by the authors. Jejaden *et al.* report end of shift sum of MA and phenylglyoxylic acids (PGA) on the day of testing and as a mean concentration over 43 weeks. About 86% of absorbed styrene is transformed into the metabolites

MA (57%) and PGA(33%)(Greim & Lehnert, 1998). Since Benignus *et al.* state that conversion to ppm atmospheric styrene was based on MA concentration, it is unclear how they translated the Jejaden *et al.* urinary metabolite data (sum mandelic + phenylglyoxylic acids) to ppm styrene. Ambiguous descriptions for data-transfer from the original literature to the model inputs do not support transparency of results, and concrete information for each included study would improve enormously the plausibility of the results. For the majority of the studies included, the relationship between MA to styrene ppm is of interest because shift-end values refer to urinary samples. For the correlation of inhaled styrene to MA, the data of five reports were pooled for figure 2, and conversions from urinary MA to inhaled styrene were based on Fields & Horstman, 1979. The literature reveals additional information on this association, which are used more often (e.g. Härkönen *et al.* 1978; Guillemin and Berode, 1988, Lauwreys and Hoet, 1993; Pekari *et al.*, 1993; ACGIH, 2001) and other values of predicted styrene ppm could have been calculated, and thus the presented styrene ppm values - after prediction from MA – were only one of many possible estimations. Thus their exposure estimations are neither transparent, nor do they use standard extrapolations (such as ACGIH) from urinary metabolite data.

Another source for possible under-estimation of exposures occurs with the authors' treatment of exposure histories; they assume a uniform exposure during the work history of individuals in the included studies with an average duration of about 8 years. Exposure effects are attributed to 8 exposure years, extrapolated from exposure data belonging to the last day of exposure before testing, but this fails to account for exposure reductions. The authors correctly reflect on "overestimating the magnitude of effect for any indicated exposure". Contemporary data suggests the magnitude of exposure reduction is great (HSE 2005, BGIA 2002) with pooling of data for the period 1985-1997 suggesting exposure reduction of about 86%, although greater reductions of exposures can be achieved (Welp *et al.* 1996).

In their analysis, the authors assume that all persons in each included study (or each subgroup within the Mutti *et al.* study) were exposed to the same concentration of styrene; however, as shown in Table 2, that is not true. There was a wide variety of exposures within each study and one cannot ascribe the effect as being caused by the mean concentration of exposure. Further the individual studies report ranges of duration of exposure from 1 year to 16 years; Benignus *et al.* treated all subjects within each study as exposed for the mean duration reported for the individual study. While such assumptions are often used for testing a hypothesis of an effect, such lumping of exposure concentrations and durations make dose-response evaluations and time-response evaluations unreliable.

Further, the authors assumed a linear regression of dose-response relations using formula [2] for the estimated value of effects. Thus, any search for a threshold of neurotoxic effects is impossible. Visual examination of the scatter plot of work-years of styrene against CCI (Figure 7) does not support an interpretation of linear dose response. Examination of Figure 4 shows that for 50 and 160 ppm-years styrene exposure there was no increase in CRT. Further doubt on the linear extrapolation of effect with time is rendered by evaluations within two of the studies. Jejaden *et al.*, 1993 found no greater effect among those exposed for 9-14 years than among those exposed for 1 year. Triebig *et al.*, 1989 reported no effect of duration of exposure based on a chronic exposure index. Thus, the linearity imposed on the mathematical model does not reflect sufficiently the empirical realities; i.e., the scatter plots provided by Benignus *et al.* do not appear to support linear relationships.

Statistical modelling should be encouraged in the field of neurobehavioral assessment of workplace exposures, but it is equally crucial to (i) perform appropriate modelling, with data that makes reasonable modelling feasible, and (ii) to interpret such modelling carefully. Meta-analysis can distance readers from the original data and therefore leave them highly dependent on the care and diligence of the analysts. Application of the pooled data to linear regression models may not be a reliable way of expressing the results Benignus *et al.* believe they found. In performing "Dose-Effect Curves" in figures 4-6, the authors may be accentuating any effects observed in the larger studies they selected, and minimising any effects observed in smaller-scale studies. When using group-mean data (as opposed to individual-participant data) the fitting of regression lines will often result in vastly inflated confidence limits. (see Prog REG and weight statement before)

The authors have assumed a linearity between dose and CRT and CCI in figures 4-6, and this linearity of effect, when forced through zero can only produce highly "steep" effects. This feature would make linear modelling inappropriate when dealing with effects generally assumed to have a threshold. The authors should have suggested alternative non-linear methods of curve-estimation. However, such modelling of CRT may perhaps suffer from the low number of observations (n = 7), but this would not be expected for the CCI data (n = 15).

It is not quite clear why exposures less than 10 ppm were used as a baseline. "Zero" exposure would be more appropriate, and thereby any exposure above zero be classified as an "exposure". This measure may have been performed in the way that it was in order to allow for the inclusion of as many studies as possible in the analysis, but this may also be perceived as more evidence that not enough suitable papers existed for such analyses, without the data being "tweaked" to suit the analysts.

Extrapolation from the models to real life situations

After the critical view on the procedures used by the authors, different restrictions of this meta-analytical study can be summarized, regarding the (1) the representativeness of the studies used, (2) the analysed dependent variables, (3) the extrapolated exposure levels, (4) the historical bias of exposures as well as (5) the assumed linearity of dose-effect relations especially in low exposure levels. Such critical points can be seen as reason to abstain from further conclusions.

The authors correctly state that color vision deficiencies that are associated with exposure to solvents appear to be primarily blue-yellow in nature. Their Discussion concerning the significance of color vision defects, however, details studies with subjects with congenital defects, which are overwhelmingly red-green type defects. Additionally, the degree of difficulty performing everyday tasks (e.g. traffic signal recognition, judging of ripeness of fruit) reported by color vision defectives in these studies varies considerably depending upon the severity of the color vision deficit. Individuals with the milder defects report few, if any, difficulties with everyday activities. Readers of this paper, however, are left with the impression that styrene exposure of 20 ppm for 8 work-years (predicting a 2.23% increase in mean CCI) can result in the same functionally significant color discrimination losses equal to those found with more severe congenital deficiencies. To place the 2.23% increase in CCI into proper perspective, a perfect score on the Lanthony test is 1.0. For a group of subjects with normal color vision, the expected mean CCI will be approximately 1.20. A 2.23% increase in mean CCI would result in an increase to only 1.23. Severe congenital red-green defectives, however, will have a mean Lanthony CCI of between 2.5 and 3.0.

Benignus *et al.* have concluded that occupational styrene exposure to 20 ppm for 8 work years would impair CRT by 6.5%, corresponding to an increase of roughly 100 ms. To illustrate a real-world effect of increased CRTs, the authors discuss the importance of CRT in automobile driving, particularly total brake reaction times. A National Highway Safety Traffic Administration study is discussed that has estimated that brake reaction times that are decreased by 100 ms can reduce a significant amount of accident-related property damage and personal injuries. Moskowitz and Fiorentino (2000) has identified 13 behavioral areas and tasks that constitute driver related skills: aftereffects, cognitive tasks, critical flicker fusion, divided attention, driving skills, perception, psychomotor tasks, CRT, SRT, tracking, vigilance, visual functions and drowsiness. It is unclear which CRT testing method most accurately simulates the CRT component of total brake reaction time. It is admirable that Benignus *et al.* wish to demonstrate the effectiveness of reducing occupational styrene exposure; however, CRT is only one component of automobile-driving behavior, and its relationship to brake reaction times is unclear. Such a discussion is speculative and beyond the scope of their paper, and should only be considered as a hypothesis rather than a request for regulatory action.

The predictive value of the models used

Benignus *et al.* (2005) refer to an article of Benignus *et al.* (1998) with similar ideas and prognoses on the consequences of toluene exposure. They presented a model for the increase of choice reaction times with toluene exposure (Figure 6 in Benignus *et al.* 1998). According to this model 120 min. of 27 ppm exposure to toluene should result in prolonged reactions of about 10%. However, various publications since the Benignus *et al.*, 1998 publication do not support this model prediction. These studies included acute effects of toluene (Neubert *et al.*, 2001, van Thriel 1999) as well as chronic effects considering current and life time weighted averages of toluene exposure (Zupanic *et al.*, 2002; Seeber *et al.* 2004, 2005, van Thriel 2000). Using different approaches and a repeated measurement design, none of these studies confirmed the model predictions. Thus, for toluene the results of a creative model-approach did not find any correspondence in the real life of toluene exposed workers.

Conclusion

The assessment of neurobehavioral effects from workplace styrene exposure remains unclear, with conflicting results reported ranging from slight effects as low as 22 ppm to no effects as high as 100 ppm. Insufficient details were provided in the Benignus *et al.*, 2005 paper to reconstruct and therefore, fully assess the validity of their meta analysis of neurobehavioral effects of styrene. However, their extrapolation of urinary styrene metabolites into air styrene exposures did not use typical extrapolation relationships, and did not account for reductions in workplace styrene exposures during the course of the studies included. Two of the four studies included in evaluation of styrene effects on CRT do not meet the accepted criteria for CRT endpoints. Their use of a linear model precludes estimation of a no-effect level, and is not justified by the data. The assumption of a cumulative (duration) effect is counter to the results of two of the four CRT studies, which explicitly looked for, but found no effect of duration of exposure on CRT. Further, any change in color discrimination from styrene exposure has no clinical impact, contrary to their assertion and no impact of styrene exposure on driving ability or automobile accidents has been demonstrated.

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Table 1: Characteristics on additional studies regarding simple and choice reaction after occupational styrene exposure. Characteristics on the additional study with CCI is superfluous since already included in an earlier meta-analysis (Paramai *et al.*, 2004)

Reference	Exposure measure	RT measure	Control?	Blind?	N	Comment
Additional studies for simple reaction time						
Cherry et al., 1980	2 exposure groups. MA conc. ppm data.	Before and after shift	Yes – matched for age	?	13 low exposed 14 high exposed	Inclusion: OK Pre-shift effects on SRT. No after-shift differences to controls
Viaene et al., 2001	Exposure hrs. (mg/m^3) conc.	NES – timing not specified	Yes – matched for age, sex, schooling	Yes	27 current exposed 90 formerly exposed 64 controls	Inclusion: OK Less than 10 years exposure with 36 ppm does not produce SRT effects
Edling et al., 1993	Styrene TWA concentrations (mean = 8.6ppm)	Testing one work day. Follow-up 2-5 weeks after exposure (vacation)	Yes - matched for age, shift and workload	?	20 exposed 20 controls	Inclusion: OK No SRT effect
Fallas et al., 1992	Styrene conc (24.3ppm). MA conc, PGA conc.	WHO-NCT battery	Yes - matched for age, ethnicity, social status	?	60 exposed 60 controls	Inclusion: OK No Rt effects
Kjellberg et al., 1979	Data available	Available, but few participants		?	7 exposed 7 controls	Inclusion: OK Styrene effects were shown, but few participants
Mergler et al., 1996	Respiratory monitoring (ppm) MA conc. in three plants	WHO-NCT and FAST	longitudinal follow-up study	?	118 baseline 57 returned at follow-up	Inclusion: possible. Changes in exposure directly reflected by changes in SRT
Edling & Ekberg 1985	Respiratory monitoring (ppm) morning MA conc,	SRT before and after shift	Yes – no matching mentioned	?	12 exposed 10 controls	Inclusion: possible. No SRT effects at TWA exposure of 25ppm ($110mg/m^3$)
Additional study for choice reaction time						
Mackay & Kelman 1986	MA conc,	CRT before and after shift	No – 3 exposure groups used	No	10 exposed	Inclusion: possible. Some CRT effects but difficult to determine effects of test-practice

reliably

Table 2. Characteristics of Cohorts for CRT Studies from Benignus et al., 2005

Reference	Number Exposed workers	Mean Age	Age Range	Mean Exposure Concentration	Concentration Range	Mean Exposure Duration	Duration Range
Jejaden et al., 1993	30	28	+/- 6	22.7 ppm 43 week 30 ppm	4-55 ppm average 20% above 50 ppm	5 yr	+/-4.5 11 persons 1 yr 9 persons 2-5 10 persons 9-14
Mutti et al., 1984	50	40	+/-11.5	14 @<25ppm 9 @25-50 14 @50-75 13 @>75	10-300 ppm	8.7 yr 8.1 7.9 9.7	+/- 4.0 4.7 4.9 5.0
Triebig et al., 1989	36	Not given	24-59	18 ppm	3-251 ppm	7 yr	1-16
Tsai et al., 1996	41	35	+/- 8.9 16-67	22 ppm	+/- 40 ppm	8.3	+/- 7.9

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Letter to the editor

Styrene and breast cancer incidence in Texas: a comment on an ecological association

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Key words: breast neoplasms, environment, risk factors

We read with interest the recent ecological study by Coyle et al. [1] in which they concluded that "styrene was an important breast carcinogen." This finding is likely an incidental one for several reasons. Ecologically speaking, the association of ambient styrene exposure in Texas and breast cancer is not supported by breast cancer rates in that state. Furthermore, there are several cohort studies of styrene workers that show no elevation of breast cancer. Air monitoring of styrene in the Houston area demonstrates that exposures are very low, especially compared to occupational exposure.

The authors assert that, because Texas ranks first among all US states for the amount of styrene released, there is support for their finding of a relationship between styrene and breast cancer incidence. If this were true and if styrene releases were a cause of breast cancer, one would expect that the rates of breast cancer in Texas would be among the highest in the nation. However, this is not the case. In fact, Texas has the lowest [2]. The overall average annual age-adjusted incidence rate for breast cancer for the period between 1997 and 2001 for Texas was much lower than the national rate (110 versus 127 per 100,000). Similarly, the rates per 100,000 among Whites (110 versus 130), Blacks (104 versus 107) and Hispanics (77 versus 87) were lower in Texas than the nation [2].

The hypothesis generated by the ecological data analyses of Coyle et al. is not supported by other studies of styrene exposure. The studies referenced by Coyle et al. [3,4] do not contain any information on exposure, only on the industrial branch in which individuals worked. Conversely, the cohort studies for which breast cancer rates were reported, representing more than 100,000 men and women with occupational exposure to styrene (Table 1) show no elevation of breast cancer. Given the stronger study design (cohort versus ecological) the hypothesis has been tested and not supported.

Furthermore, the authors' conclusions are inconsistent with dose-response principles. Based on ongoing monitoring, ambient styrene monomer exposures in the Houston area average 0.018 ppb [5], whereas exposures in reinforced plastics workers have been 50,000 ppb or greater [4,5,9]. In other words, industrial exposures are approximately 3 million fold higher than environmental

Table 1. Summary of studies of breast cancer and styrene

Number of breast cancer cases/cohort	Rate ratio	Design	Exposure	Authors
54,487 Cases (577 males)	1.11 (66.2/59.8)	Ecological	Residence in counties with reported release	Coyle et al. [1]
29,009 Cases versus, 101,254 controls	1.38 (Exposure level 3)	Case–control	Occupation listed on death certificate	Cantor et al. [6]
4 Cases among 7949 workers	0.46	Cohort	Employment in reinforced plastic industry	Coggon et al. [7]
13 Cases among 40,688 workers	0.52	Cohort	Employment in reinforced plastics industry	Kogevinas et al. [8]
6 Cases among 2492 female workers	0.57	Cohort	Employment in rubber plant	Solionova and Smulevich [9]
3 Cases among 36,691 workers	0.79	Cohort	Employment in rubber industry	Sorahan et al. [10]
14 Cases among 15,826 workers	0.62	Cohort	Employment in reinforced plastics and composites industry	Wong et al. [11]

exposures in the area of Texas. It is not biologically plausible that styrene could increase breast cancer rates among individuals living in areas with extremely low exposure potential without evidence of risk among those highly exposed in the industrial settings.

The authors have dismissed the negative results of the occupational cohort studies because women were under-represented. However, in the study by Coyle et al., breast cancer cases among men are treated equally to those of women. The statistical models in their study predicted less than 15% of the study variability. In light of the low breast cancer rates in Texas (to the US), lack of supporting evidence in occupation cohort studies, and evidence of low styrene levels from air monitoring, the current study is a textbook example of the ecological fallacy.

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Effect of the inhibition of the metabolism of 4-vinylphenol on its hepatotoxicity and pneumotoxicity in rats and mice

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Abstract

Styrene is known to be both hepatotoxic and pneumotoxic in rodents. 4-Vinylphenol (4-VP) has been shown to be a minor metabolite of styrene in some studies and is a more potent toxicant in mice than either styrene or styrene oxide. 4-VP is metabolized primarily by CYP2E1 and CYP2F2 to an unknown metabolite. The purpose of this study was to use inhibitors of these cytochromes P450 to address the question of whether the parent compound or a metabolite is responsible for 4-VP induced toxicity. Rats as well as mice were found to be susceptible to the toxicity of 4-VP. Prior treatment with either diethyldithiocarbamate or 5-phenyl-1-pentyne as inhibitors of CYP2E1 and CYP2F2 prevented or greatly decreased the hepatotoxicity of 4-VP as assessed by measuring serum sorbitol dehydrogenase and its pneumotoxicity as determined by measurements of cells, protein and lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid. Thus the hepatotoxicity and pneumotoxicity of 4-VP are due to a metabolite(s) and not the parent compound. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: 4-Vinylphenol; Liver; Lung; CYP2E1; CYP2F2

1. Introduction

Styrene is a widely used chemical with extensive human exposure, especially in the reinforced plastics industry (Miller et al., 1994). The primary route for the metabolism of styrene is via styrene oxide, and this epoxide is generally thought to be the active metabolite responsible for the toxicity of styrene (Bond, 1989). Styrene causes both hepatotoxicity and pneumotoxicity with mice generally being more susceptible than rats (Roycroft et al., 1992; Morgan et al., 1993a,b; Gadberry et al., 1996; Cruzan et al., 1997). Styrene has also been shown to cause lung cancer in mice but not in rats (Cruzan et al., 1998, 2001).

Other metabolic pathways also are involved in the metabolism of styrene (Sumner and Fennell, 1994). One of these involves ring hydroxylation to yield 4-vinylphenol (4-VP). Bakke and Scheline (1970) identified 4-VP in the hydrolyzed urine of rats dosed orally with styrene, but it amounted to only 0.1% of the administered dose. Pantarotto et al. (1978) identified small amounts of 4-VP in the urine of rats administered styrene intraperitoneally. They also found 4-hydroxymandelic acid and 4-hydroxybenzoic acid, metabolites similar to the

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end products from styrene metabolism via the styrene oxide pathway. 4-VP was determined to be a minor metabolite in the urine of workers occupationally exposed to styrene (Pfaffli et al., 1981). Compared with the more common pathway of styrene metabolism to styrene oxide and eventually to mandelic acid, the amount of 4-VP measured in the urine was 0.3% that of mandelic acid. Watabe et al. (1984) reported the formation of 4-VP using ^{14}C-labeled styrene and a rat hepatic microsomal preparation. They found that this metabolic process required NADPH suggesting that it is cytochrome P450 dependent. Similar results were found by Pantarotto et al. (1978) who also found this process to be inducible by treating rats with 3-methylcholanthrene. However, we were unable to demonstrate the formation of 4-VP from styrene in rodent microsomal preparations but did find that 4-VP was rapidly metabolized by mouse and rat hepatic and pulmonary microsomes involving CYP2E1 and CYP2F2 (Carlson et al., 2001). In a study in which rats and mice were exposed by inhalation to [ring-U-^{14}C]styrene, Boogaard et al. (2000) reported finding $^{14}CO_2$. They suggested that ring hydroxylation may be occurring followed by ring opening. On the other hand, recent studies in which human volunteers inhaled $^{13}C_8$-styrene did not reveal metabolites that could be derived from ring hydroxylation (Johanson et al., 2000).

The possible contribution of 4-VP as an active metabolite of styrene in either the acute or chronic toxicity associated with stryrene is unknown. Little information on the toxicity of 4-VP is available in the published literature. Berger et al. (1977) reported that feeding 4-VP at a concentration of 1 mg/g diet for 7–12 days resulted in a decrease in the uterine weight of the small rodent *Microtus montanus*. A report in RTECS (1986) on 4-VP indicates that 200 mg/kg administered topically to rabbits is corrosive. We recently examined the hepatotoxicity and pneumotoxicity of 4-VP in CD-1 mice (Carlson et al., 2002). Doses of 4-VP from 25 to 200 mg/kg were administered i.p. Hepatotoxicity was assessed by measuring serum sorbitol dehydrogenase (SDH) and by light microscopy. Pneumotoxicity was assessed by measuring proteins, cells and lactate dehydrogenase (LDH) activity in bronchoalveolar lavage fluid (BALF) and by light microscopy. 4-VP caused a dose dependent increase in serum SDH and mild hepatocellular swelling. It caused an increase in cell number and LDH activity in BALF. Microscopically, there was widespread and severe necrosis of the bronchioles by 12 h. Re-epithelialization of the bronchioles was evident by 48 h. These studies indicate that 4-VP is both hepatotoxic and pneumotoxic. The doses at which changes were observed (50 and 100 mg/kg) were lower than those observed in similar studies with either styrene or styrene oxide (Gadberry et al., 1996).

The metabolism of 4-VP in vitro has been measured by determining the loss of substrate in microsomal preparations (Carlson et al., 2001). 4-VP metabolizing activity in mouse liver microsomes was three times greater than that in rat liver microsomes, and activity in mouse lung microsomes was eight times greater than that in rat lung microsomes. This activity was completely absent if NADPH was not present. Studies with cytochrome P450 inhibitors indicated the involvement of CYP2E1 and CYP2F2. The latter was of particular importance in lung. The current studies were designed to investigate the hypothesis that inhibition of 4-VP metabolism could influence its toxicity, either increasing it if 4-VP itself was active or decreasing it if a metabolite was responsible for its actions. In addition, studies were carried out using rats as well as mice to determine if there were species differences in susceptibility. The inhibitors selected were diethyldithiocarbamate (DDTC) and 5-phenyl-1-pentyne (5P1P). As noted above we have used these inhibitors in vitro and demonstrated inhibition of 4-VP metabolism (Carlson et al., 2001). DDTC administered to rats and mice has been found to be protective against the toxicity induced by carbon tetrachloride, a chemical known to be dependent upon bioactivation by CYP2E1 in order for it to exert its toxic effects, by a number of investigators (Siegers et al., 1981; Masuda and Nakayama, 1982; Lauriault et al., 1992). 5P1P administration has been shown to be protective against styrene-induced pulmonary toxicity (Green et al., 2001).

To examine hepatotoxicity serum SDH elevations were used as an indicator of hepatic damage. This is a very specific and sensitive indicator of hepatic damage (Dooley, 1984). We found for 4-VP that this measurement is a more sensitive indicator of damage than is histopathology (Carlson et al., 2002). We also found this to be true for carbon tetrachloride-induced liver damage (Paustenbach et al., 1986). To examine pulmonary toxicity, proteins, cells and LDH activity in BALF were measured since we have found them to be good, dose dependent indicators of 4-VP-induced damage to lungs (Carlson et al., 2002). The dose of 150 mg/kg 4-VP used in these studies was selected based on the fact that while 100 mg/kg could produce both pneumotoxicity and hepatotoxicity, it would be difficult to show protection at that level of response. On the other hand, higher doses, e.g. 200 mg/kg, can lead to lethality (Carlson et al., 2002).

2. Materials and methods

2.1. Animals

Adult male CD-1 [Crl:CD-1 (ICR) BR] (27–35 g) mice were obtained from Charles River Laboratories (Wilmington, MA). Adult male Sprague–Dawley rats (200–250 g) were obtained from Harlan Sprague–Dawley (Indianapolis, IN). The animals were housed in group cages in an AAALAC-accredited facility in environmentally controlled rooms on a 12-h light:12-h dark cycle. Rodent laboratory chow (No. 5001, Purina Mills, Inc., St. Louis, MO) and tap water were allowed ad libitum. All animals were allowed a minimum of 1 week to adapt to the animal facilities and diet before being used in any experiment.

2.2. Chemicals

4-VP (10% in propylene glycol; purity >95%) was obtained from Lancaster Synthesis (Windham, NH). NADH, pyruvate, and tris buffer were from Sigma Chemical Co. (St. Louis, MO). Triethanolamine was from Mallinckrodt (Paris, KY) Fructose was from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade or better.

2.3. Study design

To examine the toxicity of 4-VP, groups of mice or rats were administered 150 mg/kg 4-VP per kg body weight in saline i.p. These dilutions were made immediately prior to dosing. Controls were treated with vehicle (propylene glycol in saline). Some groups received either DDTC in saline at a dose of 400 mg/kg as an inhibitor of CYP2E1 or 5P1P in corn oil at a dose of 100 mg/kg as an inhibitor of CYP2F 1 h prior to the 4-VP. For the hepatotoxicity studies, groups of ten mice or five rats were administered 150 mg/kg 4-VP. Twenty-four hours later they were anesthetized with diethyl ether. The abdominal cavity was opened, and the diaphragm was cut. Blood was obtained by cardiac puncture. For rats individual animals were used, and for mice the blood was pooled from pairs of animals for measurement of SDH.

For the pneumotoxicity studies, groups of five or six mice or rats were administered 150 mg/kg 4-VP. BALF was obtained 24 h after dosing. The animals were anesthetized with diethyl ether, and the abdominal and thoracic cavities were opened. The incision was continued to the neck region to expose the trachea. A nick was made in the trachea, and an oral feeding needle was inserted and tied in place. The lungs were perfused twice with 0.8 ml of lavage fluid for a total of 1.6 ml in mice or twice with 7.0 ml of lavage fluid for a total of 14.0 ml in rats. This fluid consisted of NaCl (145 mM), KCl (5 mM), NaH_2PO_4 (1.9 mM), Na_2HPO_4 (9.4 mM) and glucose (5.5 mM) at a pH of 7.4. These protocols and procedures were approved by the Purdue University Animal Care and Use Committee.

2.4. Biochemical analyses

Serum was prepared, and serum SDH activity was measured spectrophotometrically by the method of Gerlach (1983). Serum (0.2 ml) and NADH (12 mM) were incubated for 30 min at 30 °C in triethanolamine buffer (0.2 M, pH 7.4). The reaction was started by addition of 0.3 ml of

72% (w/v) fructose for a final reaction volume of 3.0 ml. SDH activity was measured by the decrease in absorbance of NADH at 366 nm for 2 min using a Shimadzu Model UV160U UV-visible spectrophotometer. Results are expressed as μmol/min per l serum.

In the analysis of BALF, the number of cells in 100 μl of BALF was counted using a hemocytometer. The remaining BALF was centrifuged at low speed, and the amount of protein determined using the bicinchoninic acid method (Redinbaugh and Turley, 1986). LDH activity was measured by the spectrophotometric method of Vassault (1983) in centrifuged BALF samples from individual mice. BALF fluid (0.1 ml), NADH (0.24 mM), and Tris (81 mM)/NaCl (203 mM) buffer (pH 7.2) were incubated for 15 min at 30 °C. The reaction was initiated by the addition of 0.5 ml pyruvate (9.8 mM) to make a total volume of 3.0 ml. The activity of LDH was measured 30 s after the addition of pyruvate by the decrease in absorbance of NADH at 339 nm for 2 min. Results are expressed as μmol/min per l BALF.

2.5. Statistical analysis

Values are expressed as mean ± S.E. In comparing the values, an ANOVA was utilized followed by Student Newman–Keuls' test to detect differences among the groups. In each case the level of significance selected was $P < 0.05$. In some cases, because of differences in the variances, it was necessary to log transform the data.

3. Results

Immediately following the intraperitoneal administration of 4-VP both mice and rats became very lethargic and lay prostrate in their cages, but they recovered from this condition in 2–3 h. A few of the animals, both mice and rats, died between 2 and 24 h following the 4-VP dosing. This was apparently due to CNS effects, which appeared very shortly after dosing. In neither mice nor rats treated with the inhibitors of 4-VP metabolism, DDTC for CYP2E1 or 5P1P for CYP2F, did the inhibitors have any apparent influence on this effect.

As expected, 4-VP demonstrated hepatotoxicity in mice as measured by several-fold increases in serum SDH activities (Table 1). This effect was decreased considerably if the animals were first treated with either DDTC or 5P1P. In the experiment with DDTC treatment prior to the 4-VP, the value for this group fell between those of the control and the 4-VP treatment group and was not statistically significantly different from either. For the mice treated with 5P1P prior to the 4-VP, the value was between the control and 4-VP treatment values and significantly different from both. These data suggest that inhibition of metabolism could ameliorate this effect of the 4-VP. 4-VP also proved to be hepatotoxic in rats (Table 2). As evidenced in mice, protection was observed with both inhibitors in rats. The SDH activity for the group treated with DDTC prior to the 4-VP was not different from control. In the study with 5P1P there were no significant differences among the three groups although the value for the group treated with 4-VP was five times the control value and the activity of the group treated with 5P1P prior to the 4-VP fell between the control and 4-VP alone values.

Pneumotoxicity was assessed using analysis of BALF fluid. Cells, protein, and LDH released

Table 1
Influence of DDTC and 5P1P on the effect of 4-VP (150 mg/kg) on mouse serum SDH activity

Treatment	N	SDH activity[a]
Control[b]	5	45.7 ± 6.6^{e}
4-Vinylphenol[c]	5	159.1 ± 31.8^{f}
DDTC + 4-vinylphenol[d]	4	$93.0 \pm 19.1^{e,f}$
Control[b]	5	22.1 ± 4.8^{e}
4-Vinylphenol[c]	4	205.9 ± 26.3^{f}
5P1P + 4-vinylphenol[d]	5	138.2 ± 15.9^{g}

[a] Activity is expressed as μmol/min per l.
[b] Control received propylene glycol in saline.
[c] Administered i.p. 24 h prior to sacrifice.
[d] Diethyldithiocarbamate at a dose of 400 mg/kg or 5-phenyl-1-pentyne at a dose of 100 mg/kg was administered i.p. 1 h prior to 4-VP.
[e–g] Values with different superscripts are significantly different from one another ($P < 0.05$).

Table 2
Influence of DDTC and 5P1P on the effect of 4-VP (150 mg/kg) on rat serum SDH activity

Treatment	N	SDH activity[a]
Control[b]	5	11.2±0.9[e]
4-Vinylphenol[c]	4	79.4±15.1[f]
DDTC+4-vinylphenol[d]	4	33.2±6.2[e]
Control[b]	5	10.5±3.1[e]
4-Vinylphenol[c]	5	44.7±14.1[e]
5P1P+4-vinylphenol[d]	5	25.2±6.0[e]

[a] Activity is expressed as μmol/min per l.
[b] Control received propylene glycol in saline.
[c] Administered i.p. 24 h prior to sacrifice.
[d] DDTC at a dose of 400 mg/kg or 5P1P at a dose of 100 mg/kg was administered i.p. 1 h prior to 4-VP.
[e,f] Values with different superscripts are significantly different from one another ($P < 0.05$).

from the respiratory tract into this fluid were measured. As expected from our previous study (Carlson et al., 2002), 4-VP caused increases in all three parameters in mice (Table 3). LDH activity increased to five to nine times the control value. The cell count was elevated to 18–24 times the control value. The protein concentration increased to two and one-half to three times the control. Prior treatment with either of the inhibitors demonstrated dramatic effects decreasing the values to control level. 4-VP proved to be pneumotoxic in rats as well as in mice as indicated by increases in all three endpoints (Table 4). As in the mice, prior treatment of the rats with either of the inhibitors protected the animals to the extent that the values for the combination groups did not differ from their respective controls.

4. Discussion

Styrene produces both liver damage and lung damage in experimental animals with mice usually being more susceptible to these effects than are rats (Roycroft et al., 1992; Morgan et al., 1993a,b; Gadberry et al., 1996; Cruzan et al., 1997). These toxic effects are generally attributed to the generation of styrene oxide (Bond, 1989) which has been shown to be both hepatotoxic and pneumotoxic in mice (Gadberry et al., 1996). The basis for this species difference is not entirely clear. However, we have shown that styrene is metabolized to styrene oxide to a greater extent in mouse lung than in rat lung particularly to the more toxic *R*-enantiomer and especially by the Clara cells, the target for styrene (Hynes et al., 1999).

However, an additional pathway has been identified in which, not surprisingly, the *para*-position on the styrene is hydroxylated to yield 4-VP. There is both direct evidence for the formation of this metabolite (Bakke and Scheline, 1970; Pfaffli et al., 1981; Pantarotto et al., 1978) and indirect evidence (Boogaard et al., 2000). 4-VP is metabolized to as yet unidentified metabolites

Table 3
Influence of DDTC and 5P1P on the pneumotoxicity of 4-VP (150 mg/kg) in mice

Treatment	N	LDH activity[a]	Cells[b]	Proteins[c]
Control[d]	6	64.6±11.6[g]	54±17[g]	313±19[g]
4-Vinylphenol[e]	6	324.9±52.1[h]	1278±255[h]	795±66[h]
DDTC+4-vinylphenol[f]	6	126.2±28.6[g]	315±105[g]	417±67[g]
Control[d]	6	54.6±9.9[g]	59±25[g]	328±36[g]
4-Vinylphenol[e]	5	510.5±164.4[h]	1056±373[h]	928±142[h]
5P1P+4-vinylphenol[f]	6	64.1±13.5[g]	296±139[g]	317±37[g]

[a] Activity is expressed as μmol/min per l.
[b] Cells per μl BALF.
[c] Micrograms protein per μl BALF.
[d] Control received propylene glycol in saline.
[e] Administered i.p. 24 h prior to sacrifice.
[f] DDTC at a dose of 400 mg/kg or 5P1P at a dose of 100 mg/kg was administered i.p. 1 h prior to 4-VP.
[g,h] Values with different superscripts are significantly different from one another ($P < 0.05$).

Table 4
Influence of DDTC and 5P1P on the pneumotoxicity of 4-VP (150 mg/kg) in rats

Treatment	N	LDH activity[a]	Cells[b]	Proteins[c]
Control[d]	5	15.5±1.5[g]	29±10[g]	126±2[g]
4-Vinylphenol[e]	3	384.2±200.8[h]	218±113[h]	950±451[h]
DDTC+4-vinylphenol[f]	5	65.0±34.6[g]	35±7[g]	174±54[g]
Control[d]	5	12.7±1.3[g]	53±7[g]	250±8[g]
4-Vinylphenol[e]	6	49.6±17.7[h]	207±30[g]	353±45[h]
5P1P+4-vinylphenol[f]	6	14.2±3.4[g]	119±34[g]	232±10[g]

[a] Activity is expressed as μmol/min per l.
[b] Cells per μl BALF.
[c] Micrograms protein per ml BALF.
[d] Control received propylene glycol in saline.
[e] Administered i.p. 24 h prior to sacrifice.
[f] DDTC at a dose of 400 mg/kg or 5P1P at a dose of 100 mg/kg was administered i.p. 1 h prior to 4-VP.
[g,h]Values with different superscripts are significantly different from one another ($P < 0.05$).

although it has been suggested that these may be ring-opened products (Boogaard et al., 2000). We have shown that the 4-VP is metabolized rapidly by mouse and rat hepatic and pulmonary microsomes although we have not yet been able to identify the product(s) (Carlson et al., 2001). This process requires NADPH, and the use of chemical inhibitors has identified CYP2E1 and CYP2F2 as being the most important cytochromes P450 involved. 4-VP metabolizing activity in mouse liver microsomes was three times greater than that in rat liver microsomes, and activity in mouse lung microsomes was eight times greater than that in rat lung microsomes.

Recent studies using methodologies similar to those employed in the current study have shown that 4-VP is both hepatotoxic and pneumotoxic in mice (Carlson et al., 2002). Statistically significant increases were observed in serum SDH and in cells and LDH activity in BALF at doses as low as 50 mg/kg. Thus 4-VP is an order of magnitude more potent than either styrene or styrene oxide is (Gadberry et al., 1996).

The current studies have shown that, like the mouse, the rat is also susceptible to the effects of 4-VP on the CNS, liver and lungs. In both species, treatment of the animals with DDTC or 5P1P resulted in a remarkable prevention of 4-VP-induced hepatotoxicity. DDTC is a relatively specific inhibitor of CYP2E1 (Ono et al., 1996) although studies using CYP2E1 knockout mice indicate that it may also inhibit CYP2F2-associated activities to some extent (Powley and Carlson, 2001). 5P1P is a good inhibitor of CYP2F2 especially in the lung where this isozyme is located in Clara cells (Chang et al., 1996). Green et al. (2001) recently demonstrated that the administration of 5P1P (200 mg/kg i.p.) inhibited the metabolism of styrene in vivo and prevented increased bronchiolar cell replication rates, a response to cell damage, in the lungs of CD-1 mice. However, this inhibitor is not completely specific for CYP2F since Roberts et al. (1998) have shown in in vitro studies that at high concentrations it will also inhibit the activities of CYP2E1. The overall finding though is that these inhibitors protect against the toxicity of 4-VP by inhibiting these two cytochromes P450.

Pneumotoxicity was observed in both rats and mice. The increase in cells observed in the BALF following 4-VP was very great. This was observed previously in mice not only in terms of the number of cells in BALF but also by histopathology studies which indicated microscopic lesions in the lungs of mice given 4-VP (100 mg/kg i.p.) as early as 12 h after administration (Carlson et al., 2002). The distal bronchi and bronchioles were partially lined by varying proportions of attenuated or low cuboidal epithelial cells, and their lumens contained sloughed cells and necrotic cellular debris. Protection against the pneumotoxicity was observed with both inhibitors.

The exact role of 4-VP in styrene-induced toxicity is not yet known. However, these studies and others (Carlson et al., 2002) indicate that 4-VP is more toxic than styrene or styrene oxide. Since the toxicity to both liver and lung is greatly diminished by the treatment of both species with either DDTC or 5P1P, it is likely that these toxic effects are related to a metabolite of 4-VP and not 4-VP itself.

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Styrene-7,8-oxide burden in ventilated, perfused lungs of mice and rats exposed to vaporous styrene

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Key words

Styrene; styrene-7,8-oxide; mouse; rat; ventilated, perfused lung; inhalation; mode of action.

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Abstract

Styrene (ST) is an important industrial chemical. In long-term inhalation studies, ST induced lung tumors in mice but not in rats. In order to test the hypothesis that the lung burden by the reactive metabolite styrene-7,8-oxide (SO) would be most relevant for the species-specific tumorigenicity, we investigated the SO burden in isolated lungs of male Sprague-Dawley rats and in-situ prepared lungs of male B6C3F1 mice ventilated with air containing vaporous ST and perfused with a modified Krebs-Henseleit buffer (37°C). ST vapor concentrations were determined in air samples collected in immediate vicinity of the trachea. They were almost constant during each experiment. ST exposures ranged from 50 to 980 ppm (rats) and from 40 to 410 ppm (mice). SO was quantified from the effluent perfusate. Lungs of both species metabolized ST to SO. After a mathematical translation of the obtained ex-vivo data to ventilation and perfusion conditions as they are occurring in vivo, a species comparison was carried out. At ST concentrations of up to 410 ppm, mean SO levels in mouse lungs ranged up to 0.45 nmol/g lung about 2 times higher than in rat lungs at equal conditions of ST exposure. We conclude that the species difference in the SO lung burden is too small to consider the genotoxicity of SO as sufficient for explaining the fact that only mice developed lung tumors when exposed to ST. Another cause has to be considered as driving force for lung tumor development in the mouse.

Introduction

Styrene (ST) is an important chemical of wide industrial use, particularly in the production of polymers, co-polymers and reinforced plastics. Its toxicology and metabolic fate are described in several reviews, e.g. IARC (1994); Sumner and Fennell (1994); IARC (2002); Cohen et al. (2002). After its uptake by the mammalian organism, ST is oxidized to styrene-7,8-oxide (SO) by cytochrome P450 dependent monooxygenases (CYP). SO is a directly DNA alkylating mutagen (comprehensively reviewed in IARC 1994 and IARC 2002). It induced dose dependent forestomach tumors after oral administration in mice (Lijinsky, 1986) and in rats (Conti et al., 1988; Ponomarkov et al., 1984). This metabolic intermediate of ST is further biotransformed by epoxide hydrolase (EH) and glutathione S-transferase (GST). In long-term animal

studies, ST induced lung tumors in mice but not in rats (summarized in e.g. IARC 1994; IARC 2002; Cohen et al., 2002). At ST exposure concentrations below about 250 ppm systemic bioavailability of metabolically formed SO (determined as concentration in blood) is very similar in both species as has been demonstrated in inhalation studies with rats and mice (Kessler et al., 1992 and Morgan et al., 1993, data comparatively depicted in IARC 1994). The systemic SO burden is governed predominantly by the ST and SO metabolizing capacity of the liver as shown by Csanády et al. (1994). However, in the lung, the entrance organ for vaporous ST, the SO burden of ST inhaling rodents might be considerably higher than is to be expected from systemic concentrations because lungs of rats and mice have been demonstrated to be able to form SO from ST (Salmona et al., 1976; Cantoni et al., 1978; Carlson, 1997; Mendrala et al., 1993; Nakajima et al., 1994; Hynes et al., 1999; Oberste-Frielinghaus et al., 1999). Although the occurrence of SO has been qualitatively shown in lungs of mice after dosing ^{14}C-labeled ST intraperitoneally (Löf et al., 1984), quantitatively reliable data on the SO burden in the lungs at steady state were not available so far. Assuming the SO lung burden to be most relevant for ST induced tumorigenicity and considering lung tumorigenicity occurring only in mice, one might speculate the SO levels to be considerably higher in lungs of ST exposed mice than in those of rats. In vivo, this burden cannot be determined correctly because of fast metabolism in the lung as can be estimated from the activities of GST and EH towards SO measured in lung cell fractions by Oberste-Frielinghaus et al. (1999). Therefore, the aim of the present work was to quantify the SO burden in isolated perfused rat lungs and in in-situ perfused mouse lungs ventilated with air containing vaporous ST.

Material and Methods

Chemicals

All chemicals were of analytical grade if not indicated otherwise. Styrene (purity ≥ 99%), racemic styrene-7,8-oxide (purity 97%), (1R, 2R)-(+)-1-phenylpropenoxid (PPO; purity 97%), magnesium sulfate (99% anhydrous), albumin from bovine serum (purity ≥ 96%, essentially fatty acid free), HEPES buffer (minimum 99.5%, titration), and D-(+)-glucose (purity 99.5%) were obtained from Sigma-Aldrich, Steinheim,

Germany. Acetonitril (CHROMASOLV® for HPLC) was obtained from Riedel-de Haën, Seelze, Germany, isofluran from Baxter, Unterschleißheim, Germany, Ketamin (10%) from „Wirtschaftsgenossenschaft Deutscher Tierärzte eG", Garbsen, Germany, Rompun® (2%) from Bayer, Leverkusen, Germany, n-hexane (Picograde) from Promochem, Wesel, Germany, and Liquemin® N25000 (Heparin-Natrium) from Hoffmann La Roche, Grenzach-Wyhlen, Germany. Gases used for gas chromatography were from Linde, Unterschleißheim, Germany. Diethyl maleate (purity 95%) and all other chemicals were obtained from Merck, Darmstadt, Germany.

Perfusion medium: The perfusion medium of Uhlig and Heiny (1995) was used. Krebs-Henseleit buffer (37°C) containing 2% bovine serum albumin, 0.1% glucose, and 0.3% HEPES. The pH was adjusted to 7.35-7.40.

Determination of ST: ST was measured in air samples using a gas chromatograph (GC-8A, Shimadzu, Duisburg, Germany) equipped with a flame ionization detector (FID) and a stainless steel column packed with Tenax TA, 60-80 mesh, 1.5 m x 1/8" (Shimadzu, Duisburg). Air samples of 50 µl were injected directly on column. Separation was done isothermally at 200°C using N_2 as carrier gas. Injector and detector temperatures were kept at 300°C. Retention time of ST was about 1.5 min. Chromatograms were recorded and integrated by a Shimadzu C-R5A integrator. Calibration curves were constructed three times by generating ST vapor concentrations ranging from 10 to 1000 ppm in atmospheres of closed desiccators according to Filser et al. (1993). Calibration curves were linear in the whole range. Analysis of linear regression through the origin revealed correlation coefficients of at least 0.957 between peak areas and atmospheric styrene concentrations. Each time before starting an ST exposure, a one-point calibration was carried out in the concentration range used in the actual experiment.

Determination of SO: SO was quantified by GC/FID and additionally in at least one effluent perfusate sample per exposure experiment by GC/MSD for the unequivocal identification of SO. Fresh lung perfusate samples of 2.5 ml (rat) and 1 ml (mouse) were immediately spiked with a methanolic PPO solution of 0.5 µmol/l (rat 5 µl; mouse 1 µl) and mixed with acetonitrile (rat 2.5 ml; mouse 1 ml). n-Hexane (rat 4.5

ml; mouse 2 ml) was added and the mixture vigorously shaken for 2 min. Phase separation was done by centrifugation (5 min, 5°C, 4400 rpm). The clear supernatant was collected and the remaining aqueous phase extracted once again. The combined extracts were concentrated under a gentle stream of Nitrogen to a final volume of 2.5 ml (rat) and 0.5 ml (mouse) and then stored at 4°C in a closed autosampler vial for a maximum time span of 2 days. GC/FID or GC/MSD analysis was carried out within this time frame. Under these conditions, SO and PPO were stable as had been demonstrated by Kessler et al. (1990).

GC/FID determination of SO and PPO was done by capillary gas chromatography as described in Kessler et al. (1990). An Agilent GC 6090N equipped with a direct on-column injector was used. The retention times of SO (13.3 min) and of PPO (14.0 min) were very near to those reported in the study of Kessler et al. (1990). The limit of detection was about 30 nmol/l perfusate at an injection volume of 25 µl and a signal-to-noise ratio of 3:1.

GC/MSD determination by capillary gas chromatography was done according to Bitzenhofer (1993) using a gas chromatograph HP 5890 series II equipped with a mass selective detector (HP 5970), both Agilent, Waldbronn, Germany. 1 µl of the concentrated n-hexane extract was manually injected onto a "fused silica" pre-column (length 10 m, ID 0.53 mm; from Agilent, Waldbronn, Germany) using the "Gerstel-KaltAufgabeSystem KAS 3" from Gerstel, Mühlheim an der Ruhr, Germany. The pre-column was connected to the separation column (HP-1 MS, film 0.33 µm, length 25 m, ID 0.2 mm; from Agilent, Waldbronn, Germany) by a "Miccon" press fit connector. Helium (0.8 ml/min) was used as carrier gas. Column temperature was maintained at 35°C for 0.25 min during the injection process. Then, it was heated up to 220°C with a rate of 30°C/min. The temperature of the transfer line to the MSD was 250°C. The electron ionization potential of the MSD was at 70 eV and the voltage of the electron multiplier was 2000 Volt. Retention times were 7.7 min (SO) and 8.1 min (PPO). Both substances were detected in the single ion-monitoring mode at m/z 89 and 119 (SO) and 89 and 133 (PPO) and were quantified using the m/z 89 according to Langvardt and Nolan (1991). The detection limit for SO was about 15 nmol/l perfusate at an injection volume of 1 µl and a signal-to-noise ratio of 3:1.

Calibration curves (signal area of SO to signal area of PPO versus the concentration of SO) were constructed over a concentration range from 0.1 µmol/l to 10 µmol/l

using five different SO concentrations. Analysis of linear regression through the origin revealed correlation coefficients of at least 0.999 (both GC/FID and GC/MS) between peak areas and SO concentrations. At each ST perfusion experiment, a three-point calibration curve through the origin was constructed.

Animals and Surgery

Male Spague-Dawley rats and male B6C3F1 mice were obtained from Charles River Wiga GmbH, Sulzfeld, Germany. All experimental procedures with animals were performed in conformity with the "Guide for the care and use of laboratory animals" (7th edition, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press Washington, D.C., 1996) under the surveillance of the authorized representative for animal welfare of GSF. Up to four weeks before use, two rats or five mice each were housed in the GSF-Institute of Toxicology in a macrolon type III cage placed in a IVC top-flow system (Tecniplast, Buguggiate, Italy). This system provided the animals with filtered room air. A constant 12-hour light/dark cycle was maintained in the chamber room. Animals had free access to standard chow (Nr. 1324 from Altromin; Lage, Germany) and tap water. When using the animals, body weights were in the range of 230 – 360 g (rats) and 25 – 34 g (mice).

Lungs were prepared as described in Uhlig and Wollin (1994) for rats and in von Bethmann et al. (1998) for mice with the exceptions that different narcotics were used instead of pentobarbital sodium. To rats, a mixture of 0.8 ml/kg ketamine and 1 ml/kg Rompun® was administered. Per kg body weight, mice received a mixture of 1.4 ml ketamine, 0.4 ml Rompun®, and 6.3 ml of a 0.9% saline. Immediately before intraperitoneal injection of this mixture animals were anesthetized by isoflurane inhalation.

In summary, animals were intubated and ventilated by positive pressure (rats: 80 breath/min, tidal volume approximately 2 ml; mice: 90 breath/min, tidal volume approximately 200 µl) using the ventilation-perfusion systems for rat and mouse lungs "Isolierte perfundierte Lunge Größe 2" (rat) and "Isolierte perfundierte Lunge Größe 1" (mouse) manufactured by Hugo Sachs Elektronik (HSE), March-Hugstetten, Germany. After laparotomy, they received intracardial injections of Liquemin® N25000 (0,1 ml per rat and 0.05 ml per mouse). Thereafter, the

diaphragms were removed and the animals exsanguinated. A ligature was placed around both the pulmonary artery and the aorta. The canula for the influent perfusate was inserted into the pulmonary artery and fixed by the ligature thereby closing the aorta. Immediately, the apex cordis was opened by a cut. Then the canula for the effluent perfusate was inserted into the left ventricle and also fixed by a ligature. The rat lung together with the heart were carefully isolated and suspended by the trachea in the humidified and warmed rat lung ventilation chamber. The lid was closed and the lung was immediately ventilated using negative pressure. The mouse lung was prepared in the open mouse ventilation chamber and was not separated from the body. After removing the thorax, the chamber lid was closed and negative pressure ventilation was started.

Experimental procedure

For safety reasons, all exposure experiments were carried out under the hood. The once-through perfused lungs were ventilated by negative pressure with ST vapors of defined concentrations in the inhaled air and SO was measured in the effluent perfusate. Thereafter, the perfusion and ventilation parameters were extrapolated to the species-specific in-vivo conditions and the corresponding SO lung burdens were calculated by means of the partition coefficient lung-to-perfusate of SO.

Ventilation-perfusion of rat and mouse lungs: Both above cited exposure systems from HSE, described in Uhlig and Wollin (1994) and Uhlig and Heiny (1995) for the rat as well as in von Bethmann et al. (1998) for the mouse, were modified to enable inhalation exposure to a gas of constant concentration.

Modifications of the rat system for inhalation exposure

Figure 1 presents a scheme of the mechanical parts of the isolated perfused lung system for rats including the following modifications. A T-formed hollow tube (T-tube) made in-house out of brass was cold-welded to the upper end of the pneumotachograph tube and connected via a Tygon® tubing to the ST-vapor transporting glass pipe. A constant flow of ST vapor from the "ST-stock desiccator" through the glass pipe and the horizontal duct of the T-tube was established (flow of ST vapor containing air: 15 ml/min) by means of the roller pump I that contained Tygon® tubing. The lung took its breathing air from the T-tube via the

pneumotachograph tube. In order to enable the determination of ST vapor concentrations immediately in front of the trachea, a custom made "Kel-F" hub needle (Hamilton, Bonaduz, Switzerland) was directed through a small pipe in the lid of the artificial thorax into a lateral outlet of the trachea attachment and fixed up gas-tightly by PTFE and silicone tubing. The upper end of this canula possessed a Teflon® luer lock usually closed by a plug (No. 5 in Figure 1). For measuring ST concentrations in inhaled air, the plug was exchanged by a Hamilton syringe (100 µl series 1710 TLL, obtained from Machery-Nagel, Düren, Germany), and a gas sample of 50 µl was collected and immediately injected on the column of the GC-8A.

Modifications of the mouse system for inhalation exposure

Figure 2 presents a scheme of the mechanical parts of the isolated perfused lung system for mice including the modifications enabling inhalation exposure. The original system is equipped with a moistener for inhaled air (No. 11 in Figure 2). This vial (volume about 2 cm^3), which is covered by a PTFE plug, contains two openings, one to the external air and the other one to the pneumotachograph (No. 8 in Figure 2). After drilling a hole through the PTFE plug, a small PTFE host was inserted tightly. The ST vapor was transferred from the ST-stock desiccator by the roller pump I (11.3 ml/min) into the air space of the moistener. The ventilated lung inhaled the ST vapor from the air mixture passing through the modified moistener. In order to determine the actual inhaled ST concentration, the 51 mm long needle of a Hamilton syringe (100 µl, series 1710 TLL) was inserted into the trachea through the opening of the moistener to the room air (No. 5 in Figure 2), and an air sample of 50 µl was collected and immediately injected on the column of the GC-8A.

Exposure conditions: Rat lungs (n=13) were ventilated in the negative chamber pressure mode as described in Uhlig and Wollin (1994) and in Uhlig and Heiny (1995) with the exception that inspired air was neither moistened nor warmed above room temperature. Instead of applying the recirculating buffer method of Uhlig and Wollin (1994) the less elaborate once-through perfusion technique was used. Inhaled air contained mean ST concentrations between 49 and 984 ppm (see Table 2). The exposures lasted between 32 and 43 min. At each exposure start, background SO in blood was measured but it was in no case detectable. The experimental conditions (mean ± SD) were: Perfusion flow 18 ± 1.4 ml/min (manually adjusted); tidal volume 2.2 ± 1.3 ml; breaths/min 80 (fixed) with a deep breath at every 5 min to prevent

atelectasis (see Uhlig and Wollin 1994); pulmonary resistance (the flow resistive forces in the airways) 0.46 ± 0.14 cm H_2O · sec/ml; dynamic compliance (an index of the functional stiffness of the lung) 0.20 ± 1.3 ml/cm H_2O.

In all mouse experiments (n=12), lungs were ventilated in the negative chamber pressure mode and were perfused as described in von Bethmann et al. (1998) with the exceptions that inhaled air was neither moistened nor warmed above room temperature. This air contained average ST concentrations between 39 and 407 ppm (see Table 3). The exposures lasted between 55 and 68 min. At each exposure start background SO in blood was measured but is was in no case detectable. The experimental conditions (mean ± SD) were: breaths/min: 90 (fixed) with a deep breath at every 5 min, tidal volume (210 ± 45 µl), and perfusion flow 1.0 ± 0.24 ml/min. The dynamic compliance was 0.027 ± 0.005 ml/cm H_2O.

The values of these parameters were controlled and monitored by a computer running the software ("Pulmodyn® W" from HSE) of the ventilation-perfusion system that possessed the "PLUGSYS" unit (from HSE) to amplify the raw data.

LDH release into the perfusate was measured using the test kit "LDH IFCC-Methode 37°C" (Rolf Greiner BioChemica, Flacht, Germany) in order to test for the integrity of the alveolar membranes. Mean activity (±S.D.) in the influent perfusates (background controls) was 7.1 ± 4.3 U/l (n=25). In the effluent perfusates it was 6.7 ± 4.3 U/l (n=93). The maximum value in the influent and effluent perfusate was identical: 17.6 U/l. Consequently, the lungs were considered as physiologically and biochemically intact.

Partition coefficient lung-to-perfusate of SO ($P_{L/P}$): The thermodynamic partition coefficient $P_{L/P}$ depends on the temperature and on physicochemical properties of SO, lung tissue, and perfusate. For tissue and perfusate, water and lipid contents are most relevant. $P_{L/P}$ had to be determined (at 37°C) because it enabled to calculate the actual SO concentration in the cellular tissue (C_{LSOc}) of the ST inhaling lung at steady state from measurements of SO in the vasculature (the effluent perfusate; C_{PSO}). Under this condition, C_{LSOc} is in thermodynamic equilibrium with the perfusate and can therefore be calculated by the relationship:

(1) $C_{LSOc} \approx P_{L/P}\, C_{PSO}$

About 30 min before preparing the rat lungs with the aim to determine $P_{L/P}$, the animals were administered intraperitoneally with diethyl maleate (0.6 ml/kg body weight i.p.), a glutathione-depleting compound (see Plummer et al. 1981). Thereafter, rats were narcotized and the lungs were positively ventilated and perfused in situ (see "Animals and Surgery"). Perfusion was carried out with the above described perfusion medium for 10 min with a flow of 20 ml/min in order to remove blood completely from the lung. Because only one ventilation-and-perfusion system was available and three rat lungs were required for one experiment, the lungs had to be stored before use. Therefore, they were flash frozen in liquid nitrogen and then stored in a freezer at -80°C.

After cutting the freshly thawed lungs into halves, each half was placed in a glass beaker (10 ml) together with enough physiological saline to cover it completely. Then, the beakers were placed for 5 min in a boiling water bath in order to denature the SO metabolizing enzymes. Thereafter, lung-halves were taken from the beakers, carefully dabbed with a tissue, weighed, and cut in small slices. Each sliced lung-half was placed in a glass culture tube (7 ml; with PTFE covered screw cab; K&K Laborbedarf, München, Germany) to which 1 ml of perfusion medium was added containing a defined SO concentration (1 or 10 µmol/l). The tubes were sealed and immediately incubated in a shaking water bath (37°C). Parallel SO incubations were carried out in perfusion medium alone in order to determine loss of SO by non-enzymic hydrolysis. Aliquots of 0.5 ml perfusion medium (one vial containing one lung-half represented one time point) were taken at selected time points, PPO (5 µmol/l) was added and the SO content in the liquid phase was determined by GC/MSD as described above. From the concentration-time courses of SO obtained in the incubations with or without lung, the $P_{L/P}$ was calculated by means of a two-compartment model describing distribution between compartment one (medium) and compartment two (lung) and elimination from compartment one by hydrolysis. The partition coefficient was calculated using the following equation:

(2) $P_{L/P} = (k_1V_1) / (k_2V_2)$

k_1, k_2 micro-constants describing the SO transport between both compartments

V_1 volume of compartment one (medium)

V_2 volume of compartment two (lung)

At any time point t, the concentration of SO in the medium of the vial containing both medium and lung incubate (c_{SO}) is given by:

(3) $c_{SO} = C_1 e^{\alpha \cdot t} + C_2 e^{\beta \cdot t}$

c_{SO} concentration of SO in medium at time point t

C_1, C_2 intercepts of the function

α, β rate constants of the function

These constants together with k_{el} (the elimination rate constant describing the hydrolytic disappearance of SO from medium in the incubations without lung) were used to obtain k_1 and k_2:

(4a) $k_1 = - k_{el} - (\alpha C_1 + \beta C_2) / (C_1 + C_2)$

(4b) $k_2 = [- C_1 C_2 (\alpha - \beta)^2] / [(C_1 + C_2) [C_1 (k_{el} + \alpha) + C_2 (k_{el} + \beta)]]$

The elimination rate k_{el} was determined from SO incubations with perfusion medium alone. The SO concentration-time curve in these experiments followed the e-function:

(5) $c_{SO} = C_{(0)} e^{-k_{el} \cdot t}$

c_{SO} SO concentration in the medium in SO incubations without lung at any time point t

$C_{(0)}$ SO concentration in the medium at time point t = 0

k_{el} elimination rate constant

The program "Prism" (GraphPad, San Diego, CA, USA) was used to fit corresponding e-functions through the data obtained in the SO incubations with pure medium and with medium plus lung pieces in order to obtain the values of k_{el}, C_1, C_2, α, and β. These values were inserted in Equation 4 to calculate k_1 and k_2. $P_{L/P}$ was then calculated using Equation 2.

Extrapolation from ex-vivo to in-vivo conditions: The perfusion conditions that had been used ex vivo in the ventilated perfused lungs were smaller than those occurring in vivo. This reduction was inevitable to avoid edema formation. Estimation of the in-vivo burden of the lungs is required for a species comparison of the SO lung burden between mice and rats that results from exposure to ST. In order to calculate the in-vivo burden resulting only from ST metabolism in the lung (i.e. not considering the lung burden by SO resulting from ST metabolism in the liver) the following extrapolation from the ex-vivo to the in-vivo situation was done considering the relationship expressed by equation 1:

(6) $C_{SOLiv} = P_{L/P}\, C_{SOP} \cdot (Q_{aiv} / Q_{aev}) \cdot (Q_{Lev} / Q_{Liv})$

$P_{L/P}$	partition coefficient lung/perfusate of SO*
C_{SOLiv}	SO concentration in lung in vivo [nmol/g]
C_{SOP}	SO concentration in effluent perfusate (ex vivo) [nmol/ml]
Q_{aiv}	alveolar ventilation in vivo (mouse: 25; rat: 117 [ml/min])**
Q_{aev}	actual alveolar ventilation ex vivo [ml/min] (calculated as actual tidal volume times fixed breath frequency times 2/3***)
Q_{Lev}	lung perfusion ex vivo [ml/min]
Q_{Liv}	lung perfusion in vivo (mouse: 17; rat: 83 [ml/min])**
*	determined at 37°C
**	from Arms and Travis (1988)
***	The number "2/3" represents the conversion factor from pulmonary to alveolar ventilation (e.g. Arms and Travis 1988).

The equation was derived considering the differences between in-vivo and ex-vivo conditions in the alveolar ventilations of the air containing the metabolic precursor ST (represented by Q_{aiv} and Q_{aev}) and in the perfusion flows of SO (represented by Q_{Liv} and Q_{Lev}). It renders the dependency of the SO burden in the lung from the in-vivo and ex-vivo ventilation and perfusion rates.

Calculations and statistics

Means ± standard deviations (SD) of measured data, comparison of means by the unpaired two-tailed t-test, as well as the values of kinetic parameters describing

measured concentration-time-courses were calculated using Prism 4 for Macintosh (GraphPad Software, San Diego, CA, USA).

Results

Partition coefficient lung-to-perfusate of SO ($P_{L/P}$)

$P_{L/P}$ was calculated using the parameters gained by curve fitting from 7 concentration-time-courses of SO in perfusate that were recorded up to 250 min at pH 7.4 and 37°C (between 5 and 6 data points per concentration-time-course). Four of these experiments were conducted in SO containing perfusate free of lung tissue and the other three with heat-inactivated lung tissue in the perfusate at initial SO concentrations between 0.55 an 10 µmol/l (Figure 3). For these experiments, the kinetic parameters describing the fitted curves, the rate constants of the spontaneous hydrolysis (k_{el}), and the calculated $P_{L/P}$ values are given in Table 1. The half-life ($\ln 2/k_{el}$) of the spontaneous hydrolysis at pH 7.4 was (mean ± SD; n=4) 298 ± 27 min. This value fits to that of 306 min determined earlier in potassium phosphate buffer at pH 7.0 (Filser et al., 1992). The value of $P_{L/P}$ (mean ± S.D.; n=3) of 1.29 ± 0.05 is independent on the SO concentration in the range between 0.5 and 10 µmol/l, at least.

SO burden in ST inhaling lungs

Figures 4 and 5 depict concentration-time courses that are characteristic for the inhalation studies with ventilated, perfused lungs of rats (Figure 4) and mice (Figure 5). Atmospheric ST concentrations in the air entering the lungs were maintained nearly constant during the exposure time (Figures 4A, 5A). Concentrations of metabolically produced SO in the effluent perfusate remained nearly constant from about 5 min after starting ST exposures until the end of the experiments (Figures 4B, 5B). In both figures, the straight lines represent the mean concentrations of the measured data from about 5 min after starting the respective exposure to its end.

The experimentally determined mean SO concentrations in effluent perfusate (Tables 2 and 3) were mathematically translated from the experimental to the species-specific in-vivo conditions of lung perfusion and ventilation (see Eq. 6). These

translated ("measured") lung levels of SO are depicted in Figure 6 (both species) versus the corresponding ST concentrations in inhaled air. Over the whole demonstrated concentration range of ST, the "measured" pulmonary SO burden is about 2 times lower in rat than in mouse. At ST concentrations of 100 ppm and above, the SO levels in rat lungs are higher than in mouse lungs at 40 ppm. Additionally, the SO levels in rat lungs at 350 ppm ST and above are similar to those in mouse lungs at 160 ppm ST.

Discussion

The pulmonary SO levels resulting solely from ST metabolism in the lung (Figure 6), are at least 5 times (mice) and 10 times smaller than the SO blood levels that were reached during inhalation exposure of rats and mice to equal ST concentrations of up to 250 ppm. From the blood data in rats and mice measured (Kessler et al., 1992; shown also in IARC, 1994) and using the partition coefficient lung/blood of SO (1.9; Csanády et al., 2003), a maximum pulmonary SO level of about 2.4 nmol/g can be extrapolated for a steady-state exposure of rats or mice to 160 ppm ST by neglecting production as well as metabolism of SO in the lung. Interestingly, this value is not far from the pulmonary SO levels of about 1.5 and 2.5 nmol/g predicted for both species by a physiological toxicokinetic model that contained pulmonary ST metabolism to SO and metabolic SO elimination in the lung (Csanády et al., 2003). Considering the low SO burdens obtained in the ventilated, perfused lungs, it has to be concluded that pulmonary ST metabolism is only of minor relevance with respect to the SO lung burden in vivo. For in-vivo exposure, it follows that the pulmonary SO levels are predominantly dependent on the actual SO blood levels and that not only blood but also lung levels by the alkylating SO are similar in both rodent species below 250 ppm ST. One might speculate that not the SO burden of the whole lung but that of the terminal bronchioles, the actual target of the pulmonary ST toxicity (e.g. Cruzan et al. 2001), should be considered as relevant. However, considering a 6-h exposure to 160 ppm ST, the model of Csanády et al. (2003) predicts for the bronchioles of rat lungs an SO concentration of almost 2 nmol/g (similar to the SO concentration of the whole lung) and for mice an SO concentration of almost 3 nmol/g (not much higher than in the whole lung in this species). For equal exposure conditions, the

physiologically based pharmacokinetic model of Sarangapani et al. (2002) predicts for the terminal bronchioles of rats almost the same SO concentration as does the Csanády model, but for the terminal bronchioles of mice almost 8 nmol/g are expected. In a third recently published physiologically based pharmacokinetic model concerning ST and its metabolite SO, the physiological description of the respiratory pathways did not enable to distinguish between whole lung and transitional bronchiolar region (Cohen et al., 2002). The small species difference in the SO burden of whole lungs and bronchioles predicted by our model (Csanády et al., 2003) are in agreement with studies on DNA binding of metabolites of ^{14}C-labeled ST carried out in rats and mice that were exposed by inhalation to 160 ppm ST over 6 h (Boogaard et al., 2000). In the lungs of the animals sacrificed immediately at the end of exposure, about the same extremely low N7-(hydroxyphenylethyl)guanine adduct level of ^{14}C-labeled SO of about 1 adduct/10^8 nucleotides was quantified in both species; adduct levels in mouse Clara cells, most rich in CYP450 enzymes (reviewed in Gram, 1997), were similar as in the total lung. For the whole lung of mice exposed to about 170 ppm over 6 h, a comparable guanine adduct level of 1.3 adducts/10^8 nucleotides was also reported by another group (Vodicka et al. 2001). "Covalent binding indices" calculated by Boogaard et al. (2000) in livers and lungs of the ^{14}C-ST exposed rodents were also very small and similar in rats and mice. The authors concluded "that DNA adduct formation does not play an important role in styrene tumorigenicity in chronically exposed mice".

Considering that in a long-term inhalation study (Cruzan et al., 2001) with mice exposed to concentrations of up to 160 ppm, the frequency of lung tumors had increased at ST concentrations ≥ 20 ppm, and that no tumors had been found in lungs of rats exposed up to 1000 ppm ST under comparative conditions (Cruzan et al., 1998), one has to conclude that the species-specific lung tumorigenicity of ST in mice does not result directly from the lung burden by the genotoxic metabolite SO. As already concluded earlier from the results delivered by our ST model, it is highly probable that a non-genotoxic glutathione dependent mechanism is involved (Filser et al. 2002).

Based on modeled tissue concentrations of SO and on kinetic interactions of the glutathione turnover with the SO forming and eliminating enzyme activities that had been measured in vitro (Oberste-Frielinghaus et al., 1999), our physiological

toxicokinetic model (Csanády et al., 2003) predicted a drastic GSH decrease of 40% in the bronchiolar region of mice and only a marginal one in that of rats following a 6-h inhalation exposure to 20 ppm ST. Although model predictions for the whole lung agreed with measured data (Dhawan-Robl et al., 2000; Filser et al., 2002), it cannot be ruled out that also other ST metabolites might contribute to the glutathione depletion in mouse lungs. For instance, 4-vinylphenole, a putative ST metabolite in mice (Carlson et al., 2001) and a minor one detected in urine of rats (Bakke and Scheline, 1970; Pantarotto et al., 1978; Manini et al., 2002) and humans (Pfäffli et al., 1981; Manini et al., 2002) resulted in a certain glutathione depletion in the lungs of mice, following intraperitoneal administration (Turner et al. 2005). 4-Vinylphenol was hepato- and pneumotoxic in rodents due to the formation of metabolites, probably ring-opened products (Carlson et al., 2001; Carlson 2002).

Repeated administration of mouse Clara cell toxicants as coumarin, naphthalene, or 4-ipomeanol can result in a certain tolerance to these chemicals, probably due to up-regulation of the detoxifying glutathione (Boyd et al., 1981; Born et al., 1999; West et al., 2000; Vassallo et al., 2004). However, after intermittence of daily injections of naphthalene for 4 days, elevated glutathione levels in the terminal airways of tolerant mice had declined to control levels and mice were again susceptible to naphthalene injury (West et al., 2000). Plopper et al. (2001) have investigated early events in naphthalene-induced acute Clara cell toxicity. They described a broad heterogenicity of loss of cellular glutathione and other sulfhydryls in the cell populations of the minimally susceptible lobar and proximal bronchi. However, in the most susceptible distal bronchioles, high loss of glutathione was detected in all cells. The authors concluded that at least 50% of the intracellular glutathione pool must be lost before cell organelle changes become apparent and that a loss of at least 75% is required before toxic cellular changes become irreversible. The observed cytotoxic effects should result at least directly from the glutathione depletion and not from a reactive naphthalene metabolite, because Phimster et al., (2005) demonstrated that abrupt glutathione depletion in mice resulted in Clara cell toxicity similar to naphthalene treatment with respect to Clara cell swelling, plasma membrane blebs and actin cytoskeleton disruptions (Phimister et al., 2005).

Furthermore, it is well known that severe changes in the GSH homeostasis can lead to apoptosis and cell proliferation (reviewed in e.g. Rahman et al., 1999). Together

with a depletion of glutathione in lung homogenate, such effects have been detected in Clara cells of mice repeatedly exposed to ST (Gamer et al., 2004). Following repeated SO or 4-vinylphenol administration to mice, cell proliferation, histomorphological changes and apoptosis in bronchi and terminal bronchioles were observed. It was suggested that Clara cells were primary target cells (Kaufman et al., 2005).

Considering this information, we propose the following hypothesis to explain lung tumor formation in ST exposed mice: Glutathione conjugation with the ST metabolite SO results in perturbation of the glutathione pathway in Clara cells of the terminal bronchioles because of insufficient glutathione turnover. This effect leads to cell death and regenerative proliferation. Together with the cell burdens by reactive ST metabolites as SO and possibly ring-oxidized derivatives, the finally observed tumorigenesis in mouse lungs becomes comprehensible. In the rat lung, no such effects are to be expected, since ST induced loss of glutathione is minimal in this species (see Filser et al. 2002; Csanády et al. 2003).

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Tables

Table 1. Values of the parameters describing the concentration-time courses of SO plotted in Figure 3 (pH 7.4 ; 37°C; perfusate volume V_1 = 1 ml) and hereof calculated $P_{L/P}$ values.

Dashed curves: $c_{SO} = C_0 \cdot e^{-k_{el} \cdot t}$						
Number of experiment	C_0 [µmol/l]	k_{el} [min^{-1}]	Number of experiment	C_0 [µmol/l]	k_{el} [min^{-1}]	Mean k_{el} ± SD [min^{-1}]; n=4
1	10.0	0.0022	3	2.27	0.0021	0.0023 ± 0.0002
2	4.83	0.0025	4	0.55	0.0025	

Solid curves: $c_{SO} = C_1 \cdot e^{\alpha \cdot t} + C_2 \cdot e^{\beta \cdot t}$						
Number of experiment	C_1 [µmol/l]	C_2 [µmol/l]	α [min^{-1}]	β [min^{-1}]	V_2 [ml]	$P_{L/P}$* (calculated using k_{el})
5	4.03	6.00	-0.096	-0.0026	0.51	1.34
6	0.42	0.59	-0.033	-0.0021	0.53	1.30
7	0.40	0.60	-0.188	-0.0019	0.53	1.24
					Mean $P_{L/P}$ ± SD (n=3):	1.29 ± 0.05

Curves 1, 2, 3, and 4 (dashed lines with filled symbols in Figure 3) were recorded to determine the rate constant k_{el} of the SO hydrolysis in perfusate. Curves 5, 6, and 7 (straight lines with hollow symbols in Figure 3) were obtained from measuring SO in perfusate containing heat inactivated lung tissue probes of volumes V_2 (mean values of each experiment). The values of C_0, C_1, C_2, k_{el}, a, and β were obtained by curve fitting.

*: For calculation of PL/P from these values see Equations 2 – 5.

Table 2. Mean concentrations of styrene (ST) in inhaled air and of styrene-7,8-oxide (SO) in effluent perfusate as well as tidal volumes and perfusion flows measured in the experiments with ventilated, perfused rat lungs.

ST (ppm)	SO (µmol/l)	Tidal volume (ml)	Perf. flow (ml/min)
49 ± 3*	0.063 ±0.021*	1.0	18
113 ± 10	0.11 ± 0.028	1.2	19
160 ±16	0.50 ± 0.046	3.2	17
167 ± 14	0.44 ± 0.11	2.4	19
261 ± 40*	0.61 ± 0.040*	3.7	19
265 ± 49	0.26 ± 0.058	0.95	18
335 ± 52	0.48 ± 0.10	1.2	18
378 ± 48	0.29 ± 0.017	0.76	18
454 ± 32*	1.10 ± 0.10*	3.5	19
494 ± 67	0.69 ± 0.091	2.2	20
477 ± 52	1.40 ± 0.11	2.9	15
631 ± 85	0.78 ± 0.054	1.9	18
984 ± 308	2.05 ± 0.10	5.2	21

Means ± SD represent at least 5 concentration measurements per exposure experiment (compare Figure 4).

*: Mean values of the data presented in Figure 4.

Table 3. Mean concentrations of styrene (ST) in inhaled air and of styrene-7,8-oxide (SO) in effluent perfusate as well as tidal volumes and perfusion flows measured in the experiments with ventilated, perfused mouse lungs.

ST (ppm)	SO (µmol/l)	Tidal volume (ml)	Perf. flow (ml/min)
39 ± 5	0.26 ± 0.044	0.19	1.0
42 ± 5*	0.25 ± 0.033*	0.19	1.2
80 ± 4*	0.64 ± 0.077*	0.26	1.0
158 ± 10	2.66 ± 0.53	0.21	0.50
169 ± 19	1.00 ± 0.28	0.20	1.0
172 ± 15	1.23 ± 0.08	0.21	1.0
202 ± 16	2.42 ± 0.18	0.28	1.3
255 ± 28	1.27 ± 0.31	0.15	1.2
257 ± 14*	2.22 ± 0.19*	0.18	1.1
271 ± 21	1.30 ± 0.25	0.20	1.5
374 ± 19	2.66 ± 0.33	0.21	1.0
407 ± 41*	3.16 ± 0.39*	0.21	0.80

Means ± SD represent at least 6 concentration measurements per exposure experiment (compare Figure 5).

*: Mean values of the data presented in Figure 5.

Legends to the Figures

Figure 1 Simplified scheme of the ventilated once-through perfused rat lung system from HSE with in-house made modifications enabling the inhalation of ST vapors and the gas-tight collection of perfusate samples.

Abbreviations: 1 septum for collection of ST vapor samples; 2 septum for collection of perfusate samples; 3 exit to pressure transducer (inflowing perfusate pressure); 4 exit to pressure transducer (pressure of chamber atmosphere); 5 site for collecting air samples of inhaled ST; 6 gas trap; 7 exit to venturi gauge; 8 pneumotachograph tube; 9 exit to differential pressure transducer (air flow); 10 pressure equilibrium vial; 11 trachea attachment.

Figure 2 Simplified scheme of the ventilated once-through perfused mouse lung system from HSE with in-house made modifications enabling the inhalation of ST vapors and the gas-tight collection of perfusate samples.

Abbreviations: 1 septum for collection of ST vapor samples; 2 septum for collection of perfusate samples; 3 exit to pressure transducer (inflowing perfusate pressure); 4 exit to pressure transducer (pressure of chamber atmosphere); 5 site for collecting air samples of inhaled ST; 6 gas trap; 7 venturi gauge; 8 pneumotachograph; 9 exit to differential pressure transducer (air flow); 10 pressure equilibrium vial; 11 air moistener.

Figure 3 Concentration-time courses of SO in perfusate (filled circles) and in incubations containing heat inactivated lung tissue together with perfusate (hollow circles, squares and diamants).

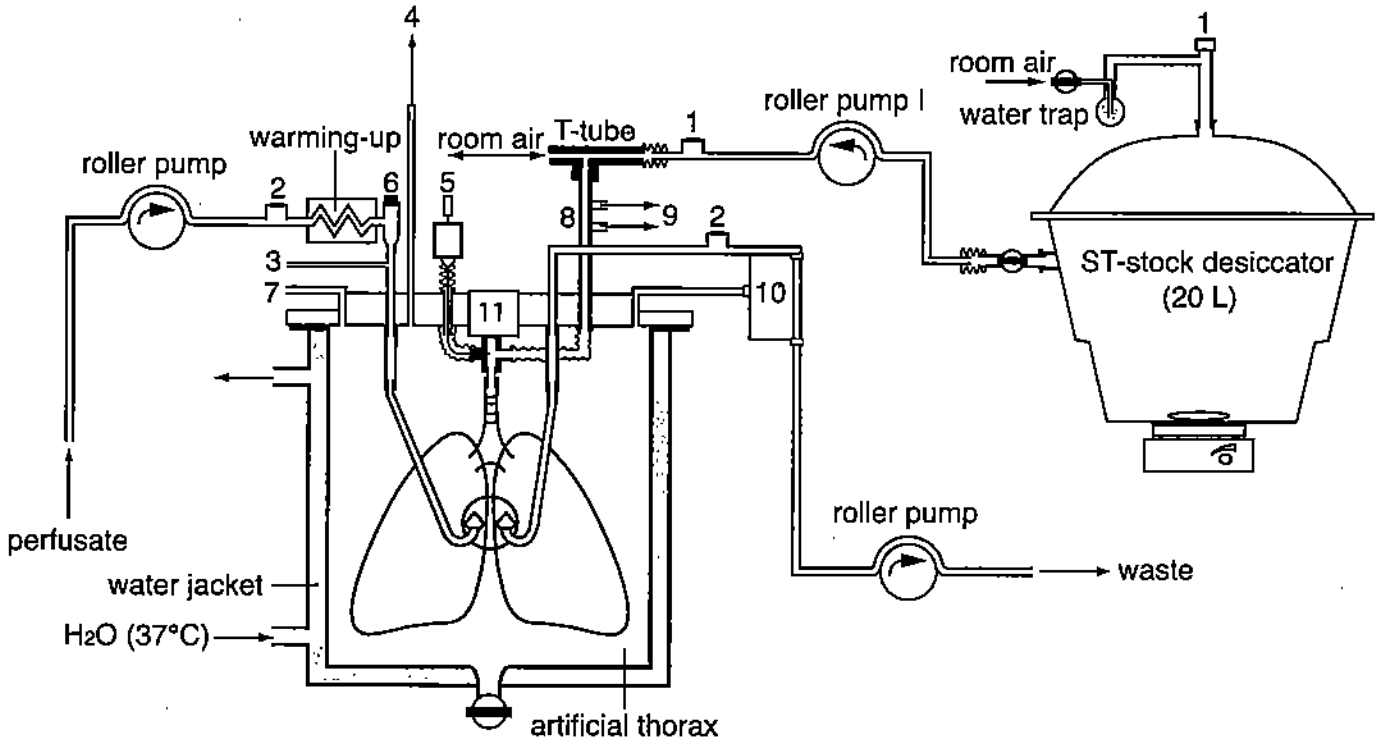
Symbols: filed circles initial concentrations 10, 4.8, 2.3, 0.55 ppm; hollow circles, initial concentration 10 ppm; hollow squares and diamantes, initial concentrations 1 ppm.

Figure 4 Concentration-time courses in three experiments with ventilated, perfused rat lungs, each at a different ST concentration in inhaled air.

ST concentrations in inhaled air are given in 3A (filled symbols) and corresponding, metabolically formed SO concentrations, determined in effluent perfusate, are presented in 3B (hollow symbols).

Figure 5 Concentration-time courses in three experiments with ventilated, perfused mouse lungs, each at a different ST concentration in inhaled air.
ST concentrations in inhaled air are given in 4A (filled symbols) and corresponding, metabolically formed SO concentrations, determined in effluent perfusate, are presented in 4B (hollow symbols).

Figure 6 "Measured" SO concentrations in lungs of rats (•) and mice (○) inhaling ST at constant concentrations of up to 630 ppm (not shown: SO level of 0.30 ± 0.015 nmol/g in the rat lung exposed to 984 ± 308 ppm ST).
Dashed lines: fitted by eye. Symbols ± bars: means ± SD of the values obtained by means of Equation 6 from the data measured in perfusate (given in Tables 2 and 3).


4
1
room air
water trap
roller pump I
warming-up
room air
T-tube
1
roller pump
2
6
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8
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11
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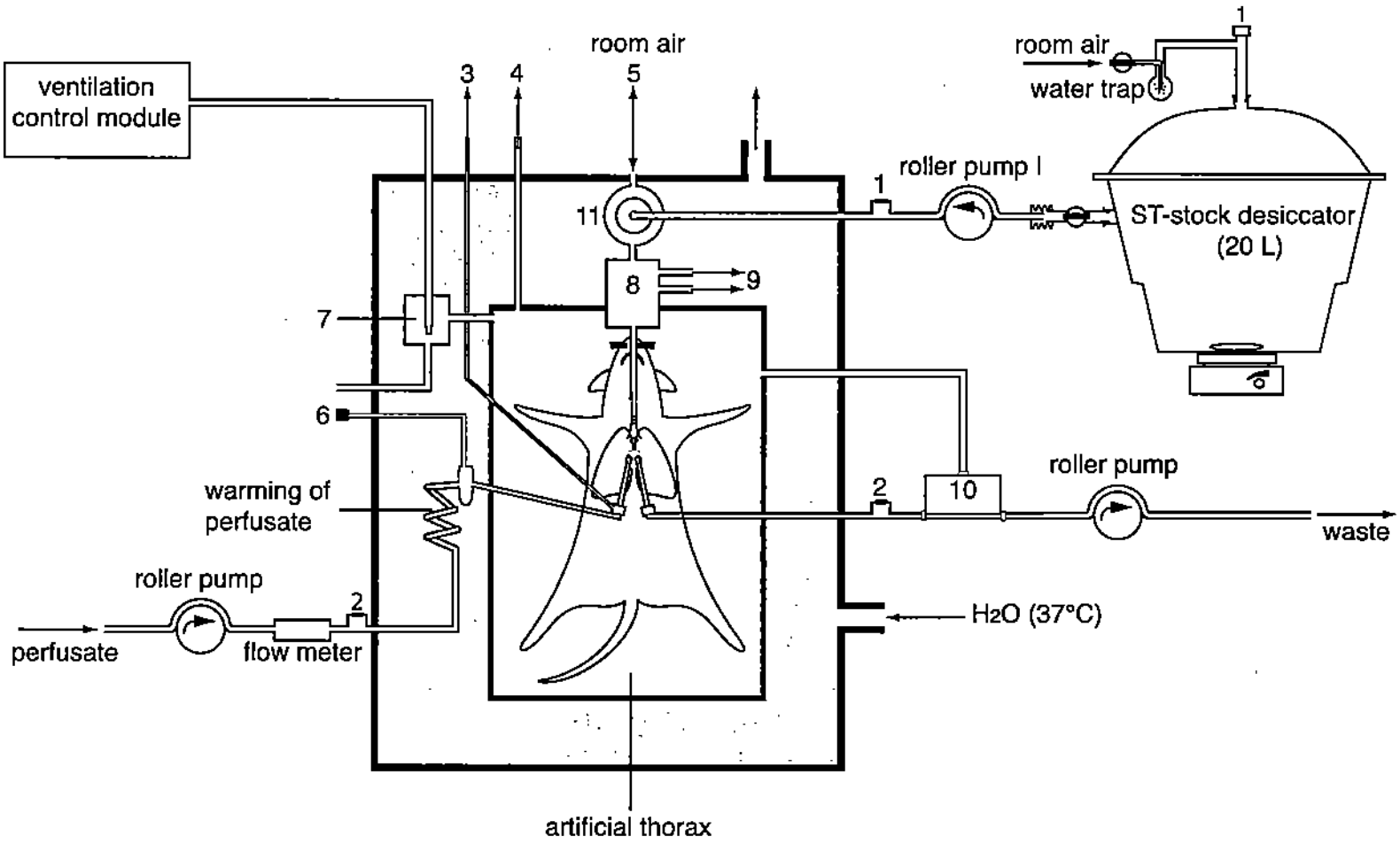
(20 L)
perfusate
roller pump
waste
water jacket
H2O (37°C)
artificial thorax

Fig.1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room air
3
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room air
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1
roller pump I
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11
ST-stock desiccator (20 L)
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7
6
warming of perfusate
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10
roller pump
waste
roller pump
2
perfusate
flow meter
H2O (37°C)
artificial thorax

Fig.2

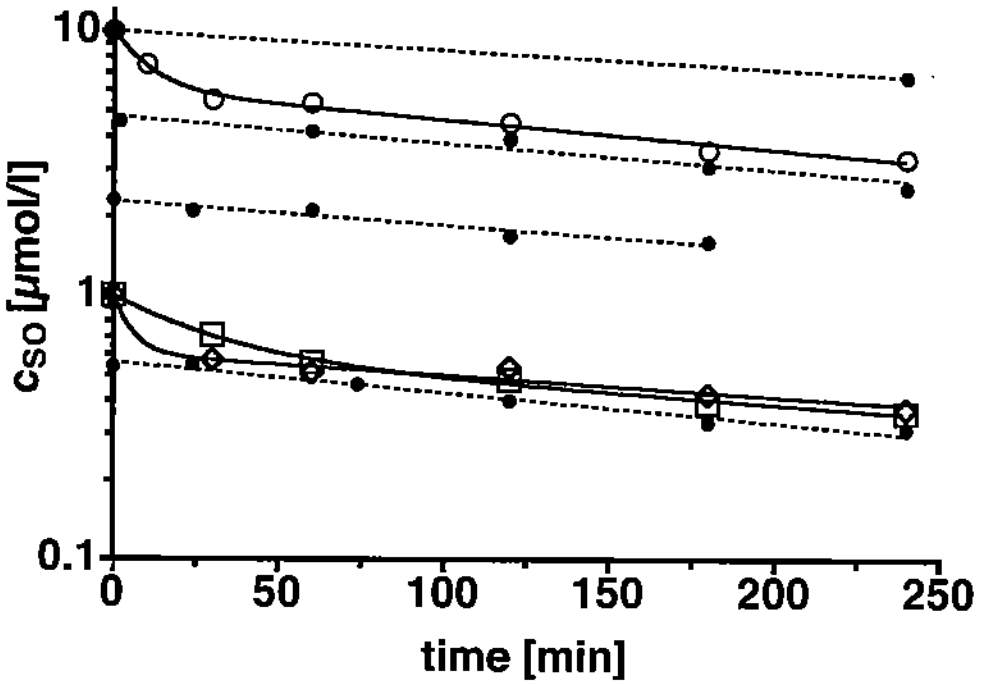


Fig.3

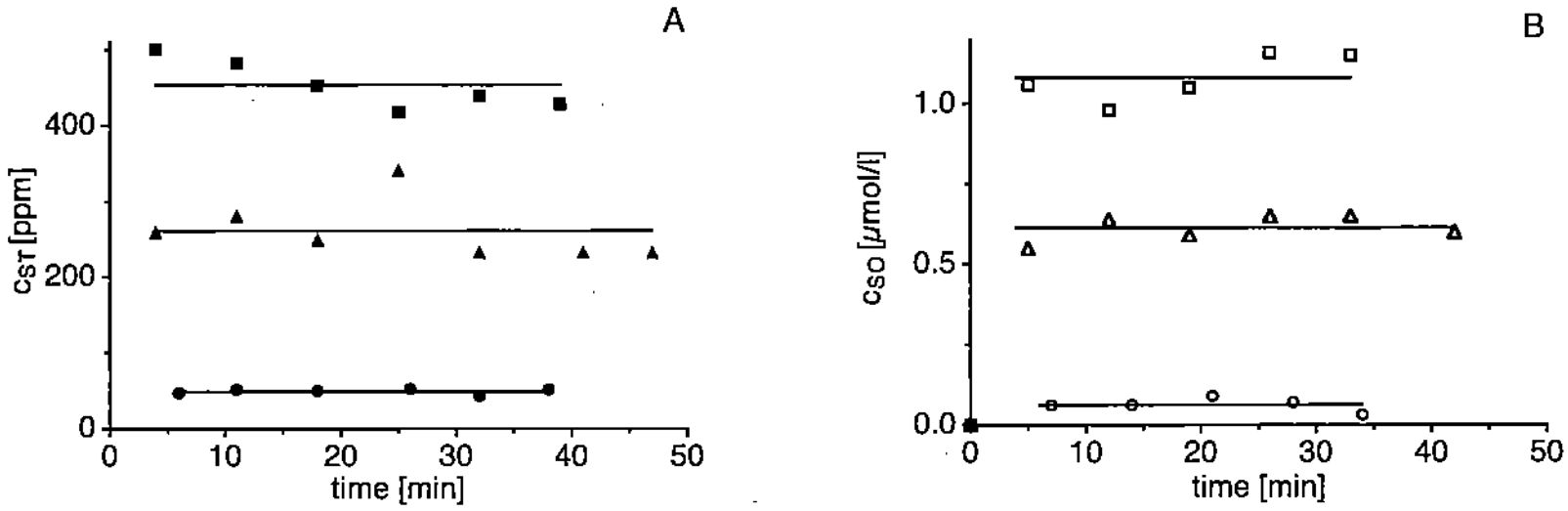
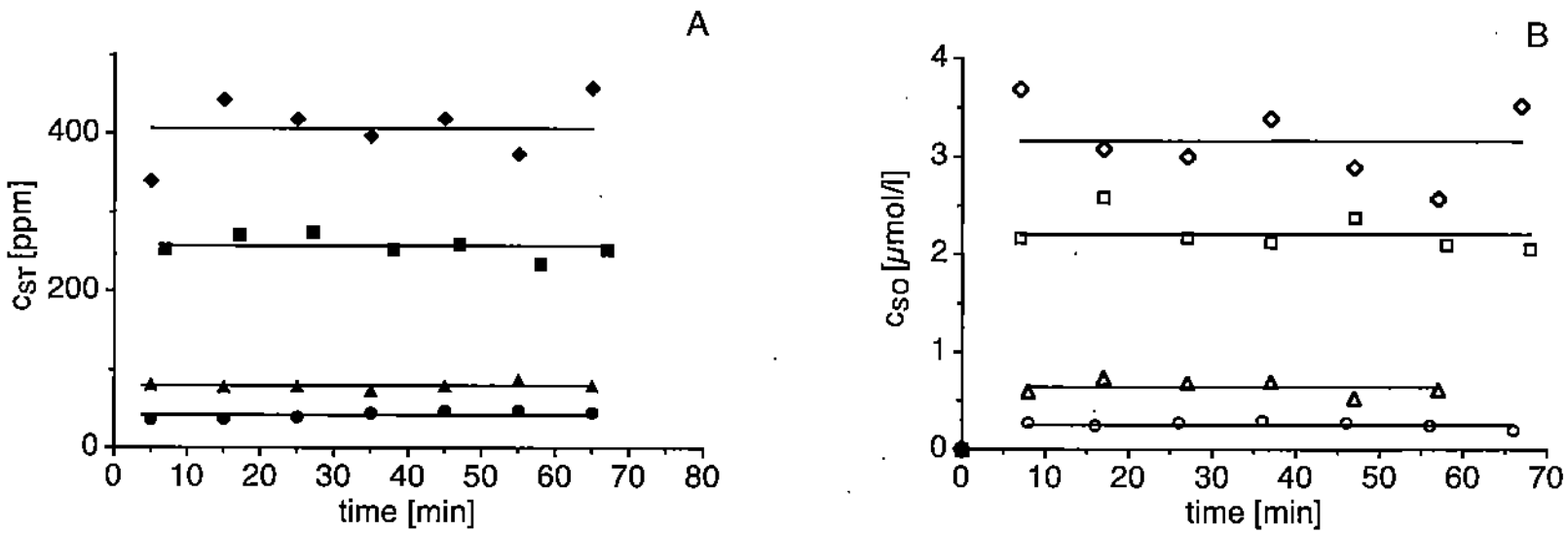

A
c_{ST} [ppm]
400
200
0
0
10
20
30
40
50
time [min]
B
c_{SO} [µmol/l]
1.0
0.5
0.0
0
10
20
30
40
50
time [min]

Fig.4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time [min]

Fig.5

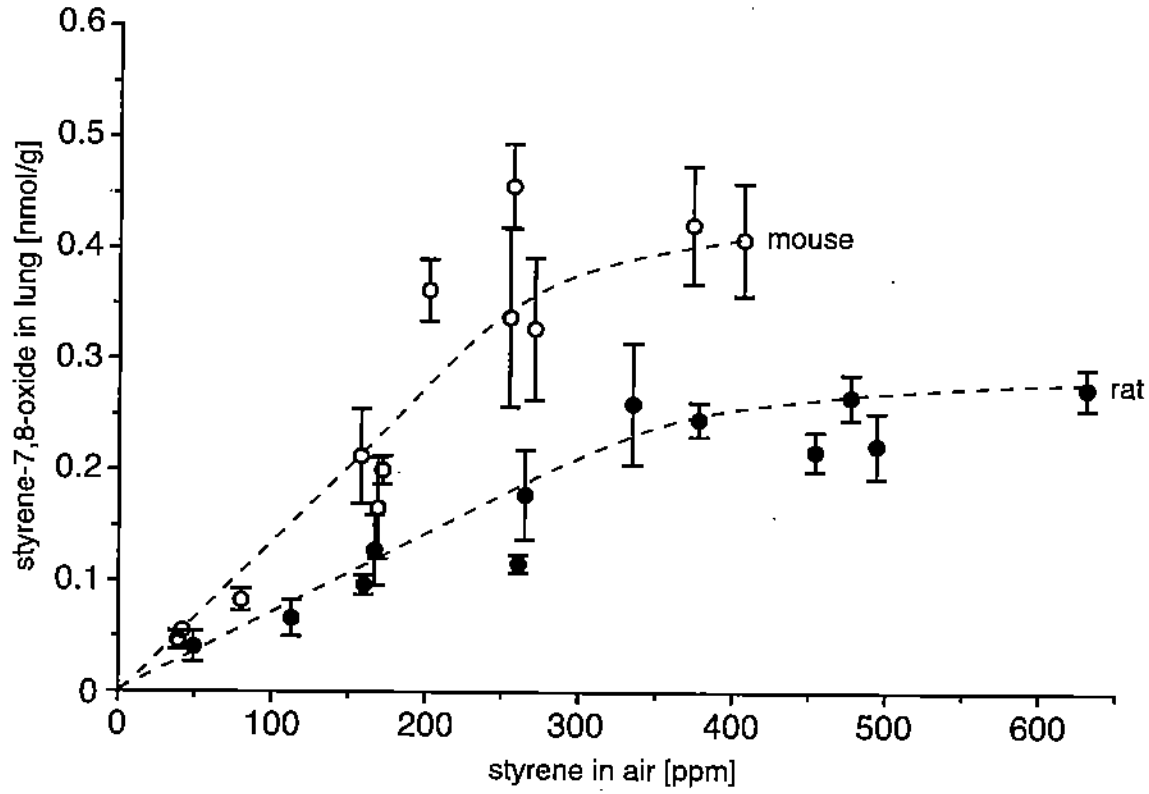
0.6
0.5
0.4
0.3
0.2
0.1
0
styrene-7,8-oxide in lung [nmol/g]
0
100
200
300
400
500
600
styrene in air [ppm]
mouse
rat

Fig.6

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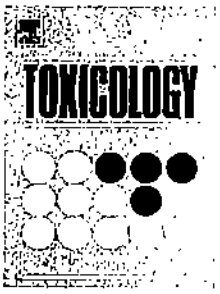

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Effects of Styrene and Styrene Oxide on Glutathione-related Antioxidant Enzymes

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Abstract

Styrene is both hepatotoxic and pneumotoxic in mice. Its mode of action is not clear, but it may be related to oxidative stress including a very large decrease in reduced glutathione (GSH). The current studies evaluated if 1) the more toxic R-styrene oxide had a greater effect on reduced GSH levels than the less toxic S-styrene oxide, 2) the ratio of reduced to oxidized forms of glutathione was altered by styrene or styrene oxide, 3) other enzymes involved in the oxidant status of the cell, namely glutathione reductase, glutathione peroxidase and □-glutamylcysteine synthetase were altered, and 4) lipid peroxidation, as measured by the determination of malondialdehyde, increased. R-Styrene oxide (300 mg/kg ip) caused greater decreases in mouse liver and lung GSH than did S-styrene oxide (300 mg/kg, ip). Styrene (600 mg/kg ip) caused decreases in both GSH and GSSG in both liver and lung. Styrene and styrene oxide did not cause significant increases in lipid peroxidation in either liver or lung. Styrene and styrene oxide had minimal effects on glutathione reductase and glutathione peroxidase in liver and lung. Styrene increased □-glutamylcysteine synthetase activity. The results suggest that while styrene and its metabolite styrene oxide cause significant decreases in GSH levels, they have little effect on the enzymes glutathione reductase and glutathione peroxidase and that in response to decreased glutathione levels there is an increase in its synthesis via induction of □-glutamylcysteine synthetase activity.

1. Introduction

Styrene is a ubiquitous chemical in the environment with primary exposures occurring in workers especially those in the reinforced plastics industry (Miller et al., 1994; Cohen et al., 2002). While a number of adverse effects have been reported in rats and mice, much of the toxicological focus has been on styrene-induced pneumotoxicity and hepatotoxicity with the mouse being a more sensitive species than the rat (Morgan et al., 1993a, 1993b; Gadberry et al., 1996; Cruzan et al., 1997). Styrene causes lung tumors in mice (Cruzan et al., 2002) but not in rats (Cruzan et al., 1998).

The primary step in the metabolism of styrene is epoxidation to form styrene oxide which is further metabolized by epoxide hydrolase to give styrene glycol which is metabolized to mandelic acid and phenylglyoxylic acid (Bond, 1989; Sumner and Fennell, 1994). Conjugation of the styrene oxide with glutathione also occurs particularly in rats and mice. Following inhalation exposure approximately 60% of the metabolites recovered from rodents are formed via the epoxide and hydrolysis pathway, and about 40% are via glutathione conjugates. A small percentage of styrene is ring metabolized to form 4-vinylphenol.

Although a number of studies have shown that styrene causes toxicity in liver and lung, the mode of action is not clear. One possibility is through oxidative stress. Styrene decreases glutathione levels in livers of mice, rats and guinea pigs with the mouse being the most sensitive species (Vainio and Makinen, 1977). Styrene and styrene oxide have also been shown by other investigators to decrease

hepatic glutathione (Srivastava et al., 1983, Beiswanger et al., 1993). While hepatic glutathione depletion happens quickly, recovery to baseline occurs within 10 to 20 hours (Katoh et al., 1989).

The effect of styrene on glutathione levels in lung has been less well studied. Exposure of rats by inhalation to 500 cm^3/m^3 styrene for 24 hours caused a 66% decrease of GSH in lung and only a 16% decrease in liver one hour after the exposure (Elovaara et al., 1990). Glutathione levels returned to normal by 24 hours. Coccini et al. (1997) exposed rats to 300 ppm styrene 6 hours/day, 5 days/week for 2 weeks and found significant decreases in non-protein sulfhydryl content immediately after the last exposure. In addition, single ip injections of styrene (400 mg/kg) or styrene oxide (200 mg/kg) resulted in significant decreases after 2 hours. They also found decreases in pulmonary (-40%) and hepatic (-35%) nonprotein sulfhydryl content immediately following inhalation of 300 ppm styrene, 5 days per week, for 2 weeks (Coccini et al., 1998). As in the liver, other studies have demonstrated the greater sensitivity of mice compared to rats to styrene-induced pulmonary GSH depletion. Mouse lung demonstrated a significant decline in GSH when exposed by inhalation to 80 ppm styrene 6 hr/day for two days with rats requiring 300 ppm to obtain a significant decrease (Filser et al., 2002; Csanady et al., 2003). Gamer et al. (2004) found that glutathione in mouse lung was significantly decreased due to inhalation of 160 ppm styrene for 6 hours per day for 21 days but not to 40 ppm. Kaufmann et al. (2005) found that styrene oxide at a

dose of 100 mg/kg given ip daily for 3 days caused glutathione depletion in the bronchiolar epithelium of mice.

We recently compared styrene and styrene oxide for their abilities to decrease glutathione levels in a dose and time dependent manner (Turner et al., 2005). Styrene (600 mg/kg, 5.8 mmols/kg ip) caused decreased GSH levels in both liver and lung within one hour. A maximum was seen at three hours with return to control levels by 12 hours. Lower doses also caused changes in a dose dependent fashion. For styrene oxide, similar findings were observed with a dose of 300 mg/kg (2.5 mmols/kg). GSH levels in liver, but not lung, returned to control by 6 hours. Again a dose response was found for both tissues. In general, the lung was more affected by these agents than was liver. The decreases in GSH suggest the possibility that the toxicity of styrene in lung and liver may be related to a profound but reversible oxidative stress in these tissues. The current studies were designed to determine if 1) the more toxic R-styrene oxide had a greater effect on reduced glutathione levels than the less toxic S-styrene oxide, 2) the ratio of reduced to oxidized forms of glutathione was altered by styrene or styrene oxide, 3) other enzymes involved in the oxidant status of the cell, namely glutathione reductase, glutathione peroxidase and □-glutamylcysteine synthetase, the rate limiting enzyme in glutathione synthesis, were altered, and 4) lipid peroxidation, as measured by the determination of malondialdehyde, was associated with styrene or styrene oxide administration.

2. Materials and methods

2.1. Animals and chemicals

Styrene and styrene oxide were from Aldrich Chemical Co. (Milwaukee, WI). Trichloroacetic acid (TCA) was from Fisher (Fair Lawn, NJ). GSH, GSSG, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), bathophenanthrolinedisulfonic acid (BPDS), 1-fluoro-2,4-dinitrobenzene (FDNB, also called 2,4-dinitrofluorobenzene), iodoacetic acid (IAA), thiobarbituric acid, 1,1,3,3-tetraethoxypropane EDTA, NADPH, glutathione reductase, tetrabutylammonium phosphate (TBAP), adenosine 5'-triphosphate (ATP), L-glutamic acid, EDTA, magnesium chloride, Tris, sucrose, boric acid, L-serine, 5-sulfosalicylic acid (SSA), □-glutamyl cysteine (□-GC), glutathione (GSH), *N*-ethylmorpholine (NEM), and monobromobimane (MBB) were obtained from Sigma (St. Louis, MO). γ-Glutamyl glutamate was obtained from Bachem Biosciences (King of Prussia, PA). All other chemicals were reagent grade or better.

CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Laboratories (Wilmington, MA). They were housed in group cages in environmentally controlled rooms on a 12 hr light:dark cycle. Rodent laboratory chow (No. 5001, Purina Mills, Inc., St. Louis, MO) and tap water were allowed ad libitum. All animals were allowed a minimum of one week to adapt to the animal facilities and diet before being used in any experiment.

2.2. Treatments

Groups of 4 mice (22-28 grams) were administered styrene (600 mg/kg, 5.8 mmols/kg) in corn oil, styrene oxide 300 mg/kg (2.5 mmols/kg) in corn oil, or corn oil

as control vehicle ip. In three cases an extra animal was added to give groups of five mice, and in one case a sample was not usable giving a group of 3 mice. To determine the effect of styrene (600 mg/kg, ip) on GSH and GSSG levels, groups of mice were sacrificed at 2, 6, or 12 hours after styrene administration. These times were selected based on our previous studies on the time course of GSH depletion (Turner et al., 2005). In comparing the R- and S- enantiomers of styrene oxide on GSH levels, groups of animals were administered 300 mg/kg styrene oxide ip, and measurements were made at 2, 6 and 12 hours after administration since in our earlier studies GSH levels in the treated animals were decreased by 2 hours, were still decreased at 6 hours, and returned to control levels by 12 hours. In the lipid peroxidation studies, mice were treated with styrene (600 mg/kg, ip) and measurements made at 1, 3, 6, 12 and 24 hours, with styrene oxide (300 mg/kg, ip) and measurements at 1, 3 and 12 hours to determine the time course relative to GSH levels or with 4-vinylphenol (100 mg/kg), a minor toxic metabolite of styrene which is more potent a hepatotoxicant and pneumotoxicant than styrene or styrene oxide (Carlson et al., 2002; Vogie et al., 2004), and measurements made at 3 and 12 hours where any changes that might be expected to occur would be at the earlier time point with return to control by the later time point. Glutathione reductase measurements were made 3, 6 and 12 hours after styrene oxide (300 mg/kg, ip) administration, and glutathione peroxidase measurements were made 1, 3, 6, and 12 hours after styrene oxide (300 mg/kg, ip). These times were selected to include the times when GSH was expected to be decreased and when recovery of GSH levels

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to control levels was expected based on our previous studies. When □-glutamylcysteine synthetase activity was measured, groups of 4 or 5 mice were administered styrene (600 mg/kg ip), and measurements were made 6 and 24 hours later. This was the time period during which GSH levels were returning to control in our previous studies (Turner et al., 2005). In all cases, the dosing solutions were prepared immediately before administration and were given in a volume of 1 ml/100 g body weight.

2.3. Assay for reduced glutathione

Entire lungs and livers (approximately 0.20 to 0.28 grams and 1.2 to 1.9 grams respectively) were removed and weighed, and deproteinized homogenates were prepared by homogenizing the tissues in 2 ml and 3 ml respectively of cold 0.1 M potassium phosphate buffer, pH 7.5, followed by the addition of one-half volume of 10% TCA. Homogenates were centrifuged at 10,000 x g in a refrigerated centrifuge for 5 minutes to prepare the supernatants for assay. The assay was that of Speisky et al. (1986, 1989). Briefly, the deproteinized supernatants were combined with a solution of DTNB in 0.1 M potassium phosphate buffer giving a final concentration of 10 mM DTNB. Samples were mixed by vortexing, and the absorbance was determined at 464 nm. A standard curve was generated ranging from 0 to 625 nmols GSH and was used to calculate the tissue GSH levels. Amounts were normalized per gram of tissue.

2.4. HPLC assay for reduced and oxidized glutathione

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Reduced and oxidized glutathione levels were measured using the procedure of Fariss and Reed (1987) as modified in Current Protocols in Toxicology Online (Mustacich, 2003). Deproteinized homogenates were prepared by homogenizing the livers or lungs of mice in 0.1 M potassium phosphate buffer, pH 7.5, followed by acid extraction with one-half volume of 10% perchloric acid containing 1 mM bathophenanthrolinedisulfonic acid. Homogenates were centrifuged at 15,000 x g for 5 minutes to obtain the supernatant. The thiols in the supernatant were reacted for 5 minutes with 100 mM iodoacetic acid in 0.2 mM *m*-cresol purple to form S-carboxymethyl derivatives which were treated with 2 M KOH / 2.4 M $KHCO_3$ for 1 hour at room temperature. They were then further derivatized with 1-fluoro-2,4-dinitrobenzene for 1 hour at room temperature, followed by >8 hours at 4°C. □-Glu-glu was used as an internal standard. The DNP derivatives were separated using a 3-aminopropyl column with UV detection at 365 nm. The mobile phase was maintained at 80% of Solvent A (consisting of 80% methanol and 20% water) plus 20% of Solvent B (0.8 M sodium acetate in 64% methanol) for 8 minutes, followed by a 20-minute linear gradient to 1% A, 99% B, at a flow rate of 1.0 ml/minute. The mobile phase was kept at this level for 12 minutes. GSH and GSSG were used as standards. Amounts were normalized per gram of tissue.

2.5. Assay for malondialdehyde

The measurement of malondialdehyde determined as thiobarbituric acid reacting substances (TBARS) was used as an indicator of lipid peroxidation. The method is that of Buege and Aust (1978) as modified in Current Protocols in

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Toxicology Online (Reilly and Aust, 2003) with some additional changes based on published methods by Uchiyama and Mihara (1978) and Ohkawa et al. (1979). Deproteinized homogenates were prepared by homogenizing the livers or lungs of mice in 0.1 M potassium phosphate buffer, pH 7.5, and then immediately placing 1 ml of each homogenate into 2 ml of a mixture of 0.37% thiobarbituric acid, 15% trichloroacetic acid, and 0.2 N hydrochloric acid. The mixture was heated to boiling for 15 minutes, and the TBARS were extracted using 3 ml of n-butanol/pyridine (15:1 v/v). Absorbance of the organic phase was determined at a wavelength of 535 nm, and concentrations were determined using a standard curve generated from the hydrolysis of 1,1,3,3-tetraethoxypropane. Amounts were normalized per gram of tissue.

2.6. Assay for glutathione reductase

Glutathione reductase activity was measured using the procedure of Mannervik (2003). Livers and lungs were homogenized in 0.2 M potassium phosphate buffer, pH 7.0, followed by centrifugation at 10,000 x g for 20 minutes. The supernatant was then added to a mixture containing 1.78 mM EDTA in 178 mM potassium phosphate buffer, 1 mM GSSG, and 0.1 mM NADPH in 10 mM Tris-HCl in a final volume of 1 ml. The linear decrease in absorbance of the NADPH at 340 nm was measured using a Shimadzu spectrophotometer at 340 nm for two minutes. A blank was prepared without tissue. Activity was normalized per mg protein.

2.7. Assay for glutathione peroxidase

Glutathione peroxidase activity was measured using the procedure described by Esworthy et al. (2003). Liver and lung were homogenized in 50 mM sodium phosphate buffer, pH 7.0 and centrifuged at 10,000 x g for 20 minutes. The sample was then combined with 31.5 mM sodium phosphate buffer, 1 mM GSH, 0.2 mM □-NADPH, 11.25 mM sodium azide, and 10 units glutathione reductase in a final volume of 1.0. After allowing the incubation mixture to equilibrate for a few minutes, 0.238 mM hydrogen peroxide was added. The linear decrease in absorbance of NADPH was measured at 340 nm for one minute following a lag time of 30 seconds. Activity was normalized per mg protein.

2.8. Assay for □-glutamylcysteine synthetase

Supernatant protein was prepared by homogenizing mouse lung in TES/SB buffer containing 0.25 M sucrose, 20 mM Tris, 1 mM EDTA, 20 mM boric acid and 1 mM L-serine. Homogenization was done on ice. Homogenates were centrifuged at 10,000 x g for 15 min at 4°C. Standards of □-GC and GSH were prepared by serial dilutions. Baseline samples were prepared with GLCL reaction mixture containing 20 mM ATP, 100 mM L-glutamic acid, 1 mM EDTA, 20 mM magnesium chloride, and 200 mM Tris. To this mixture was added the supernatant, water, and 200 mM SSA. Standards were prepared the same way, using the standard solution instead of supernatant. Assay samples were prepared with GLCL reaction mixture plus supernatant which were then pre-incubated for 5 min at 37°C. Reactions were initiated by adding 5 mM L-cysteine, and samples were incubated for 10 min at 37°C. Reactions were stopped by adding 200 mM SSA. Samples and standards

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were derivatized by adding 0.2 M NEM/0.02 M KOH plus 25 mM MBB, followed by incubation in the dark at room temperature for 30 min. Reactions were stopped with 200 mM SSA, followed by microcentrifuging at top speed for 2 min. Samples and standards were analyzed by HPLC, using a C-18 reversed phase column and fluorescence detection with excitation at 375 nm, and emission at 475 nm. The aqueous mobile phase was 1 mM TBAP adjusted to pH 3 with 10 % phosphoric acid. The organic phase was 100% methanol. The starting point for the gradient was 95% aqueous phase, 5% organic phase. The gradient proceeded to 80% aqueous by 1 min, 70% aqueous by 10 min, 40% aqueous by 12 min, and was held there until 14 min. Then it returned to 95% aqueous by 16 min and was held there until the program ended at 21 min. Activity was normalized per mg protein.

2.9. Statistical analysis

Values are expressed as mean ± S.E. The numbers of animals in each group are indicated in the tables. In most cases, samples were run in duplicate. In comparing between two groups, Student's t test was used. In comparing multiple values, an ANOVA was utilized followed by Student Newman-Keuls' test to detect differences among the groups. In each case the level of significance selected was $p < 0.05$.

3. Results

While our previous studies were informative with respect to dose and time, they did not demonstrate what is happening with respect to the ratio of reduced glutathione to oxidized glutathione following styrene or styrene oxide

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administration (Turner et al., 2005). Therefore, selected studies, based on the above, were done in which reduced glutathione and oxidized glutathione levels were determined in liver and lung using an HPLC method. The results observed were similar to those obtained in our previous study. GSH levels in liver were found to be greatly decreased following administration of 600 mg/kg styrene at both 2 and 6 hours (Table 1). There was no statistically significant difference from control at 12 hours. There was no increase in oxidized glutathione (GSSG). In fact, there was a decrease at the 6 hour time period. As with GSH, GSSG levels returned to normal by 12 hours. GSH levels were decreased in lung at 2 and 6 hours after styrene administration (Table 2) similar to what was observed in the previous study. GSSG levels were decreased by 6 hours and remained so at 12 hours. Here, as in the results on the enzyme assays described below, there was considerable variation among the control values. This may well be related to the diurnal variations associated with glutathione, and thus the corresponding controls at each time point are critical.

In previous studies it was shown that the R-enantiomer of styrene oxide is more toxic than is the S-enantiomer (Gadberry et al., 1996), so it was of interest in view of our previous finding of the racemic mixture causing a depletion of GSH (Turner et al., 2005) to determine if there was a difference between the two enantiomers in their ability to cause depletion of GSH. When administered to mice at a dose of 300 mg/kg, R-SO caused a significantly greater decrease in GSH in liver and in lung than did S-SO two hours after administration (Table 3). Six hours after

administration, GSH levels in the livers of animals given R-SO were still decreased, but they were back to normal in S-SO treated mice. At this time, GSH levels were not decreased in lung. At 12 hours post-administration, there were no decreases with either enantiomer.

Studies were also carried out to examine the effects of styrene and styrene oxide on lipid peroxidation in liver and lung by measuring malondialdehyde using the thiobarbituric acid method. Mice were treated with either styrene (600 mg/kg ip) or racemic styrene oxide (300 mg/kg ip) similar to the treatments used in the glutathione assays. Styrene had a small and transient effect (Table 4) while styrene oxide treatment did not cause any increases (Table 5). This lack of a positive effect is somewhat surprising in view of the great decreases we had observed in reduced glutathione levels in these tissues and the positive effects with lipid peroxidation observed in other studies (Srivastava et al., 1983; Katoh et al., 1989; Dare et al., 2004). The reason for this is not clear. Lipid peroxidation was also not increased following the administration of the styrene metabolite 4-vinylphenol (Table 6).

Further studies were carried out to better characterize the effect of styrene on other enzymes involved in the oxidant state of the cell related to glutathione. To do this the active metabolite styrene oxide was used. The activity of glutathione reductase, important in converting GSSG to GSH, was measured. Styrene oxide had no effect in liver and lung at 3 and 6 hours (Table 7). There was a decrease noted in the liver at 12 hours. The effect of styrene oxide on glutathione peroxidase,

important in maintaining low levels of peroxides in cells, was also measured. There was a great deal of variability within groups, but the results indicate that styrene oxide had no consistent effect on this enzyme (Table 8).

As noted above and in our previous study (Turner et al., 2005), styrene and styrene oxide caused significant decreases in GSH levels in both liver and lung soon after administration, but these levels returned to control values within 6 to 24 hours. To determine if such treatment stimulated an increase in GSH synthesis, measurements were made on □-glutamylcysteine synthetase. Styrene administration (600 mg/kg ip) caused increased glutamylcysteine formation, the rate limiting step in glutathione synthesis, after 6 and 24 hours (Table 9).

4. Discussion

While the ability of styrene and its metabolites to cause hepatotoxicity and pneumotoxicity is well known (Morgan et al., 1993a, 1993b; Gadberry et al., 1996; Cruzan et al., 1997), the mode of action is not. One possibility that has been suggested is that styrene causes cytotoxicity that may be related to oxidative stress. Lung cells in particular are very susceptible to oxidative stress. GSH is present at high concentrations (approximately 3 mM) in cells where it plays a major role in protecting them against oxidative damage. It also provides the reducing milieu needed to maintain the integrity of cell membranes, participates in a number of metabolic pathways such as the synthesis of leukotrienes and is involved in detoxification reactions (see Rahman et al., 1999 for a review). In addition,

glutathione is excreted into the extracellular fluid lining the alveolar epithelial surface where it also appears to play a protective role.

Styrene has been shown in a number of studies to cause a decrease in reduced glutathione levels, possibly resulting in oxidative stress, in both liver and lung with the mouse appearing to be a sensitive species. (Vainio and Makinen, 1977; Srivastava et al., 1983; Beiswanger et al., 1993). The effect occurs very rapidly and with a return of GSH to control levels between 12 and 24 hours (Katoh et al., 1989; Beiswanger et al., 1993). These observations were supported by our previous work which demonstrated that styrene and styrene oxide caused significant dose and time dependent decreases in GSH in both liver and lung with rapid return to control levels within 6 to 12 hours (Turner et al., 2005). Interestingly, Marczynski et al. (1997) detected 8-hydroxydeoxyguanosine, a marker of oxidative damage to DNA, in white blood cells of workers occupationally exposed to styrene. These authors have proposed oxidative stress to be the basis of genotoxicity in humans (Marczynski et al., 2000).

To determine if the level of GSH had decreased as a result of its conversion to GSSG, the levels of both were measured using a HPLC method. Control levels were similar to those we reported using a colorimetric assay as were the decreases due to styrene and styrene oxide administration (Turner et al., 2005). This would indicate that the effect was not due simply to the oxidation of the reduced glutathione. This finding is not too surprising. Considerable work has been done for many years on styrene metabolism which indicates that styrene oxide is conjugated with

glutathione with the eventual excretion of mercapturic acids (Leibman, 1975; Seutter-Berlage et al., 1978; Mendralla et al., 1993; see Sumner and Fennell, 1994 for a review). There are also stereochemical relationships in this activity (Dostal et al., 1987; Hiratsuka et al., 1989; Linhart et al., 2000) with the R-SO reacting faster. Another important factor involved in the cellular status of glutathione is glutathione reductase which is important in converting the oxidized form back to the reduced form. The activity of this enzyme was not consistently decreased by styrene oxide.

To further examine the role of styrene oxide in the depletion of glutathione, the two enantiomers were compared. R-styrene oxide caused a greater and longer lasting depletion than did S-styrene oxide. This is in keeping with our previous observation that R-styrene oxide is more toxic than S-styrene oxide is (Gadberry et al., 1996).

Glutathione peroxidase plays an important role in protecting the cell from oxidative damage through the general reaction ROOH + 2GSH □ GSSG + H_2O. The activity of this enzyme was not generally altered by the administration of 300 mg/kg of racemic styrene oxide.

Because there is a rapid reversal of the GSH depleting action of styrene and styrene oxide (Turner et al., 2005), the hypothesis was tested that there was an increase in the biosynthesis of GSH in mouse lung in response to styrene administration. □-Glutamylcysteine synthetase (glutamate cysteine ligase) is the first and rate limiting step in the synthesis of GSH (Shi et al., 1994; Rahman et al.,

1996; Tian et al., 1997; Mari and Cederbaum, 2000). It has been shown to be increased in mouse Clara cells in response to naphthalene (West et al., 2000) and in rat lung to monocrotaline (Yan and Huxtable, 1996). It has also been shown to be responsive *in vitro* in human alveolar epithelial cells exposed to cigarette smoke (Rahman et al., 1996), in rat lung epithelial L2 cells exposed to 2,3-dimethoxy-1,4-naphthoquinone (Shi et al., 1994) and buthionine sulfoximine (Tian et al., 1997), and in HepG2 cells exposed to diethyl maleate (Sekhar et al., 1997). As hypothesized, there was an increase in the activity of this enzyme activity.

The literature on whether or not styrene increases lipid peroxidation is mixed. Dare et al. (2004) demonstrated that SK-N-MC neurons exposed to styrene oxide exhibited a lower mitochondrial Ca^{++} capacity and loss of mitochondrial membrane potential in addition to lipid peroxidation after 12 hours. Srivastava et al. (1983) and Katoh et al. (1989) also reported lipid peroxidation in the livers of rats administered styrene or styrene oxide. However, Gamer et al. (2004) found only a slight increase in malondialdehyde in mouse lung after a single exposure to 160 ppm styrene. Increases were not observed after 5 or 20 exposures or after a single exposure to 40 ppm. Coccini et al. (1996) exposed rats to 326 ppm of styrene (6 h daily, 5 days a week, for 2 weeks) and found a 50% depletion of hepatic GSH but did not find an increase in lipid peroxidation in liver or brain. Moreover, Dlugosz et al. (2005) found that styrene alone did not increase malondialdehyde in the plasma of workers although the combination of styrene and ethylene glycol did. In our current studies, lipid peroxidation was not generally associated with styrene

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administration with minor increases in the liver being observed at 3 and 12 but not at 6 hours and with no increases in the lung. No increases were observed with styrene oxide or the minor metabolite 4-vinylphenol. Thus lipid peroxidation as measured by TBARs does not appear to play an important role in the toxicity of styrene. Thus it is not entirely clear at this time why, despite the decrease in GSH levels, there is no increase in lipid peroxidation despite the loss of GSH. The mechanism by which styrene exerts its effects is not entirely clear. We have, however, noted an increase in reactive oxygen species (ROS) in Clara cells incubated with styrene and its metabolites (unpublished observation).

In summary, we have confirmed that styrene oxide causes significant deficits in GSH in both liver and lung with the R-enantiomer being more active in terms of both potency and time to recovery. This decrease is not accompanied by an increase in oxidized glutathione (GSSG). Other enzymes involved, namely glutathione reductase and glutathione peroxidase, were generally not affected by styrene oxide in either liver or lung. Lipid peroxidation does not appear to play a major role in cell damage even at doses of styrene, styrene oxide or 4-vinylphenol known to cause significant injury to liver and lung. In response to decreased glutathione there is an increase in synthesis via induction of □-glutamylcysteine synthetase activity

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Table 1. Effect of styrene on reduced and oxidized glutathione levels in CD-1 mouse liver.

	Control			Styrene[a]		
Hours	N[b]	GSH	GSSG	N[b]	GSH	GSSG
2	4	5.64 ± 0.33[c]	0.47 ± 0.08	4	0.80 ± 0.29[d]	0.36 ± 0.08
6	4	4.32 ± 0.41	0.70 ± 0.16	4	1.08 ± 0.31[d]	0.22 ± 0.04[d]
12	4	3.56 ± 0.24	0.56 ± 0.07	4	2.45 ± 0.48	0.40 ± 0.15

[a]600 mg/kg, ip

[b]Number of animals

[c]μmols/gram liver; for each time and measurement, value is mean ± S.E. for N mice

[d]Significantly different from control, $p < 0.05$

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Table 2. Effect of styrene on reduced and oxidized glutathione levels in CD-1 mouse lung.

	Control			Styrene[a]		
Hours	N[b]	GSH	GSSG	N[b]	GSH	GSSG
2	4	1.98 ± 0.10[c]	0.47 ± 0.07	4	0.88 ± 0.06[d]	0.37 ± 0.06
6	4	1.79 ± 0.06	0.56 ± 0.19	4	0.85 ± 0.05[d]	0.16 ± 0.05[d]
12	4	1.81 ± 0.15	0.66 ± 0.26	4	1.35 ± 0.17	0.11 ± 0.04[d]

[a]600 mg/kg, ip

[b]Number of animals

[c]μmols/gram liver; for each time and measurement, value is mean ± S.E. for N mice

[d]Significantly different from control, $p < 0.05$

Table 3. Comparison of R-styrene oxide (R-SO) and S-styrene oxide (S-SO) on glutathione levels in mouse liver and lung.

	Glutathione Levels[a]					
Time	Liver			Lung		
(hours)	Control	R-SO[b]	S-SO[b]	Control	R-SO[b]	S-SO[b]
2	8.34 ± 0.55[c]	2.75 ± 0.54[d]	4.81 ± 0.27[e]	1.73 ± 0.08[c]	0.73 ± 0.04[d]	1.35 ± 0.12[e]
6	6.12 ± 0.56[c]	3.13 ± 0.35[d]	5.85 ± 0.29[c]	2.04 ± 0.18[c]	1.98 ± 0.18[c]	2.81 ± 0.16[d]
12	4.90 ± 0.28[c]	4.21 ± 0.58[c]	5.66 ± 0.33[c]	1.71 ± 0.05[c]	1.86 ± 0.09[c]	2.34 ± 0.19[d]

[a]micromoles/gram tissue; for each time and measurement, value is mean ± S.E. for 4 mice

[b]300 mg/kg ip

[c-e]For each tissue and time, values with different superscripts are significantly different from one another, $P < 0.05$

Table 4. Effect of styrene on lipid peroxidation in mouse liver and lung.

		Liver			Lung	
		Control	Styrene[a]		Control	Styrene[a]
Hours	N[b]	MDH	MDH	N[b]	MDH	MDH
1	4	13.1 ± 0.3[c]	11.6 ± 0.8	4	58.1 ± 7.2	54.4 ± 8.2
3	4	13.7 ± 1.2	18.8 ± 1.5[d]	4	48.0 ± 6.6	46.1 ± 6.5
6	4	13.9 ± 1.2	13.6 ± 0.5	4	66.6 ± 6.0	67.7 ± 5.5
12	4	15.7 ± 0.6	26.0 ± 4.7[d]	4	68.8 ± 8.2	43.2 ± 6.5[d]
24	4	9.0 ± 1.3	8.7 ± 0.5	4	44.7 ± 2.1	47.1 ± 5.8

[a]600 mg/kg, ip

[b]Number of animals

[c]μmols malondialdehyde/gram tissue; for each time and measurement, value is mean ± S.E. for N mice.

[d]Significantly different from control, $p < 0.05$

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Table 5. Effect of styrene oxide on lipid peroxidation in mouse liver and lung.

	Liver			Lung		
		Control	Styrene Oxide[a]		Control	Styrene Oxide[a]
Hours	N[b]	MDH	MDH	N[b]	MDH	MDH
1	4	17.6 ± 1.8[c]	15.1 ± 0.7	4	57.3 ± 5.7	65.3 ± 2.1
3	4	30.3 ± 0.7	27.8 ± 1.1	4	89.8 ± 10.0	97.2 ± 14.2
12	4	29.6 ± 2.3	25.8 ± 1.5	5	137.1 ± 14.7	95.5 ± 5.6[d]

[a]300 mg/kg, ip

[b]Number of animals

[c]μmols malondialdehyde/gram tissue; for each time and measurement, value is mean ± S.E. for N mice

[d]Significantly different from control, $p < 0.05$

Table 6. Effect of 4-vinylphenol on lipid peroxidation in mouse liver and lung.

	Liver			Lung		
		Control	4-Vinylphenol[a]		Control	4-Vinylphenol[a]
Hours	N[b]	MDH	MDH	N[b]	MDH	MDH
3	4	4.9 ± 0.8[c]	5.0 ± 0.6	4	20.6 ± 2.7	17.8 ± 0.5
12	4	24.5 ± 2.3	22.6 ± 2.3	4	81.2 ± 4.7	90.4 ± 8.0

[a]100 mg/kg, ip

[b]Number of animals

[c]µmols malondialdehyde/gram tissue; for each time and measurement, value is mean ± S.E. for N mice.

Table 7. Effect of styrene oxide on glutathione reductase (GR) activity in mouse liver and lung.

	Liver			Lung		
Time		Control	Styrene Oxide[a]		Control	Styrene Oxide[a]
(hours)	N[b]	GR	GR	N[b]	GR	GR
3	4	11.68 ± 0.31[c]	12.17 ± 0.90	4	11.09 ± 0.93	9.01 ± 1.18
6	4	12.12 ± 0.24	11.10 ± 1.03	4	8.20 ± 0.09	8.54 ± 1.00
12	4	18.54 ± 0.94	11.08 ± 0.67[d]	4	11.83 ± 1.42	8.75 ± 0.81

[a]300 mg/kg, ip

[b]Number of animals

[c]μmols NADPH oxidized/mg protein/ min; for each time and measurement, value is mean ± S.E. for N mice

[d]Significantly different from control, $p < 0.05$

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Table 8. Effect of styrene oxide on glutathione peroxidase (GP) activity in mouse liver and lung.

	Liver			Lung		
Time		Control	Styrene Oxide[a]		Control	Styrene Oxide[a]
(hours)	N[b]	GP	GP	N[b]	GP	GP
1	4	83 ± 24[d]	184 ± 78	4	31.0 ± 13.2	22.6 ± 3.3
3	3&4[c]	249 ± 23	230 ± 15	4	21.7 ± 7.2	62.7 ± 10.9[e]
6	4	197 ± 35	234 ± 48	4	46.6 ± 3.7	33.7 ± 8.5
12	4	105 ± 15	50 ± 19	4	19.1 ± 5.1	16.6 ± 4.2

[a]300 mg/kg, ip

[b]Number of animals

[c]Three for control and four for styrene oxide

[d]μmols NADPH oxidized/mg protein/ min; for each time and measurement, value is mean ± S.E. for N mice

[e]Significantly different from control, $p < 0.05$

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Table 9. Effect of styrene on □-glutamylcysteine synthetase activity in mouse lung.

Time	N[b]	Control	N[b]	Styrene[a]
6 hours	5	342 ± 85[c]	5	581 ± 79
24 hours	4	198 ± 66	4	520 ± 55[d]

[a]600 mg/kg, ip

[b]Number of animals

[c]pmols □-glutamylcysteine/mg protein/min, for each time and measurement, value is mean ± S.E. for N mice

[d]Significantly different from control, $p < 0.05$

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Comparison of Styrene and its Metabolites Styrene Oxide and 4-Vinylphenol on Cytotoxicity and Glutathione Depletion in Clara Cells of Mice and Rats

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Abstract

Styrene is a widely used compound in the manufacturing industry. In mice and rats, it is both hepatotoxic and pneumotoxic. It causes lung tumors in mice, but not in rats. The Clara cell is the main target for the toxicity of styrene and its metabolites, and it also has the greatest activity for styrene metabolism. Therefore, Clara cells isolated from CD-1 mice and Sprague-Dawley rats were used to compare the cytotoxicities induced by styrene and its metabolites. The cytotoxicity of styrene was greater *in vitro* than that of its metabolites styrene oxide (racemic, R- and S-) and 4-vinylphenol in contrast with what has been observed *in vivo* in previous studies on hepatotoxicity and pneumotoxicity. Susceptibility of rats to styrene and its metabolites are 4-fold less than that observed with mice. Glutathione levels were also measured in mice following addition of the chemicals *in vitro* and treatment of the CD-1 mice *in vivo*. Decreases in glutathione concentrations were seen even at doses which did not cause the death of mouse Clara cells. Significant decreases in glutathione were observed 3 hours after treatment with racemic SO and R-SO. At 12 hours, rebound effects were seen for all compounds, with all but R-SO rebounding above controls. These studies suggest that *in vitro* cytotoxicity of styrene and its metabolites does not strictly follow *in vivo* effects and that decreases in mouse glutathione levels may be related to oxidative stress.

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1. Introduction

Styrene is widely used in the manufacturing of plastics, resins, and rubbers. The general population can be exposed through transfer from polystyrene disposable cups and containers, cigarette smoke, and ambient air. However, exposure is highest for workers in the reinforced plastics industry (IARC, 2002). Styrene has been classified as possibly carcinogenic (IIB) in humans, and styrene oxide, its major metabolite, as probably carcinogenic (IIA) (IARC, 1994). Styrene is converted to styrene-7,8-oxide (SO) via the cytochrome P450 monooxygenase system. It is generally thought that styrene toxicity is caused by its transformation to SO, an electrophilic epoxide (Bond, 1989). SO exists in two enantiomeric forms – R and S. Alternatively, the ring hydroxylated metabolite 4-vinylphenol may be involved in styrene toxicity (Carlson et al., 2002; Cruzan et al., 2005). Although it is a minor metabolite, it is a more potent hepatotoxicant and pneumotoxicant than is styrene oxide.

While mice are susceptible to the toxicity associated with styrene and its metabolites, rats don't exhibit the same degree of response. Gadberry et al. (1996) showed that both styrene and SO are hepatotoxic and pneumotoxic in mice based on changes in serum SDH (sorbitol dehydrogenase), and GGT (gamma-glutamyltranspeptidase) and LDH (lactate dehydrogenase) levels in bronchioalveolar lavage fluid. Consistent, dose-dependent effects were seen with styrene. An increased incidence of lung tumors in CD-1 mice was found in long term inhalation studies with styrene (Cruzan et al., 2001). Rats, however, do not show an increased frequency of lung tumors over controls (Cruzan et al., 1998). It has been shown that mice preferentially form the R-enantiomer of SO (Carlson, 1997a), which is more mutagenic in the Salmonella assay than the S-enantiomer is (Sinsheimer et al., 1993). Mouse Clara cells produce approximately 15 times more R-SO than do rat Clara cells (Hynes et al., 1999), with rats preferentially forming S-

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SO, which is less toxic (Foureman et al., 1989; Watabe et al., 1981; Gadberry et al., 1996). The mode of action by which styrene causes lung tumors in mice, but not in rats, is not yet fully understood. One possibility is cytotoxicity associated with oxidative stress.

Glutathione (γ-glutamylcysteinylglycine) (GSH) plays an important role in maintaining the correct balance between oxidants and antioxidants in cells, therefore preventing cellular damage. GSH is a major antioxidant in cells and is also important in the detoxification of toxic or injurious compounds including styrene. In an inhalation study in which the conducting zones of lungs of mice were exposed to 20 ppm styrene for 6 hours, glutathione levels decreased by 40% (Kaufmann et al., 2005). Both styrene and SO have been shown to decrease GSH levels in liver and lung homogenates in a dose dependent manner (Turner et al., 2005). Decreases in GSH levels in lung homogenates after administration of styrene and SO were generally more profound than those found in liver homogenates (Turner et al., 2005). These data are also in agreement with the findings of Garner et al. (2004) that styrene treatment caused depletion of glutathione levels in mouse lung homogenates. GSH conjugation of styrene and SO is one route of detoxification, with approximately 40% of the metabolites recovered being conjugated to GSH (Sumner and Fennel, 1994).

4-Vinylphenol (4VP), a ring metabolite of styrene, has been shown to be more hepatotoxic and pneumotoxic than both styrene and SO at lower doses (Carlson et al., 2002), yet depletion of GSH after administration of 4VP was less than that observed with styrene and SO (Turner et al., 2005).

The main target of styrene toxicity appears to be the Clara cell in the lung (Cruzan et al., 1997; Cruzan et al., 2001; Green et al., 2001; Kaufmann et al., 2005). Repeated inhalation exposure of mice to styrene resulted in Clara cell proliferation and decreased cytoplasm staining

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along with cell crowding and hyperplasia in the terminal bronchioles (Green et al., 2001; Gamer et al., 2004). The Clara cell also has the highest activity in the lung for metabolizing styrene (Hynes et al., 1999). For these reasons, our study focused on the direct cytotoxicity of styrene and its metabolites in Clara cells, as well as the changes in GSH levels due to treatment with these agents.

2. Materials and Methods

2.1 Animals and Chemicals

Adult male CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and adult male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All animals were housed in group cages in environmentally controlled rooms on a 12 h light:12 h dark cycle. Rodent laboratory chow (No. 5001, Purina Mills, Inc., St. Louis, MO) and tap water were allowed ad libitum. All animals were allowed a minimum of one week to adapt to the animal facilities and diet before being used in any experiment.

Styrene, racemic styrene oxide, and (R) and (S)-styrene oxide were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Vinylphenol (10% in propylene glycol: purity>95%) and 5-phenyl-1-pentyne were obtained from Lancaster Synthesis (Windham, NH). N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES), Tris[hydroxymethyl]amino-methane (Trizma Base), mouse IgG, diethyldithiocarbamate (DDTC), 30% IgG free bovine serum albumin (BSA), gentamicin solution, ethylene glycol-bis-[β-aminoethyl ether]-N,N,N',N' tetra acetic acid (EGTA), nitro blue tetrazolium, hematoxylin solution, 5-sulfosalicyclic acid

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dihydrate, 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB), and methylene green were obtained from Sigma Chemical Co. (St. Louis, MO). Lyophilized porcine pancreatic elastase, approximately 3.6 units per mg, was obtained from Worthington Biochemical Corp. (Freehold, NJ). Bovine serum was obtained from Life Technologies (Gibco) (Grand Island, NY). Penicillin/streptomycin solution (5000 U pen./ 5 mg strept./ml), Dulbecco's modified eagle medium, and F-12 nutrient mixture (HAM) were obtained from Invitrogen Corp. (Frederick, MD). Nylon mesh (40 um) was obtained from Small Parts, Inc. (Miami Lakes, FL). Nembutal (sodium pentobarbital) was obtained from Abbott Laboratories (North Chicago, IL). Heparin (1,000 U/ ml) was obtained from Elkins-Sinn Inc. (Cherry Hill, NJ). The Total Glutathione Quantification Kit was obtained from Dojindo Molecular Technologies (Gaithersburg, MD). All other chemicals used were reagent grade or better.

2.2 Experimental Design

Clara cell isolation procedure

The procedure is an adaptation of Malkinson et al. (1993). Buffer A, used for perfusion, storage of lungs prior to digestion, and as part of the lavage solution, contained 133 mM NaCl, 5 mM KCl, 2.7 mM sodium phosphate buffer, 10 mM HEPES, 5.6 mM glucose, and gentamicin (0.10 μg/ml of solution). The lavage solution was made by adding 3 ml 0.1 M EGTA to 146 ml Buffer A. Buffer B, used as part of the digestion solution and for rinsing during the filtering process, contained 129 mM NaCl, 5 mM KCl, 2.6 mM sodium phosphate buffer, 10 mM HEPES, 1.9 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5.6 mM glucose, and gentamicin (0.10 μg/ml solution).

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Kreb's/Ringer/HEPES (KRH) Buffer (pH 7.4), used for rinsing cells off the IgG plate and suspending the cells, contained 145 mM NaCl, 5.4 mM KCl, 1.4 mM $CaCl_2$, 29 mM HEPES, 2.8 mM $MgSO_4$, 1.1 mM K_2HPO_4, 6.35 mM glucose, and 1.15 mM sodium ascorbate.

Four to six mice or one rat were anesthetized with pentobarbital and heparin in saline. The trachea was exposed and cannulated with a stainless steel feeding needle tied in place. The lungs were perfused with gravity-fed buffer A via the right ventricle into the pulmonary artery to remove blood from the lungs. The lungs and heart were removed en bloc. The lungs were lavaged eight times with buffer A/EGTA and then digested at 37°C with elastase solution (4.3 U/ml buffer B, 4 infusions at 5 minutes each for a total of 20 minutes). Following digestion, the lungs were cut away from the trachea and heart, pooled, and minced to approximately $1 mm^3$ pieces. For the *in vitro* cytotoxicity and GSH studies, the mouse lungs were pooled. For the *in vivo* procedure used to measure GSH levels, lungs of the mice were not pooled. The minced lungs were placed in 8 ml of calf serum at 37 °C for 2-5 min for neutralization of the elastase. The mixture was filtered through cotton gauze and then 40 μm nylon mesh. The resulting solution was layered on top of 8 ml of calf serum in a 50 ml conical centrifuge tube and centrifuged at 90 x g for 20 min. Macrophages were removed by panning, using a plastic Petri dish coated with IgG. Following incubation at 37°C for one hour, the Petri dish was rinsed thoroughly with KRH buffer to transfer cells to a 15 ml conical centrifuge tube, which was then centrifuged for 20 min at 90 x g. The supernatant was discarded, and the cell pellet was resuspended in KRH buffer for use in the styrene/styrene metabolite toxicity studies.

Cell counting and cell viability were determined with trypan blue and a hemocytometer. All nucleated cells were counted. After counting the cells, approximately 500,000-750,000 cells, suspended in KRH, were placed in glass vials containing Hams F12, fetal bovine serum, and

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either DMSO or styrene/styrene metabolites, and then incubated for 3 hours at 37°C. Cell counting and cell viability were determined again after the 3 hour incubation using trypan blue and a hemocytometer.

For enrichment determinations, nitro blue tetrazolium (NBT) staining (Devereux and Fouts, 1980) was performed for the identification of Clara cells. For identification of type II cell contamination, the modified Pap staining procedure was used. Because these cell preparations were found to be highly enriched (approximately 90% for mice), they are described as Clara cells in this paper, and corrections have not been made for purity.

Protection Experiments

5-Phenyl-1-pentyne (5P1P) and diethyldithiocarbamate (DDTC) were added at varying concentrations *in vitro* to Clara cells, isolated as described above, prior to adding 5 mM styrene. 5P1P or DDTC was added to a mixture of approximately 500,000 – 750,000 cells and KRH, Hams F12, and fetal bovine serum ten minutes prior to adding either DMSO or styrene. DMSO was added to the controls since it was used as the solvent for the inhibitors. Cells were then incubated for 3 hours at 37°C, and cell counting and cell viability were determined after the three hours using trypan blue and a hemocytometer.

GSH Assay Procedure

In vitro: Clara cells were isolated as described above and incubated for 3 hours at 37°C in a mixture of approximately 500,000-750,000 cells and KRH, Hams F12, fetal bovine serum, and 0.1 mM, 0.5 mM, 1.0 mM styrene/styrene metabolite or DMSO for the controls. DMSO was

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added to the controls since it was used as the solvent for the styrene and metabolites. Cells were then counted and cell viability determined using trypan blue and a hemocytometer. Glutathione content of the cells was measured using the Dojindo Total Glutathione Quantification Kit following the directions for the high sensitivity method. A 5% 5-sulfosalicyclic acid (SSA) solution was prepared. Cells were collected and centrifuged at 200 x g for 10 min at 4°C. The supernatant was discarded, and the cells were washed with phosphate buffered solution (PBS) and then centrifuged at 200 x g for 10 min at 4°C. The supernatant was discarded, and 10 mM HCl was added to the cells. The cells were lysed by freezing and thawing (x 2). Then 5% SSA was added to the lysed cells, and the sample was centrifuged at 8000 x g for 10 min. The supernatant was used for the total glutathione assay. Enzyme working solution, coenzyme working solution, and the samples were added to each well. The 96 well plate was incubated at 30°C for 10 min. Substrate working solution was added, and the plate was incubated again for 20-40 min at 37°C. Absorbance was read at 405 nm using a microplate reader, and glutathione concentrations were determined for sample solutions using a calibration curve.

In vivo: Mice were injected ip with 600 mg/kg styrene, 300 mg/kg racemic styrene oxide, R-SO, or S-SO, or 100 mg/kg 4-vinylphenol either 3 h or 12 h prior to the isolation of Clara cells as described above. Control mice were injected with corn oil since the styrene and its metabolites were dissolved in it. These times and doses were based on our previous studies (Turner et al., 2005). Measurements of glutathione levels were done using the same method as described above in the *in vitro* section.

2.3 Statistical Analysis

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Three to 6 animals per group were used for the *in vivo* studies. For the *in vitro* studies there were not always enough cells to test all the chemicals (styrene, racemic styrene oxide, R-styrene oxide, S-styrene oxide and 4-vinylphenol) at the same time. However, a control was always run with each replicate of the experiment. Therefore, there were a high number of controls and a high variability in the number of replicates for each chemical. Values are means ± standard errors. Data were compared using an analysis of variance (ANOVA) followed by the Student-Newman-Keuls' test for significance using the Instat program. The selected level of significance was $p < 0.05$. In some studies, log transformations were performed on the data prior to comparisons being made. The standard error in some of the groups was large due to a high degree of variability in some of the studies, and log transformations were used to normalize the data. These studies are identified in the legends.

3. Results

3.1 Cytotoxicity

In vitro experiments using freshly isolated Clara cells from CD-1 mice showed styrene to be the most toxic and S-SO and 4VP to be the least toxic of the compounds tested (Figure 1). LC50 values ranged from 1.72 to 4.84 mM (Table 1). Inhibitors of styrene metabolism were used to examine the importance of CYP2F2 and CYP2E1 in the bioactivation of styrene in mouse Clara cells. 5P1P, mainly a CYP2F2 inhibitor, was added to the Clara cell preparations before incubation with styrene, and the result was a significant protection of the Clara cells from the cytotoxicity of styrene (Figure 2). This was concentration dependent since without the

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addition of 5P1P, styrene toxicity at a concentration of 5 mM averaged 16% cell survival after a 3 hour incubation period. With the addition of 150 μM 5P1P, cell survival increased to almost 45%. Higher concentrations of 5P1P could not be used due to its own cytotoxicity.

DDTC, a CYP2E1 inhibitor, was also added to mouse Clara cell preparations prior to incubation with styrene. However, even at concentrations up to 500 uM DDTC, no protection against styrene-induced cytotoxicity was observed (Figure 3). Cell survival was determined with results ranging from 3% to 6%. With 200 μM DDTC alone, cell survival rates were near 91%, with no significant decreases seen as the DDTC concentration was increased.

Significant Clara cell cytotoxicity in the CD-1 mice was seen at 1 to 10 mM styrene and its metabolites. However, with the Sprague-Dawley rats, concentrations approximately four times greater were needed to produce the same level of toxicity (Figure 4). Concentrations of 40 mM were needed to produce the same level of toxicity in rats as observed with 10 mM concentrations in mice. As with the mice, however, styrene was the most toxic of the compounds tested in rats, and 4VP and S-SO were the least toxic.

NBT and Pap stain results in rats yielded an average of 50.5% positive results for the NBT stain for Clara cells and an average of 47.5% positive results for the Pap stain for type II alveolar cells. In mice, NBT staining demonstrated an average of 87.3% Clara cells, and the Pap staining showed an average of 7.6% alveolar type II cells.

3.2 Glutathione Measurement in vitro

Glutathione levels in mouse Clara cells were significantly decreased after a three hour incubation with 1.0 mM styrene and all its metabolites and at 0.5mM for racemic SO and 4VP

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(Table 2). No significant decrease in glutathione was seen at 0.1 mM for any of the compounds. There was a very large variation in glutathione levels with 0.1 mM styrene, leading to a higher SEM therefore making 0.1 mM styrene statistically insignificant. Cell survival rates for cytotoxicity at 1.0 mM ranged from 68% to 85%, and cells exposed to 0.5 mM and 0.1 mM of these compounds had, on average, higher survivability rates.

3.3 Glutathione Measurements in vivo

Glutathione levels of mouse Clara cells were measured 3 hours and 12 hours after treatment of mice with styrene or one if its metabolites (Table 3). These time periods were selected based on *in vivo* studies demonstrating that styrene and styrene oxide caused significant decreases in GSH levels in mouse lung homogenates at 1-3 hours, with repletion by 12 hours (Turner et al., 2005). At 3 hours, significant decreases in Clara cell glutathione levels compared to the controls were seen after administration of 300 mg/kg R-SO and racemic SO. A slight decrease was also seen with treatment of 600 mg/kg styrene, but it was not considered statistically significant. No significant decrease was observed following treatment with 300 mg/kg S-SO or 100 mg/kg 4VP.

When Clara cell glutathione measurements were made 12 hours after treatment, a rebound effect was seen with glutathione in mice treated with racemic SO rebounding back to the control levels, and statistically significant increases from controls were observed with S-SO, R-SO, styrene, and 4VP.

4. Discussion

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The Clara cell is the main site of toxicity and bioactivation for styrene to styrene oxide, with type II alveolar cells contributing much less to the metabolism of styrene to SO (Hynes et al., 1999; Carlson, 2000). Mouse Clara cells have an abundant amount of CYP2F2, which is the main cytochrome P450 that metabolizes styrene (Carlson 1997b; Hynes et al., 1999; Cruzan et al., 2002). The current studies indicate that the increase in the concentration of styrene and its metabolites needed to produce the same level of toxicity in the rat Clara cells as seen in the mouse supports previous findings that rats don't have the same degree of vulnerability to styrene and its metabolites as mice do. This is likely related to the fact that styrene metabolizing ability is much higher in mouse Clara cells compared to rat Clara cells (Hynes et al., 1999). Rats, like humans, also have much lower levels of the corresponding CYP2F found in mice, CYP2F4 and CYP2F1, respectively (Wheeler et al., 1992; Buckpitt et al., 1995; Green et al., 1997). In addition, rats and humans are able to detoxify styrene and its metabolites better than mice do, using epoxide hydrolase and glutathione S-transferases (Sumner and Fennel, 1994; Green et al., 2001). Another reason for the observed differences in cytotoxicity between the mouse and rat may in part reflect the purity of Clara cell preparations isolated from each. In mice, Clara cell purity averaged 85-90%, whereas for rats it averaged 45-55%.

The cytotoxicity of styrene *in vitro* in mouse and rat Clara cells is equal to or greater than its metabolites, whereas *in vivo* styrene is less potent than styrene oxide or 4-vinylphenol (Gadberry et al., 1995; Carlson et al., 2002). This may be due to the styrene being bioactivated *in vitro* by the cytochromes P450 in the Clara cell, with the resulting product(s) reacting immediately within the cell to cause it to lose its viability.

Determination of the cytochromes P450 involved in the bioactivation of styrene to SO has been made using inhibitor studies. Although CYP2E1 is an important contribution to styrene

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metabolism in liver, in mouse lung CYP2F2 is the primary isoform involved (Carlson 1997b; Hynes et al., 1999). When Clara cells were incubated with 5PIP, an inhibitor of CYP2F2 and to a lesser extent CYP2E1, styrene at a concentration that would normally kill a high percentage of cells, protection was observed. However, after incubation with DDTC, a CYP2E1 inhibitor, no protection was observed, even at high concentrations, supporting the concept that CYP2F2, not CYP2E1, is the major isoform involved in styrene metabolism in Clara cells. Cruzan et al. (2002) also concluded that the pulmonary toxicity of styrene is attributable to metabolites generated by CYP2F isoforms.

In vitro studies showed a dose dependent decrease of glutathione levels in the mouse Clara cells compared to controls. There was little difference among the chemicals tested. Decreases in glutathione levels were seen at concentrations where significant cytotoxicity was not seen.

Three hours after treatment of mice, there was 72% decrease in glutathione in Clara cells from mice treated with racemic SO and a 54% decrease in those treated with R-SO (Table 3). The decrease in glutathione following treatment with racemic SO was significantly different from all other compounds except R-SO. The lowest decrease seen was with S-SO, which also had a low level of toxicity. This significant depletion of GSH levels shows the greater relative toxicity of R-SO and racemic SO in Clara cells. This is in agreement with cytotoxicity data that show that R-SO and racemic SO are more toxic than S-SO. R-SO also causes a greater decrease in glutathione levels in mouse lung homogenates than does S-SO (unpublished data). The R enantiomer of SO is more mutagenic in the Salmonella Assay (Pagano et al., 1982; Sinsheimer et al., 1993) and more acutely pneumotoxic than is the S enantiomer (Gadberry et al., 1996). These past and current data also show that the R enantiomer of SO is more likely to be what is causing

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the toxicity and decreased glutathione levels seen with racemic SO. Styrene is not the only compound where a link was found between decreases in glutathione and Clara cell toxicity. West et al. (2000) noted that decreases in glutathione levels in Clara cells correlated with injury due to naphthalene.

4VP is a much more potent hepatotoxicant and pneumotoxicant than either styrene or SO (Carlson et al., 2002; Vogie et al., 2004). However, studies using pulmonary microsomes showed glutathione depletion following 4VP administration to be less than that observed with styrene or SO (Turner et al., 2005). Similarly, our current *in vivo* Clara cell data showed no significant decrease in glutathione levels in Clara cells after 3 hours. Glutathione is the primary defense against oxidative stress, an imbalance between oxidants and antioxidants, and depletion of glutathione levels is likely to lead to oxidative stress. The dramatic rebound of glutathione levels seen after 12 hours may be attributed to an increase in the rates of glutathione synthesis. Controls remained fairly constant between 3 and 12 hours, so it seems that the increase in glutathione levels is directly related to the compound being administered versus any type of diurnal glutathione cycle. With S-SO, levels didn't change very much at 3 hours, but nevertheless, they increased dramatically after 12 hours.

In conclusion, the Clara cells of the mouse and rat are the main site of toxicity for styrene and its metabolites. Clara cells in the rat, however, are 4-fold less susceptible to the cytotoxicity produced by styrene and its metabolites than are Clara cells in the mouse. The cytotoxicity of styrene *in vitro* is greater than that of its metabolites which is the opposite of what has been observed *in vivo*. This could possibly be due to styrene being bioactivated by the Clara cells, resulting in cell death. Decreases in glutathione levels were seen even when cytotoxicity was not, which may be a cause of oxidative stress in the cells. The finding that R-SO caused a

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greater decrease in GSH levels than did S-SO supports other data indicating this is the more toxic of the two enantiomers.

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Figure Legends

Fig. 1. *In vitro* toxicity after 3 hr incubation. Clara cells from mice were incubated for 3 hrs with varying concentrations of styrene or its metabolites. (Racemic styrene oxide (Rac-SO); R-styrene oxide (R-SO); S-styrene oxide (S-SO); 4-vinylphenol (4VP)). Cytotoxicity was determined by trypan blue exclusion. Bar equals mean ± S.E. with an average of 8-9 samples per group.

Fig. 2. Clara cells isolated from mouse lung were incubated for 3 hrs with and without increasing concentrations of 5P1P (5-phenyl-1-pentyne) prior to the addition of 5 mM styrene. Cytotoxicity was determined by trypan blue exclusion. Bar equals mean ± S.E. with an average of 4-5 samples per group.

Fig. 3. Clara cells isolated from mouse lung were incubated for 3 hrs with and without increasing concentrations of DDTC (diethyldithiocarbamate) prior to the addition of 5 mM styrene. Cytotoxicity was determined by trypan blue exclusion. Bar equals mean ± S.E. with an average of 3-4 samples per group.

Fig. 4. *In vitro* Clara cell toxicity after 3 hr incubation. Clara cells from rats were incubated for 3 hrs with varying concentrations of styrene or its metabolites. (Racemic styrene oxide (Rac-SO); R-styrene oxide (R-SO); S-styrene oxide (S-SO); 4-vinylphenol (4VP)). Cytotoxicity was determined by trypan blue exclusion. Bar equals mean ± S.E. with an average of 5-6 samples per group.

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Table 1. Cytotoxicity of styrene and its metabolites in mouse Clara cells

Chemical	LC50 (mM)
Styrene	1.721
Racemic styrene oxide	2.344
R-Styrene oxide	3.243
S-Styrene oxide	4.842
4-Vinylphenol	3.500

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Table 2. Glutathione levels shown as % of control after 3 hr mouse Clara cell incubation.

Incubation with Clara Cells for 3 hrs	n	GSH (% of control)[a]
1.0 mM Styrene	5	78 ± 4[b]
0.5 mM Styrene	5	80 ± 5
0.1 mM Styrene	5	70 ± 8
1.0 mM Rac SO	6	61 ± 6[b]
0.5 mM Rac SO	8	76 ± 8[b]
0.1 mM Rac SO	5	86 ± 6
1.0 mM R-SO	11	65 ± 4[b]
0.5 mM R-SO	10	80 ± 8
0.1 mM R-SO	5	96 ± 8
1.0 mM S-SO	7	73 ± 4[b]
0.5 mM S-SO	4	81 ± 3
0.1 mM S-SO	4	87 ± 6
1.0 mM 4VP	6	64 ± 6[b]
0.5 mM 4VP	5	53 ± 3[b]
0.1 mM 4VP	5	73 ± 13

[a] Values represent mean ± S.E. Control represents cells with the vehicle dimethylsulfoxide (DMSO).

[b] Significantly different compared to controls, $P < 0.05$

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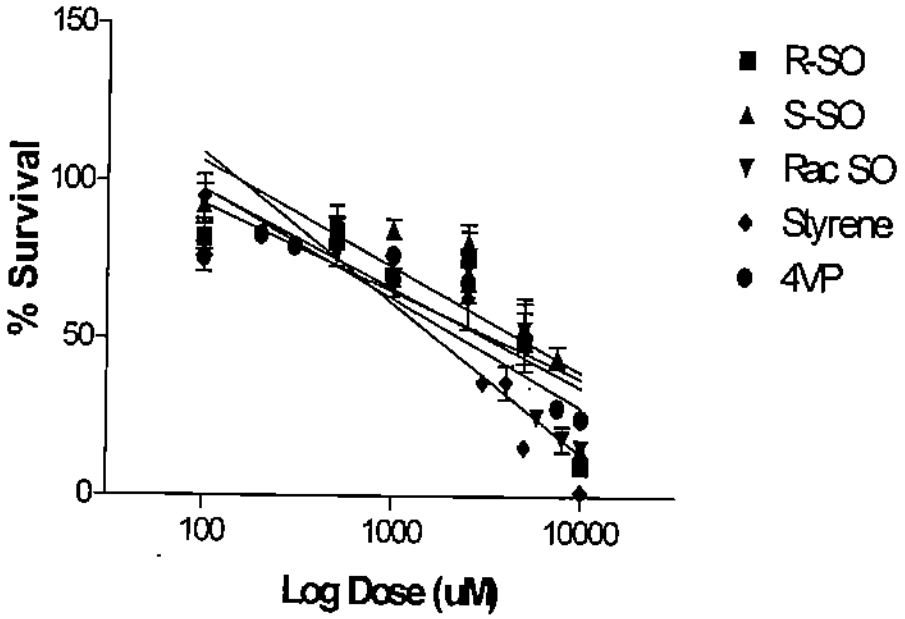
Table 3. Mouse Clara cell glutathione measurements 3 hrs and 12 hrs after administration of styrene or its metabolite.

Treatment	n	3 hr GSH Conc.(pmols)/200,000 cells[a]	N	12 hr GSH Conc.(pmols)/200,000 cells[a]
Corn oil Control	14	226 ± 30[b]	16	211 ± 26[b]
600mg/kg Styrene	4	163 ± 29[b,c]	6	569 ± 60[c,d]
300mg/kg Rac-SO	5	64 ± 14[d]	6	283 ± 67[b,d]
300mg/kg R-SO	6	104 ± 20[c,d]	5	379 ± 5[c,d]
300mg/kg S-SO	8	234 ± 54[b]	7	726 ± 107[c]
100mg/kg 4VP	6	320 ± 74[b]	6	434 ± 65[c,d]

[a] Values represent mean ± S.E.

[b,c,d] For each column, values with different superscripts are significantly different from one another, $P < 0.05$. Newman-Keuls test for statistical significance was used to compare all means. The logs of the GSH data were used for statistical comparisons.


150
100
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% Survival
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R-SO
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Rac SO
Styrene
4VP

Fig. 1.

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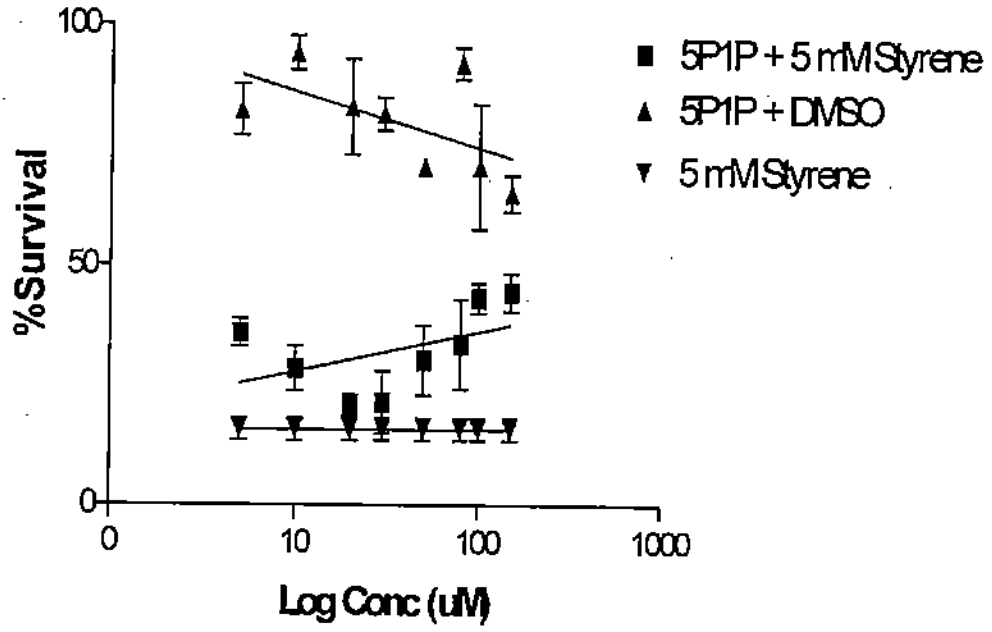
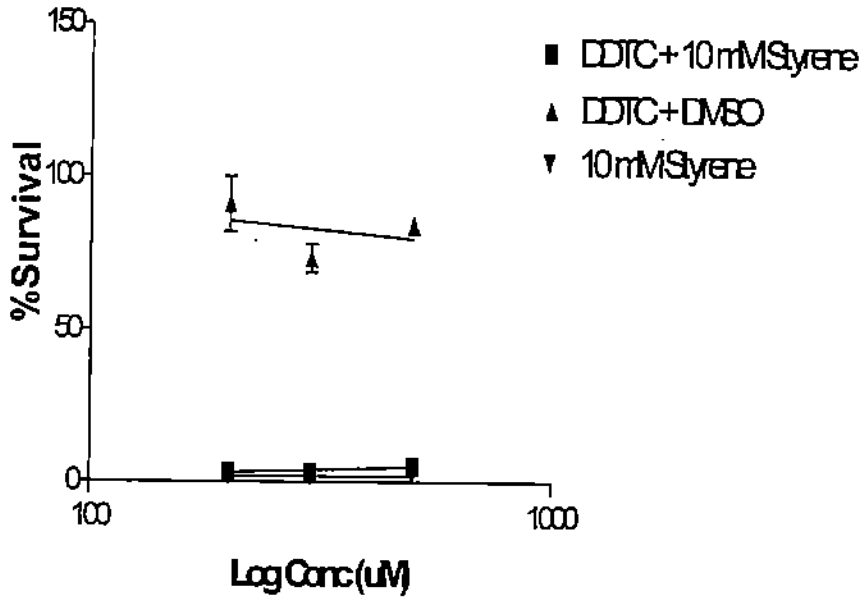


Fig. 2.


150
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%Survival
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DDTC+10mMStyrene
DDTC+DMSO
10mMStyrene

Fig. 3

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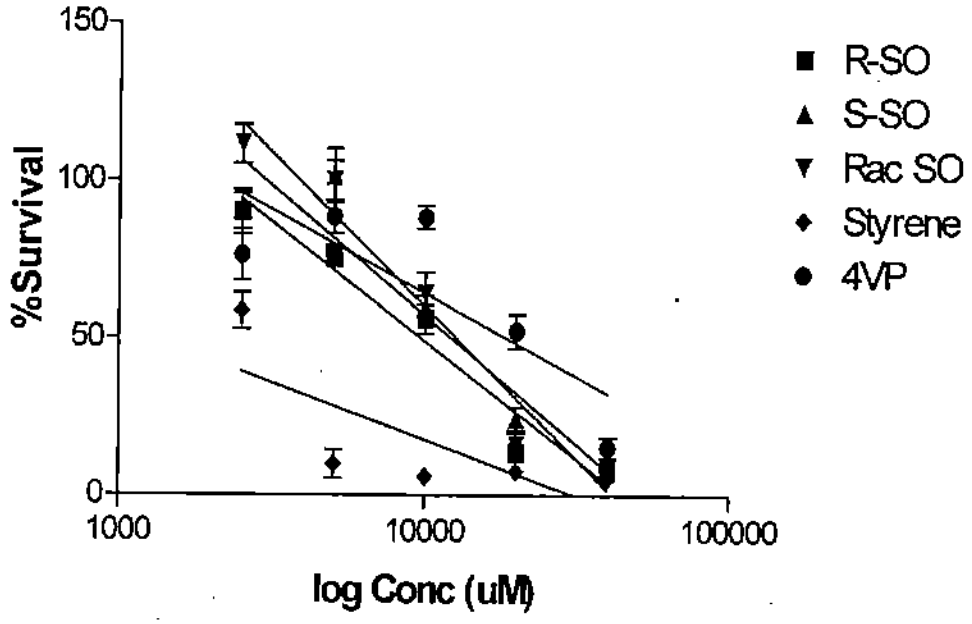

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Comparison of the depletion of glutathione in mouse liver and lung following administration of styrene and its metabolites styrene oxide and 4-vinylphenol

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Abstract

Styrene is hepatotoxic and pneumotoxic in mice. Its major metabolite styrene oxide and its minor, but potent, metabolite 4-vinylphenol cause similar toxicities. Styrene and styrene oxide cause decreases in reduced glutathione levels in tissues. The current studies examined styrene and styrene oxide in a time and dose-dependent manner and 4-vinylphenol in a time dependent fashion. Styrene (600 mg/kg, 5.8 mmol/kg ip) caused decreased GSH levels in both liver and lung within one hour. A maximum was seen at three hours with return to control levels by 12 h. Lower doses also caused changes in a dose-dependent fashion. For styrene oxide, similar findings were observed with a dose of 300 mg/kg (2.5 mmol/kg). GSH levels in liver, but not lung, returned to control by 6 h. Again a dose response was found for both tissues. While 4-vinylphenol (100 mg/kg, 0.83 mmol/kg) was administered at a dose known to be more hepatotoxic and more pneumotoxic than styrene or styrene oxide and it caused decreased GSH levels, the degree of depletion was less compared to styrene and styrene oxide. In general the lung was more affected by these agents than was liver. The decreases in GSH suggest the possibility that the toxicity of styrene in lung and liver may be related to a profound but reversible oxidative stress in these tissues.
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Keywords: Styrene; Styrene oxide; 4-Vinylphenol; Glutathione

1. Introduction

Styrene is widely utilized for a number of purposes with significant human exposure possible, especially for workers in the reinforced plastics industry (Miller et al., 1994; Cohen et al., 2002). In rodents, styrene causes both liver and lung damage with the mouse being a more sensitive species than the rat (Morgan et al., 1993a, 1993b; Gadberry et al., 1996; Cruzan et al., 1997). The greatest concern, however, is whether styrene causes cancer in humans. IARC (2002) classi-

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fies it as a possible human carcinogen. It causes lung tumors in mice (Cruzan et al., 2001) but not in rats (Cruzan et al., 1998).

Styrene oxide formed from styrene may be detoxified via microsomal epoxide hydrolase to give styrene glycol, which is further metabolized to mandelic acid and phenylglyoxylic acid (Bond, 1989; Sumner and Fennell, 1994). Particularly in rats and mice, glutathione conjugation of styrene oxide also occurs. Following inhalation exposure approximately 60% of the metabolites recovered from rodents are formed via the epoxide and hydrolysis pathway, and about 40% are via glutathione conjugates.

It is not entirely clear how styrene causes toxicity in the liver and lung. One possibility is through oxidative stress. Styrene administration has been shown to decrease glutathione levels in liver. Vainio and Makinen (1977) administered ip doses of styrene ranging from 150 to 1000 mg/kg to hamsters, guinea pigs, rats and mice, and found dose-dependent decreases in nonprotein sulfhydryl content (primarily GSH) 3 h later. The mouse appeared to be the most sensitive species. Srivastava et al. (1983) administered single ip doses of styrene ranging from 50 to 600 mg/kg to rats and found a dose-dependent decrease in GSH three hours later. Beiswanger et al. (1993) injected rats ip with styrene oxide (250 mg/kg) and found a decrease in hepatic GSH after 2 h, which rebounded to control level by 8 h. They found a similar pattern in the cerebellum, cerebral cortex, and hippocampus.

A number of studies suggest that recovery of hepatic glutathione occurs fairly rapidly. Das et al. (1981) administered styrene orally to rats at doses of 270, 450 and 900 mg/kg daily for 7 days and found a decrease in GSH only at the highest dose when measurements were made 24 h later. Katoh et al. (1989) administered styrene (300 mg/kg) or styrene oxide (300 mg/kg) ip to rats 3 times per week for 1 week. Both chemicals caused significant decreases in hepatic GSH and GSSG at 2 h after administration with recovery between 10 and 20 h.

Few studies have examined the effect of styrene or its metabolites on glutathione content in lung. Elovaara et al. (1990) exposed rats in a single 24 h inhalation exposure to 500 cm^3/m^3 styrene and found a 66% decrease of GSH in lung and only a 16% decrease in liver one hour after exposure. Tissue glutathione levels returned to normal by 24 h. Coccini et al. (1997) exposed rats to 300 ppm styrene 6 h/day, 5 days/week for 2 weeks and found significant decreases in non-protein sulfhydryl content immediately after the last exposure. They also found that single ip injections of styrene (400 mg/kg) or styrene oxide (200 mg/kg) resulted in significant decreases after 2 h. Interestingly, they found no changes when rats were given up to 400 mg/kg/day for 3 days and measurements were made 24 h after the last dose. They also found decreases in pulmonary (−40%) and hepatic (−35%) nonprotein sulfhydryl content immediately following inhalation of 300 ppm styrene, 5 days per week, for 2 weeks (Coccini et al., 1998). Other studies have demonstrated that mice are more sensitive than rats to pulmonary GSH depletion with mice showing a significant decline when exposed by inhalation to 80 ppm styrene 6 h/day for two days with rats requiring 300 ppm to obtain a significant decrease (Filser et al., 2002; Csanady et al., 2003).

The toxicity of styrene is generally associated with its biotransformation to styrene oxide (Bond, 1989). However, another metabolite of interest is the ring hydroxylated metabolite 4-vinylphenol, which has been found in the urine of both rats (Bakke and Scheline, 1970; Pantarotto et al., 1978) and humans (Pfaffli et al., 1981; Manini et al., 2003). Although only a small percentage of styrene forms this metabolite, it is much more potent as both a hepatotoxicant and pneumotoxicant than is styrene or styrene oxide (Carlson et al., 2002; Vogie et al., 2004). There is no published information available on the metabolism of 4-vinylphenol although it has been hypothesized that it may involve a ring-opened pathway (Boogaard et al., 2000). The purpose of the current study was to determine the dose dependence and time course for the depletion of reduced glutathione by styrene in mouse liver and lung and compare it to the effects of the two metabolites styrene oxide and 4-vinylphenol.

2. Materials and methods

2.1. Animals and chemicals

4-VP (10% in propylene glycol; purity >95%) was obtained from Lancaster Synthesis (Windham, NH). Styrene and styrene oxide were from Aldrich Chemical Co. (Milwaukee, WI). Trichloroacetic acid (TCA) was from Fisher (Fair Lawn, NJ). Glutathione (>98%) and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) were

from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or better.

CD-1 [Crl:CD-1 (ICR) BR] mice were obtained from Charles River Laboratories (Wilmington, MA). They were housed in group cages in environmentally controlled rooms on a 12 h light:12 h dark cycle. Rodent laboratory chow (No. 5001, Purina Mills, Inc., St. Louis, MO) and tap water were allowed ad libitum. All animals were allowed a minimum of one week to adapt to the animal facilities and diet before being used in any experiment.

2.2. Experimental design

Groups of 4 to 6 male mice (22–28 grams) were administered styrene (600 mg/kg, 5.8 mmol/kg) in corn oil or corn oil as control vehicle ip. To determine the time course for GSH depletion, they were sacrificed at 1, 3, 6, 12 or 24 h. For examining the effect of dose, mice were treated with 0, 200, 400 or 600 mg/kg (0, 1.9, 3.8 or 5.8 mmol/kg) ip styrene and sacrificed after 3 h. Similar experiments were carried out with styrene oxide. It was administered ip at a dose of 300 mg/kg (2.5 mmol/kg), and measurements were made at 1, 2, 6 and 12 h after dosing. For the dose response study, the doses selected were 0, 75, 150 and 300 mg/kg (0.63, 1.25, and 2.5 mmol/kg), and the time was 2 h. For 4-vinylphenol, which is water soluble, the vehicle was saline. The mice were administered 100 mg/kg ip and were sacrificed after 1, 2 or 6 h. Control animals received saline. In all cases, the dosing solutions were prepared immediately before administration and were given in a volume of 1 ml/100 g body weight).

2.3. Assay for reduced glutathione

Entire lungs and livers (approximately 0.20 to 0.28 grams and 1.2 to 1.9 grams, respectively) were removed and weighed, and deproteinized homogenates were prepared by homogenizing the tissues in 2 ml and 3 ml, respectively of cold 0.1 M potassium phosphate buffer, pH 7.5, followed by the addition of one-half volume of 10% TCA. Homogenates were centrifuged at 10,000 × g in a refrigerated centrifuge for 5 min to prepare the supernatants for assay. The assay was that of Speisky et al. (1986, 1988). Briefly, the deproteinized supernatants were combined with a solution of DTNB in 0.1 M potassium phosphate buffer giving a final concentration of 10 mM DTNB. Samples were mixed by vortexing, and the absorbance was determined at 464 nm. A standard curve was generated ranging from 0 to 625 nmol GSH and was used to calculate the tissue GSH levels.

2.4. Statistical analysis

Values are expressed as mean ± S.E. The numbers of animals in each group are indicated in the tables. In comparing between two groups, Student's t test was used. In comparing multiple values, an ANOVA was utilized followed by Student Newman–Keuls' test to detect differences among the groups. In each case the level of significance selected was $P < 0.05$.

3. Results

When styrene was administered to mice at the relatively high dose of 600 mg/kg ip, there was a very rapid and dramatic decrease in reduced glutathione levels in both liver and lung by one hour (Table 1). The greatest effect was observed after three hours, and there was some recovery evident by 6 h. By 12 h, there was no difference between the control and treated groups for either liver or lung.

To determine the dose response relationship between styrene and reduced glutathione levels, groups of mice were administered 200, 400 or 600 mg/kg styrene ip, and measurements were made after 3 h, the time of maximum effect in the 600 mg/kg study. As previously shown, the highest dose of styrene caused significant decreases in GSH in both liver and lung (Table 2). The two lower doses also caused decreases in GSH levels in both tissues in a dose-dependent manner.

Since styrene oxide is considered to be the metabolite responsible for its hepatotoxicity, time and dose response relationships were evaluated for this metabolite. As with the parent compound, styrene oxide (300 mg/kg ip) also caused a rapid and severe decrease in GSH levels with significant effects noted by 1 h and a maximum effect at 2 h (Table 3). For liver, there was a recovery of the GSH concentration in the treated group to control levels by 6 h, but significant reduction was still present in the lung at that time. Lung GSH levels returned to normal by 12 h.

Table 1
Glutathione depletion in CD-1 mouse liver and lung following styrene[a] administration

Hours	Lung				Liver			
	No.	Control	No.	Styrene	No.	Control	No.	Styrene
Glutathione concentration[b]								
1	4	9.64 ± 0.41	5	2.84 ± 0.37[c]	4	3.07 ± 0.06	5	2.34 ± 0.11[c]
3	5	8.13 ± 0.29	5	1.80 ± 0.18[c]	5	1.69 ± 0.07	5	0.66 ± 0.03[c]
6	5	6.08 ± 0.45	6	2.30 ± 0.22[c]	5	2.73 ± 0.15	6	1.81 ± 0.08[c]
12	5	6.81 ± 0.42	5	7.06 ± 0.54	5	4.71 ± 0.24	5	4.90 ± 0.24
24	5	8.81 ± 0.40	6	7.98 ± 0.60	5	2.48 ± 0.11	6	3.05 ± 0.13[c]

[a] 600 mg/kg, ip.
[b] μmol/g liver or lung; value is mean ± S.E.
[c] Significantly different from control, $P < 0.05$.

Table 2
Dose response for glutathione depletion by styrene[a] in the CD-1 mouse

Dose	Number	Liver	Lung
Glutathione concentration[b]			
Control	5	7.05 ± 0.38 a	2.01 ± 0.07 a
200 mg/kg	5	4.85 ± 0.28 b	1.23 ± 0.10 b
400 mg/kg	5	2.30 ± 0.33 c	0.48 ± 0.05 c
600 mg/kg	5	1.51 ± 0.34 c	0.51 ± 0.05 c

Within each column, values with different letters (a–c) are significantly different from each other, $P < 0.05$.
[a] Administered ip to groups of 5 mice 3 h prior to termination.
[b] μmol/g liver or lung; value is mean ± S.E.

In the dose response studies, styrene oxide at 300 mg/kg ip produced the expected response (Table 4). When the dose was decreased to 150 mg/kg there were still significant differences between treated and control groups in both liver and lung. However, when the lowest dose of 75 mg/kg was administered there was only a decrease in lung GSH with liver GSH being no different from control.

Table 4
Dose response for glutathione depletion by styrene oxide[a] in the CD-1 mouse

Dose	Number	Liver	Lung
Glutathione concentration[b]			
Control	4	5.70 ± 0.39 a	0.92 + 0.13 a
75 mg/kg	4	5.02 ± 0.85 a	0.68 ± 0.09 a
150 mg/kg	4	4.20 ± 0.43 a	0.32 ± 0.11 b
300 mg/kg	4	1.07 ± 0.13 b	0 c

Within each column, values with different letters (a–c) are significantly different from each other, $P < 0.05$.
[a] Administered ip to groups of 4 mice 2 h prior to termination.
[b] μmol/g liver or lung; value is mean ± S.E.

Since the styrene metabolite 4-vinylphenol is much more potent in producing liver and lung toxicity than is either styrene or styrene oxide (Carlson et al., 2002; Vogie et al., 2004), it was administered to mice at a dose of 100 mg/kg ip, which is known to be both more hepatotoxic and pneumotoxic than styrene or styrene oxide. Although there were significant decreases in hepatic GSH levels at 1 and 2 h, these were very minimal com-

Table 3
Glutathione depletion in CD-1 mouse liver and lung following styrene oxide[a] administration

Hours	Liver				Lung			
	No.	Control	No.	Styrene oxide	No.	Control	No.	Styrene oxide
Glutathione concentration[b]								
1	4	8.64 ± 0.67	4	1.21 ± 0.16[c]	4	1.56 ± 0.22	4	0.65 ± 0.32[c]
2	4	7.77 ± 0.55	4	1.76 ± 0.12[c]	4	1.40 ± 1.00	4	0
6	8	6.35 ± 0.90	8	4.85 ± 0.53	4	1.41 ± 0.07	4	0.51 ± 0.10[c]
12	4	6.02 ± 1.16	3	5.48 ± 0.31	4	2.67 ± 0.14	3	2.35 ± 0.11

[a] 300 mg/kg, ip.
[b] μmol/g liver or lung; value is mean ± S.E.
[c] Significantly different from control, $P < 0.05$.

Table 5
Glutathione depletion in CD-1 mouse liver and lung following 4-vinylphenol[a] administration

Hours	Liver				Lung			
	No.	Control	No.	4-VP	No.	Control	No.	4-VP
Glutathione concentration[b]								
1	4	9.48 ± 0.88	4	6.26 ± 0.28[c]	4	0.92 ± 0.09	4	0.61 ± 0.11
2	4	9.97 ± 0.25	4	8.81 ± 0.19[c]	4	2.37 ± 0.19	4	1.86 ± 0.03[c]
6	4	4.33 ± 0.34	4	5.62 ± 0.28[c]	4	0.95 ± 0.38	4	1.33 ± 0.41

[a] 100 mg/kg, ip.
[b] μmol/g liver or lung; value is mean ± S.E.
[c] Significantly different from control, $P < 0.05$.

pared to styrene and styrene oxide (Table 5). Similarly GSH depletion in lung was observed, but again this was less than that observed with styrene and styrene oxide. Higher doses could not be used because they are lethal.

4. Discussion

Styrene has been shown in a number of studies to cause a decrease in reduced glutathione levels, possibly resulting in oxidative induced stress in both liver and lung (Vainio and Makinen, 1977; Srivastava et al., 1983; Beiswanger et al., 1993). This may be related to a major portion of their metabolism through conjugation with glutathione. Interestingly, the effect appears to occur very rapidly but with a return of GSH to control levels between 12 and 24 h (Katoh et al., 1989; Beiswanger et al., 1993). This could explain why (Das et al., 1981) found that 900 mg/kg caused GSH depletion in liver at 24 h but not with lower doses.

Of particular interest is the lung as a potential target tissue, especially since it is the site of tumors in mice (Cruzan et al., 2001). Limited studies have also indicated that glutathione levels are also decreased in this organ in rats (Elovaara et al., 1990; Coccini et al., 1997) and mice (Filser et al., 2002; Csanady et al., 2003).

The current studies support the results of the previous studies in that styrene administration caused a dose-dependent decrease in GSH in both tissues. The effect was observed as early as one hour after administration with a peak effect observed at 3 h. This effect was short lived with a return to control levels by 12 h. Styrene oxide also gave a dose-dependent response in both tissues with a return to control by 12 h. As might be expected, effects due to styrene oxide were noted at lower doses than with the parent compound. In both cases the degree of GSH depletion and the length of time it was decreased were greater in lung than in liver. This finding is in agreement with the studies of Elovaara et al. (1990) who exposed rats to a single 24 h inhalation exposure of 500 cm^3/m^3 styrene and found a 66% decrease in GSH in lung and only a 16% decrease in liver one hour following exposure.

4-Vinylphenol is a minor metabolite of styrene and yet it is an order of magnitude more potent than styrene or styrene oxide in producing pneumotoxic and hepatotoxic effects in mice (Carlson et al., 2002; Vogie et al., 2004). However, while 4-vinylphenol did cause significant decreases in GSH in both liver and lung, these changes were small in comparison to styrene and styrene oxide. The dose could not be increased due to lethality at higher doses. The metabolic pathway for 4-vinylphenol is unknown although it has been suggested that it may be via ring opening (Boogaard et al., 2000). As such it may not involve conjugation with GSH leading to depletion and thus may act via another mechanism.

The role of the decrease in GSH in causing the toxicity to both the liver and lung is as yet unclear. However, the data suggest that a very profound but transient effect occurs. Now that the current studies have identified time and dose dependence, further studies are needed to characterize this oxidative stress in both tissues including an evaluation of the enzymes, which control GSH levels in these tissues.

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4-Vinylphenol-Induced Pneumotoxicity and Hepatotoxicity in Mice

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ABSTRACT

4-Vinylphenol (4-hydroxystyrene, 4-ethenylphenol, 4-VP) occurs naturally in some foods and has been used as a flavoring agent in food products. It is used synthetically in the production of polymers and resins. It has also been reported to be a minor metabolite of styrene in rats and humans. Varying doses of 4-vinylphenol were administered ip to mice. Hepatotoxicity was assessed by measuring serum sorbitol dehydrogenase (SDH) and by light microscopy. Pneumotoxicity was assessed by measuring proteins, cells, and lactate dehydrogenase activity in bronchoalveolar lavage fluid (BALF) and by light microscopy. 4-VP caused a dose-dependent increase in serum SDH and mild hepatocellular swelling. It caused an increase in cell number and lactate dehydrogenase activity in BALF. Microscopically, there was widespread and severe necrosis of the bronchioles by 12 hours. Re-epithelialization of the bronchioles was evident by 48 hours. These studies indicate that 4-vinylphenol is both hepatotoxic and pneumotoxic.

Keywords. 4-Vinylphenol; liver; lung; pneumotoxicity; hepatotoxicity.

INTRODUCTION

The compound 4-vinylphenol (4-hydroxystyrene, 4-ethenylphenol) is a naturally occurring chemical found in coffee, peanuts, and wild rice (2, 25); 4-vinylphenol (4-VP) has been used as a flavoring agent in a wide variety of food products (6). It is also used synthetically in the production of polymers and resins. Of particular interest is that 4-VP has been identified as a metabolite of styrene. Bakke and Scheline (1) reported finding 4-VP in the hydrolyzed urine of rats dosed orally with styrene. It amounted to 0.1% of the administered dose. Pantarotto et al (17) identified small amounts of 4-VP in the urine of rats administered styrene intraperitoneally. They also found 4-hydroxymandelic acid and 4-hydroxybenzoic acid similar to the end products from styrene metabolism via the styrene oxide pathway. Also, 4-VP was determined to be a minor metabolite in the urine of workers occupationally exposed to styrene (18).

In a short communication, Watabe et al (24) reported the formation of 4-VP using ^{14}C-labelled styrene and a rat hepatic microsomal preparation. They suggested that the 4-VP was formed via the 3,4-oxide and was further metabolized to 4-hydroxystyrene-7,8-glycol. In recent studies we were unable to demonstrate the formation of 4-VP from styrene in rodent microsomal preparations but found that 4-VP was rapidly metabolized by mouse and rat hepatic and pulmonary microsomes involving CYP2E1 and CYP2F2 (7). In a recent study in which rats and mice were exposed by inhalation to [ring-U-^{14}C]styrene, Boogaard et al (5) reported finding $^{14}CO_2$. They suggested that ring hydroxylation may be occurring followed by ring opening. Recent studies in which human volunteers inhaled $^{13}C_8$-styrene were unable to find products that could be derived from ring hydroxylation (14).

Styrene is both hepatotoxic and pneumotoxic in rodents (11, 15, 16), and its toxicity is usually thought to be associated with the production of styrene oxide (4). Furthermore, although styrene administration does not cause tumors in rats (9), lung tumors have been observed in mice by Ponomarkov and Tomatis (19) and Cruzan et al (8). Cruzan et al (10) examined the effect of inhaled styrene on the lungs of mice. In mice exposed to 50 to 200 ppm styrene, histopathology revealed a decreased eosinophilia of the bronchial epithelium accompanied by focal crowding of nonciliated cells. They also found an increase in cell proliferation (BrdU labeling) at 2, 5, and 13 weeks of exposure. A similar picture was observed by Green et al (13) in mice exposed to 40 and 160 ppm styrene for up to 10 days. There was a loss of cytoplasm and focal crowding of nonciliated epithelial cells in the bronchioles that was accompanied by an increase in cell replication rates. Evidence of necrosis and loss of cells, believed to be Clara cells, from the large bronchioles was seen immediately following a 6-hour exposure to 40 ppm styrene. The possible contribution of 4-VP as an active metabolite of styrene in either the acute or chronic toxicity of styrene is unknown.

Little information on the toxicity of 4-VP is available in the published literature. Berger et al (2) reported that feeding 4-VP at a concentration of 1 mg/g diet for 7 to 12 days resulted in a decrease in the uterine weight of the small rodent *Microtus montanus*. The RTECS report (21) on 4-VP indicates that 200 mg/kg administered topically to rabbits is corrosive. The purpose of our studies was to characterize the possible hepatotoxicity and pneumotoxicity associated with 4-vinylphenol in mice by making clinical pathology measurements and using histopathology. These endpoints and this species were selected because the lung and liver are known targets of styrene toxicity in mice (8, 9, 10, 11, 13, 15, 16) whereas in rats the pneumotoxicity is not observed (10, 13). Male mice were used because they are more susceptible to the hepatotoxicity of styrene than are female mice (16) and were used in our previous studies on 4-vinylphenol metabolism (7) and styrene toxicity (11).

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METHODS

Animals and Reagents: Adult male CD-1 [Crl:CD-1 (ICR) BR] (27 to 35 g) mice were obtained from Charles River Laboratories (Wilmington, MA). The animals were housed in group cages in an AAALAC-accredited facility in environmentally controlled rooms on a 12-hour light:dark cycle. Rodent laboratory chow (No. 5001, Purina Mills, Inc., St. Louis, MO) and tap water were allowed ad libitum. All animals were allowed a minimum of 1 week to adapt to the animal facilities and diet before being used in any experiment.

The 4-VP (10% in propylene glycol) was obtained from Lancaster Synthesis (Windham, NH). NADH, pyruvate, and tris buffer were from Sigma Chemical Co. (St. Louis, MO). Triethanolamine was from Mallinckrodt (Paris, KY). Fructose was from Fisher Scientific (Fair Lawn, NJ). All other chemicals were reagent grade or better.

Study Design: Because of the paucity of data regarding the toxicity of 4-VP, initial range finding studies were carried out in groups of 4 mice administered either 200 or 400 mg/kg 4-VP. To examine the toxicity of 4-VP, groups of mice were administered various doses of 4-VP in water IP. These dilutions were made immediately prior to dosing. For the hepatotoxicity studies, groups of 12 mice were administered 50, 100, or 200 mg/kg 4-VP. Twenty-four hours later they were anesthetized with diethyl ether. The abdominal cavity was opened, and the diaphragm was cut. Blood was obtained by cardiac puncture, and pooled from pairs of animals for measurement of sorbitol dehydrogenase (SDH). For the pneumotoxicity studies, groups of 6 to 8 mice were administered 25, 50, or 100 mg/kg 4-VP. The highest dose used was less than in the hepatotoxicity studies because of the high mortality observed at the 200 mg/kg dose in those studies. Bronchoalveolar lavage fluid (BALF) was obtained 24 hours after dosing. The mice were anesthetized with sodium pentobarbital, and the abdominal and thoracic cavities were opened. The incision was continued to the neck region to expose the trachea. A nick was made in the trachea, and a small oral feeding needle was inserted and tied in place. The lungs were perfused twice with 0.8 ml of lavage fluid for a total of 1.6 ml. This fluid consisted of NaCl (145 mM), KCl (5 mM), NaH_2PO_4 (1.9 mM), Na_2HPO_4 (9.4 mM) and glucose (5.5 mM) at a pH of 7.4. These protocols and procedures were approved by the Purdue University Animal Care and Use Committee.

Biochemical Analyses: Blood volumes of 1.0–1.5 ml were obtained by pooling samples from 2 mice, and serum was prepared. Serum SDH activity was measured spectrophotometrically by the method of Gerlach (12). Serum (0.2 ml) and NADH (12 mM) were incubated for 30 minutes at 30°C in triethanolamine buffer (0.2 M, pH 7.4). The reaction was started by addition of 0.3 ml of 72% (w/v) fructose for a final reaction volume of 3.0 ml. SDH activity was measured by the decrease in absorbance of NADH at 366 nm for 2 minutes. Results are expressed as μmol/min/L serum.

When cell counts were made, the number of cells in 100 μl of BALF was counted using a hemocytometer. The remaining BALF was centrifuged at low speed, and the amount of protein determined using the bicinchoninic acid method (17).

Lactate dehydrogenase (LDH) activity was measured by the spectrophotometric method of Vassault (22) in centrifuged BALF samples from individual mice. BALF fluid (0.1 mL), NADH (0.24 mM), and Tris (81 mM)/NaCl (203 mM) buffer (pH 7.2) were incubated for 15 minutes at 30°C. The reaction was initiated by the addition of 0.5 mL pyruvate (9.8 mM) to make a total volume of 3.0 ml. The activity of LDH was measured 30 seconds after the addition of pyruvate by the decrease in absorbance of NADH at 339 nm for 2 minutes using a Shimadzu Model UV160U UV-visible spectrophotometer. Results are expressed as μmol/min/L BALF.

Histopathology: To further evaluate the hepatotoxicity and pneumotoxicity of 4-VP, groups of mice were administered 0, 10, or 100 mg/kg 4-VP IP and were sacrificed by cervical dislocation 12, 24, and 48 hours after dosing. Lung and liver were removed. The lungs were perfused and fixed with 10% neutral buffered formalin as was the liver. Fixed tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin following standard histopathology techniques.

Statistical Analysis: Each assay was replicated as indicated in the individual tables. Values are expressed as mean ± SE. In comparing the values, an ANOVA was utilized followed by Student Newman-Keuls' test to detect differences among the groups. In each case the level of significance selected was $p < 0.05$.

RESULTS

In the initial dose range finding studies, mice that were administered 400 mg/kg 4-VP IP died in less than 3 hours. At 200 mg/kg, the animals became very lethargic, and some

TABLE 1.—Hepatotoxicity of 4-vinylphenol assessed 24 hours after dosing.

Treatment	N	Serum sorbitol dehydrogenase[a]
Control	6	19 ± 6^b
4-Vinylphenol 50 mg/kg	6	51 ± 13^c
4-Vinylphenol 100 mg/kg	4	92 ± 13^d
4-Vinylphenol 200 mg/kg	2	524 ± 11^e

[a] μmols/min/L.
[b–e] Values with different superscripts are significantly different from each other ($p < 0.05$).

TABLE 2.—Protein concentration in BALF 24 hours following 4-vinylphenol.

Treatment	N	BALF protein[a]
Control	8	287 ± 6^b
4-Vinylphenol 25 mg/kg	5	277 ± 25^b
4-Vinylphenol 50 mg/kg	6	364 ± 47^b
4-Vinylphenol 100 mg/kg	6	349 ± 29^b

[a] Micrograms protein per ml BALF.
[b] No statistically significant differences among the groups ($p > 0.05$).

TABLE 3.—Number of cells in BALF 24 hours following 4-vinylphenol.

Treatment	N	Cells in BALF[a]
Control	6	54 ± 24^b
4-Vinylphenol 25 mg/kg	5	22 ± 4^b
4-Vinylphenol 50 mg/kg	6	172 ± 41^c
4-Vinylphenol 100 mg/kg	4	1.347 ± 168^d

[a] Cells per μl BALF.
[b–d] Values with different superscripts are significantly different from each other ($p < 0.05$). Values were log transformed prior to analysis.

TABLE 4.—Lactate dehydrogenase activity in BALF 24 hours following 4-vinylphenol.

Treatment	N	BALF lactate dehydrogenase[a]
Control	5	41 ± 4^b
4-Vinylphenol 25 mg/kg	4	53 ± 11^b
4-Vinylphenol 50 mg/kg	6	137 ± 29^c
4-Vinylphenol 100 mg/kg	3	226 ± 29^d

[a] μmols/min/L.
[b–d] Values with different superscripts are significantly different from each other ($p < 0.05$).

died within 24 hours. No mouse survived beyond 40 hours. The animals at this high dose exhibited slight tremors.

When hepatotoxicity was assessed by measuring serum SDH 24 hours after administering 4-VP, all doses, 50, 100, and 200 mg/kg, increased this serum enzyme activity in a dose-dependent manner (Table 1). No lower dose was used because the variations in response indicated that a very large number of animals would have been needed to establish a statistically significant difference if one exists.

When mice were treated with 4-VP and the BALF collected 24 hours later, there was no increase in protein (Table 2). However, there was a significant increase in the number of cells found in the fluid (Table 3). Although no increase was observed in the group receiving 25 mg/kg 4-VP, at 50 mg/kg the number of cells was $3\frac{1}{2}$ times the number in the control BALF, and at 100 mg/kg it was 25 times that of control. Pneumotoxicity was also suggested by the increases in lactate dehydrogenase released into BALF. Again there was a dose-dependent increase at 50 and 100 mg/kg, but at 25 mg/kg there was no significant difference from control (Table 4).

No gross lesions were noted in the major abdominal or thoracic organs of any of the control or treatment group animals. Microscopically, lesions were noted in the lungs from all of the animals in the 3 (12-hour, 24-hour, and 48-hour) high-dose (100 mg/kg) groups. The lesions in the earliest high dose group (at 12 hours) were limited to the distal bronchial and bronchiolar epithelium that was characterized as completely necrotic (Figure 1). In the 24-hour high-dose group animals, the distal bronchi and bronchioles were partially lined by varying proportions of attenuated or low cuboidal epithelial cells, and their lumens contained sloughed cells and necrotic cellular debris (Figure 2). In the 48-hour high-dose group, the distal bronchi and bronchioles were lined by a low cuboidal epithelium, and there was little cellular debris within the lumens (Figure 3). In the 48-hour group animals, there was very little evidence of epithelial cell degeneration or necrosis. In the high-dose groups at all three time periods, a mild to moderate inflammatory cell infiltrate, neutrophils and fewer macrophages, was associated with some of the affected airways, and it extended into the adjacent alveolar spaces. Liver lesions were nonspecific and consisted of mild centrilobular hepatocellular swelling.

DISCUSSION

The purpose of these studies was to assess the hepatotoxicity and pneumotoxicity of 4-VP, a chemical of interest as a natural product, an agent used in the production of polymers and resins, and a putative metabolite of styrene.

The contribution of 4-VP as a metabolite of styrene to the toxicity of styrene is problematical. In a previous in vitro study we were unable to identify it as a metabolite of styrene (7), but it has been reported in a limited number of studies as a minor urinary metabolite in rodents (1, 17) and humans (18). It is interesting to note that newer studies by Boogaard et al (5) also suggest that this pathway is operative in mice since $^{14}CO_2$ was found to be a metabolite in animals given

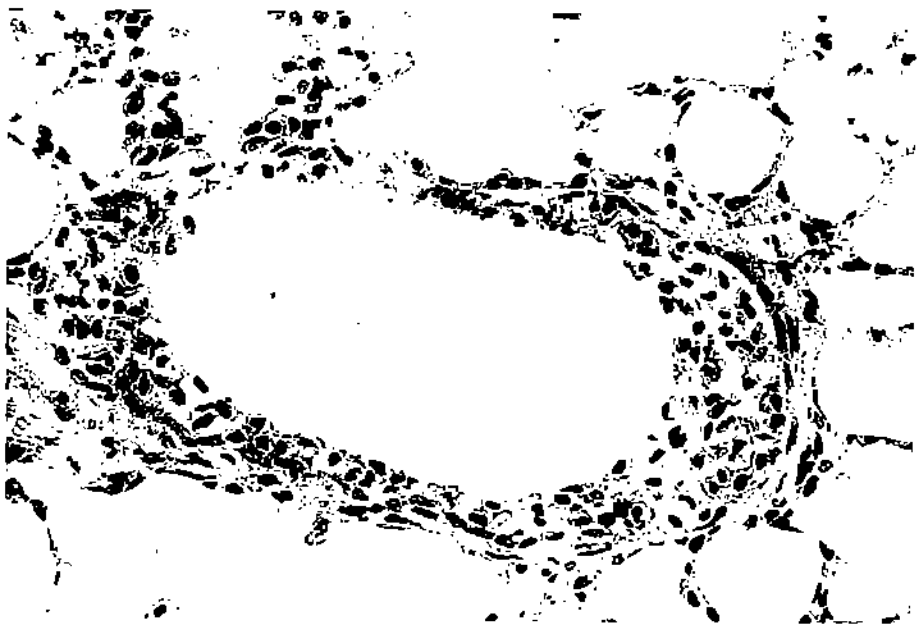

FIGURE 1.—Lung from mouse dosed with 100 mg/kg 4-vinylphenol sacrificed at 12 hours. Exposure to 4-vinylphenol produced acute pneumotoxicity of the distal bronchi and bronchioles characterized by degeneration and necrosis of the epithelium. H&E. ×560.

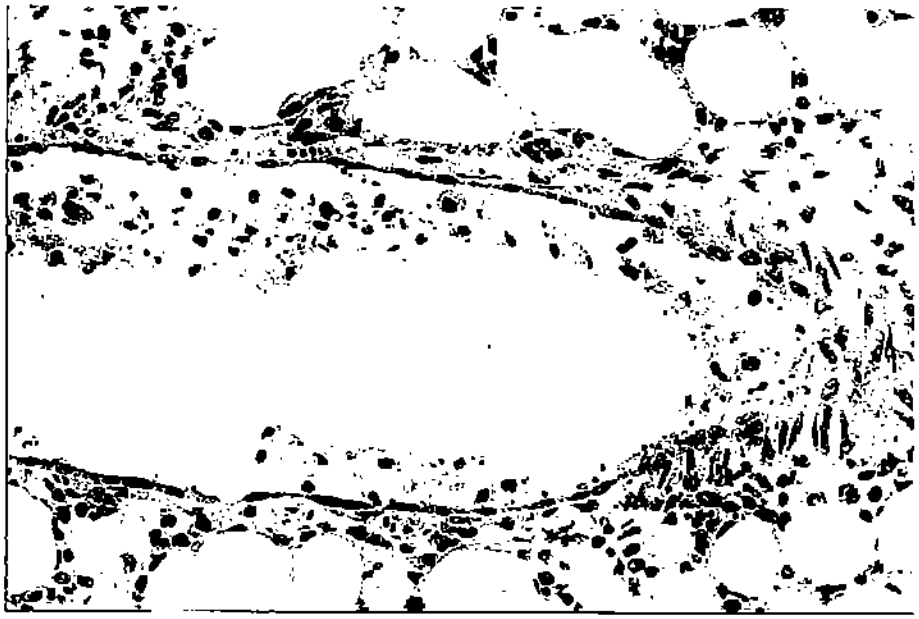

FIGURE 2.—Lung from mouse dosed with 100 mg/kg 4-vinylphenol sacrificed at 24 hours. At 24 hours postexposure to 4-vinylphenol, the distal bronchi and bronchioles were characterized by necrotic debris, sloughed epithelial cells, and an attenuated epithelium. H&E. ×560.

ring labeled ^{14}C-styrene. The authors reasoned that the only way to release this ring carbon was via an initial epoxidation followed by ring opening. On the other hand, Johanson et al (14) found no evidence of metabolites that would be derived from ring labeling in human volunteers inhaling $^{13}C_8$-styrene.

The studies reported here indicate that increases in serum SDH were observed in doses as low as 50 mg/kg. In contrast, doses of styrene as high as 500 to 1,000 mg/kg were needed to cause a similar effect (11). Even for styrene oxide, doses of approximately 300 mg/kg were needed. Thus, 4-VP is an order of magnitude more potent than either styrene or styrene oxide. The BALF studies indicate that 4-VP is also pneumotoxic. Increases in cell numbers as well as the release of lactate dehydrogenase into these lungs were readily observed at a 4-VP dose of 50 mg/kg. Again 4-VP was more potent than either styrene or styrene oxide (11). However, it

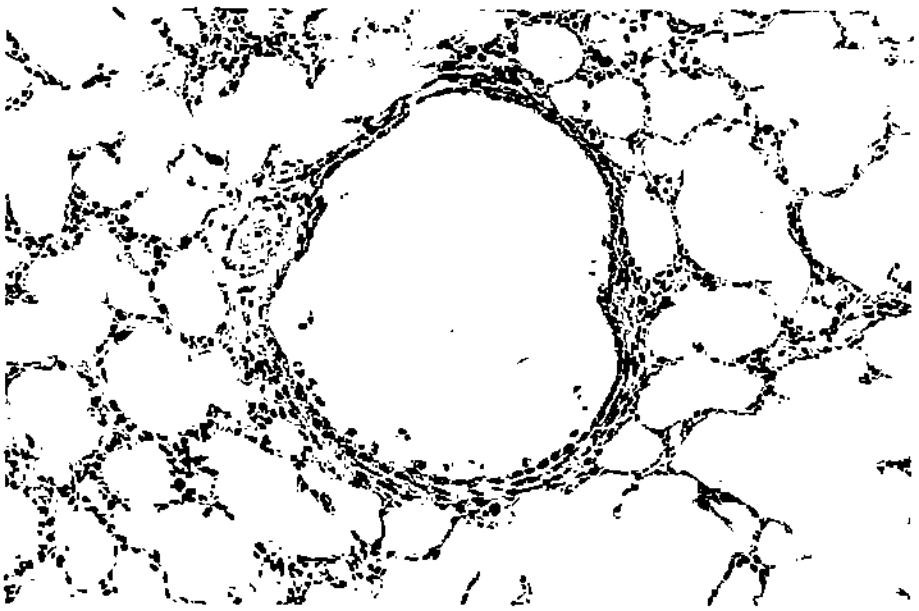

FIGURE 3.—Lung from mouse dosed with 100 mg/kg 4-vinylphenol sacrificed at 48 hours. By 48 hours postexposure to 4-vinylphenol, the distal bronchi and bronchioles were characterized by a low cuboidal to attenuated epithelium and a paucity of necrotic cellular debris. H&E. ×224.

still remains unknown if 4-VP contributes to the toxicity of styrene.

The toxic effects of 4-VP on the lung were substantiated by the microscopic findings of severe necrosis of the distal bronchi and bronchioles by 12 hours followed by a re-epithelialzation with attenuated or low cuboidal cells by 24 hours. By 48 hours there was minimal evidence of cell degeneration or necrosis. This lesion in the bronchioles appears to be similar to that described by Van Winkle et al (23) for naphthalene. They described the swelling, vacuolation, and exfoliation of Clara cells leading to denuding of some regions of the basal lamina in the distal airways within 24 hours followed by repair of the normal architecture.

Interestingly, when the liver was examined by light microscopy there was minimal damage due to 4-VP. The liver lesions were nonspecific with only mild swelling of the centrilobular hepatocytes. In previous studies with styrene administered by IP injection, we observed hepatocellular damage around the central vein with hepatocytes demonstrating a loss of cytoplasmic staining and mild swelling (11). Extensive hepatic damage has also been demonstrated in the studies of Morgan et al (16) who reported finding severe congestion and necrosis in the livers of mice exposed to styrene (250 or 500 ppm) by inhalation for 14 days. This was accompanied by increases in serum alanine aminotransferase and sorbitol dehydrogenase.

In summary, the administration of 4-VP caused both hepatic and pulmonary damage as evidenced by increases in serum enzymes and increases in cells and lactate dehydrogenase activity in BALF. The microscopic lesions were predominantly in the distal bronchi and bronchioles of the lung where extensive necrosis occurred with substantial recovery by 48 hours.

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Original articles

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Mortality from nonmalignant diseases of the respiratory, genitourinary and nervous systems among workers exposed to styrene in the reinforced plastics and composites industry in the United States

by Otto Wong, ScD,[1,2] Lisa S Trent, MS[1]

Wong O, Trent LS. Mortality from nonmalignant diseases of the respiratory, genitourinary and nervous systems among workers exposed to styrene in the reinforced plastics and composites industry in the United States. *Scand J Work Environ Health* 1999;25(4):317—325.

Objectives Mortality from diseases of the nervous system and nonmalignant diseases of the respiratory and genitourinary systems was examined for workers exposed to styrene.

Methods Altogether 15 826 styrene-exposed workers in 30 plants in the reinforced plastics and composites industry were included. Vital status was ascertained through 31 December 1989. Individual exposure estimates were developed based on job functions, existing industrial hygiene data, process changes, engineering controls, work practices, and the use of personal protective equipment. Analyses were based on cause-specific standardized mortality ratios (SMR) and the Cox proportional hazards model. Mortality data were analyzed by latency, duration of exposure, average exposure, cumulative exposure, and process category.

Results For diseases of the nervous system, the SMR was 0.56 [95% confidence interval (95% CI) 0.31—0.95]. Mortality from nonmalignant genitourinary diseases was not increased (SMR 0.87, 95% CI 0.46—1.50). Latency, duration of exposure, average exposure, cumulative exposure, and process category showed no association between styrene exposure and these 2 types of disease. A small increase in mortality from nonmalignant respiratory diseases was found (SMR 1.21, 95% CI 0.98—1.47), mainly due to "other nonmalignant respiratory diseases" (SMR 1.40, 95% CI 1.04—1.84). The highest increase occurred for short exposure duration (SMR 1.79 for <1 year's exposure) or low exposure (SMR 2.15 for <10 ppm-years); there were no increased risks in the high exposure categories. The Cox proportional hazard model revealed no association between styrene exposure and the diseases.

Conclusions No relationship was found between mortality from any of the diseases examined and any of the styrene exposure indices. The findings were compared with those reported in a European study of styrene-exposed workers.

Key terms cohort study, epidemiology, occupational health.

Currently there are 2 large-scale mortality cohort studies of workers exposed to styrene, the European study conducted by the International Agency for Research on Cancer (IARC) and its collaborators and the United States (US) study by Applied Health Sciences. The IARC study was based on a multicentric investigation consisting of several cohorts from Denmark, Finland, Italy, Norway, Sweden, and the United Kingdom (1, 2). It consisted of 40 683 workers in the reinforced plastics industry in these 6 European countries. According to the investigators, no excess was observed for mortality from all causes, all cancers, lung cancer, or other major epithelial cancers (1, 2). Mortality from neoplasms of the lymphatic and hematopoietic tissues was also not elevated. In the 1993 or 1994 report (1, 2), Kogevinas et al reported no increased mortality from mental disorders [standardized mortality ratio (SMR) 1.07], diseases of the nervous system (SMR 0.79), diseases of the respiratory system (SMR 0.81), or diseases of the genitourinary system (SMR 0.97).

In 1996, 3 papers based on the IARC study were published by Welp et al (3—5), reporting mortality analyses from nonmalignant respiratory diseases, nonmalignant

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genitourinary diseases, diseases of the nervous system, and mental disorders. For nonmalignant respiratory diseases, Welp et al (3) drew the following conclusion: "Mortality from pneumonia was associated with intensity of exposure to styrene, but this may have been due to chance. Mortality from bronchitis, emphysema, and asthma was not associated with styrene exposure [p 499]". With regard to nonmalignant genitourinary diseases, it was concluded that mortality "increased as the average intensity of exposure increased [p 226]" (4). The authors also commented that "This finding indicates that other data should be scrutinized [p 223]." Finally, Welp and her colleagues (5) reported that "mortality from diseases of the central nervous system increased with time since first exposure, duration of exposure, average level of exposure, and cumulative exposure to styrene [p 623]." The increase in mortality from the central nervous system was primarily influenced by the increased mortality from epilepsy. Welp et al stated that "mortality from epilepsy increased monotonically with all styrene exposure indicators, while associations for degenerative diseases of the central nervous system were generally weaker [p 623]". The authors concluded that "These findings suggest that, in addition to the known acute effects, exposure to styrene may contribute to chronic diseases of the central nervous system [p 623]".

The other large-scale mortality study was based on workers in the United States (6). Mortality of a cohort of 15 826 male and female workers exposed to styrene at 30 participating plants in the reinforced plastics and composites industry in the United States has recently been updated and reported by Wong et al (7). The primary analyses were conducted using the University of Pittsburgh OCMAP program, which provided a standard set of causes of death. Except for nonmalignant respiratory diseases, causes of death discussed in the 1996 papers by Welp et al were not part of the standard OCMAP analysis, and, therefore, not presented for the US cohort in the Wong et al (7) report. The present report summarizes new analyses for mortality from diseases of the nervous system, nonmalignant diseases of the respiratory system, and nonmalignant diseases of the genitourinary system in the US cohort.

Subjects and methods

The cohort consisted of 15 826 male and female employees at 30 reinforced plastics plants in the United States. To be included in the cohort, an employee must have worked in areas with styrene exposure for a minimum of 6 months between 1 January 1948 and 31 December 1977. The vital status of the cohort was ascertained through 31 December 1989. Sources of vital status information included personnel records maintained at the participating plants, the Death Master File of the Social Security Administration, the National Death Index of the National Center for Health Statistics, and the data base of a commercial retail credit bureau. With the use of information from these sources, death certificates were obtained from individual state health departments. Causes of death were coded according to the revision of the International Classification of Diseases (ICD) in effect at the time of death.

Person-years of observation started after 6 months of exposure to styrene and ended on the date of death or 31 December 1989, whichever was earlier. For those lost to follow-up (unknown vital status), person-years were counted up to the last date of contact. Person-years were classified by age (5-year groups), gender, and calendar years (5-year groups). Expected deaths were based on US national age- gender- cause- race- and year-specific death rates, and cause-specific standardized mortality ratios (SMR) were computed using the University of Pittsburgh OCMAP computer program. Since mortality rates for the causes of death included in our report are not part of the regular mortality rates in the OCMAP program, the rates were requested from the University of Pittsburgh. Because race information was missing from employment records for most of the cohort, the entire cohort was assumed to be white (7).

The Cox proportional hazards model was also used in the analysis. One important advantage of the Cox model was that the actual exposure data could be used instead of grouped data. An additional advantage was that only internal cohort data were used, whereas SMR values were based on comparisons with an external population. The actual computation was performed using SAS PHREG software. The independent variables used in these models included age, gender, duration of exposure, average exposure, and cumulative exposure.

The 30 reinforced plastics plants in the study manufactured various products, including sheet molding compounds, bulk molding compounds, tanks, pipes, ducts, boats, panels, auto-parts, trays, and small miscellaneous parts. The first year of styrene exposure at the individual plants ranged from 1948 to 1968. At the time of the original epidemiologic study, a parallel exposure assessment investigation was conducted (6, 7).

The exposure classification scheme used in the study was developed in several stages. First, for each plant, a list of job titles was generated based on employment records collected in the study. This list consisted of job or department (or location) titles or both as they appeared in the personnel records. The initial list consisted of a large number of entries, since the same job or department title could have been recorded slightly differently over the years. With assistance from the participating plants, these duplications were consolidated.

The consolidated list (by plant) was provided to the industrial hygiene team for exposure assessment. Individual plants were visited by a field survey team, and a detailed industrial hygiene assessment was conducted at each plant to measure current (around 1980) exposure levels of styrene and other substances. Information on job functions, work practices, past industrial hygiene measurements, process changes and modifications, engineering controls, and personal protection equipment at each plant was also obtained. Using the information collected, the industrial hygienists developed a list of 19 job categories with homogeneous exposures. Each job title was assigned to 1 of the 19 job categories.

A job-exposure matrix was developed for each plant, and a current 8-hour time-weighted-average (8-hour TWA) and an exposure range were assigned to each job category. Based on information on changes over time, historical TWA values were likewise estimated. Job categories in which typical TWA estimates for styrene were low included finish and assembly (5 ppm), storage and shipping (5 ppm), office and other nonproduction (2 ppm), injection molding (4 ppm), field service (5 ppm), preform production (7 ppm), and pultrusion (5 ppm). Job categories with moderate TWA values (20—45 ppm) included molding compound production, gel coating, and winding. A typical styrene exposure in the spray-up or lay-up process category was 60 ppm, with a range of 5 to 120 ppm that reflects considerable differences among facilities and the nature of specific work activities. In terms of job titles, on the average, laminators were exposed to the highest levels (8-hour TWA of 80 ppm). Overall, the average 8-hour TWA values for the majority of jobs was 10 ppm or less.

Two quantitative styrene exposure indices were developed. Based on the employment history of each cohort member (through the end of 1977) and the exposure estimates derived from the job-exposure matrix for that particular plant, a TWA was assigned to each job in a worker's employment history. A cumulative exposure in ppm-years, calculated as the sum of the products of the TWA and duration of exposure of each job, was developed for each cohort member. In order to compare the results to those in the IARC study, an average exposure in parts per million (cumulative exposure divided by duration of exposure) was also calculated for each cohort member.

In addition, based on a consideration of both exposure estimates and processes and job activities, 6 process categories were created, each with a distinct and relatively homogeneous exposure profile to styrene in combination with other chemicals. The 6 process categories are as follows, along with examples of their component job categories: (i) open-mold process (examples: spray-up/lay-up, winding, gel coating, stringing and fitting, laminating), (ii) mixing and closed-mold process (examples: cutting, weighing, pressing, mixing, pultrusion, inject molding, casting), (iii) finish and assembly (examples: finishing, storing and shipping, repairing), (iv) plant office and support (examples: general and nonproduction, quality control, office and others), (v) maintenance and preparation (examples: maintenance, utility, mold preparation), and (vi) supervisory and professional (examples: supervisors' and engineers' tasks).

Table 1 summarizes the descriptive statistics of the cohort. In terms of follow-up, the vital status of only 547 cohort members (3.5%) remained unknown at the end of 1989. Of the 1628 workers identified to have died, death certificates were obtained for all but 42 (2.56%). These 42 deaths were included in the overall SMR calculations, which have been reported previously (7), but not in the cause-specific SMR calculations. In terms of exposure, close to one-quarter of the cohort (23.5%) had more than 100 ppm-years of cumulative exposure, and one-third (34.2%) had an average exposure of 20 ppm or higher. It should be pointed out that the employment histories were not updated after 1977, the closing date of the original study. Therefore, all work and exposure assignments were truncated in 1977.

Table 1. Descriptive statistics of the cohort of workers exposed to styrene in the reinforced plastics and composites industry.

Description	Workers	
	N	%
Total cohort	15 826	100.0
Men	11 958	75.6
Women	3868	24.4
Duration of employment (years) as of 31 December 1977		
0.5—0.9	3712	23.5
1.0—1.9	3528	22.3
2.0—4.9	4326	27.3
≥5.0	4260	26.9
Cumulative exposure (ppm-years) as of 31 December 1977		
<10.0	3778	23.9
10.0—29.9	4119	26.0
30.0—99.9	4210	26.6
≥100.0	3719	23.5
Average intensity of exposure (ppm) from hire to 31 December 1977		
<5.0	2970	18.8
5.0—9.9	3702	23.4
10.0—19.9	3736	23.6
20.0—59.9	3161	19.9
≥60.0	2257	14.3
Vital status as of 31 December 1989		
Alive	13 651	86.2
Dead	1628	10.3
With death certificates	1586	97.4
Without death certificates	42	2.6
Unknown	547	3.5

Results

Table 2 shows the observed deaths, SMR values, and 95% confidence intervals (95% CI) for selected causes of death for the entire cohort and by latency (time since first exposure). For diseases of the nervous system, there were 14 deaths in the entire cohort, significantly fewer than the 24.7 expected. The corresponding SMR was 0.56 (95% CI 0.31—0.95). Only 2 deaths in the cohort were attributed to epilepsy, comparable to the 2.7 expected (SMR 0.73, 95% CI 0.09—2.66). For diseases of the genitourinary system, there were 13 observed deaths, comparable to the 14.8 expected (SMR 0.87, 95% CI 0.46—1.50). There were only 2 deaths from nephritis, slightly fewer than the 3.7 expected (SMR 0.54, 95% CI 0.06—1.96). Altogether 97 deaths were coded as nonmalignant respiratory diseases, more than the expected 80.2. However, the increase was not statistically significant (SMR 1.21, 95% CI 0.98—1.47). Within the broad category of nonmalignant respiratory diseases, 23 deaths were from pneumonia, comparable to the 24.9 expected (SMR 0.92, 95% CI 0.58—1.38). For the subcategory "bronchitis, emphysema and asthma," there were 23 deaths, slightly more than the 18.1 expected (SMR 1.27, 95% CI 0.80—1.91). There was a statistically significant increase in mortality for the subcategory "other nonmalignant respiratory diseases." A total of 51 deaths in the subcategory were observed, compared with 36.3 expected (SMR 1.40, 95% CI 1.04—1.84). Thirty-six of these deaths (71%) were from "chronic airway obstruction, not otherwise specified" (9th ICD 496), which included "chronic nonspecific lung disease," "chronic obstructive lung disease," and "chronic obstructive pulmonary disease, not otherwise specified."

Although not shown in table 2, an analysis was also performed separately by gender. All 14 deaths from diseases of the nervous system in the cohort occurred among the male workers, compared with 19.5 expected (SMR 0.71, 95% CI 0.39—1.20). Among the women, no death was attributed to diseases of the nervous system, whereas 5.1 were expected. Another cause of death for which the 2 genders differed was nonmalignant respiratory diseases. For the men, the SMR for nonmalignant respiratory diseases was 1.24 (84 observed, 95% CI 0.98—1.53), whereas that for the women was 1.04 (13 observed, 95% CI 0.55—1.78). For other causes of death, mortality was similar between the 2 genders.

Mortality analysis by latency (time since first exposure to styrene) is also presented in table 2. The increase in mortality from nonmalignant respiratory diseases was the most prominent 20 years after the first exposure (SMR 1.44, 95% CI 1.05—1.92), whereas there was no increase within the first 10 years (SMR 0.65, 95% CI 0.32—1.17). In the group with a latency of 10—19 years, the SMR for nonmalignant respiratory diseases was 1.27 (95% CI 0.90—1.73).

Table 3 depicts mortality by duration of exposure. Short-term workers with less than 1 year of exposure had the highest mortality from nonmalignant respiratory diseases (SMR 1.41, 95% CI 0.86—2.18), whereas for those with at least 10 years of exposure there was no increase (SMR 0.97, 95% CI 0.57—1.54). No significant increase was observed for any specific causes of death in any of the groups by duration of exposure. No pattern by length of exposure was evident for any specific causes of death in table 3. In particular, among those with 10 or more years of exposure, no increased mortality was seen with the exception of nonmalignant genitourinary diseases. Six such deaths were observed among those with 10 or more years of exposure (SMR 2.04, 95% CI 0.74—4.44). However, the increase was not statistically significant. A similar analysis based on length of employment was also performed (not shown). The results were very similar, indicating that length of employment and length of exposure were the same or very similar for most of the cohort members.

The results of the analysis by cumulative exposure are shown in table 4. For mortality from diseases of the nervous system, no increase or pattern was seen with regard to cumulative exposure. For mortality from nonmalignant respiratory diseases, a significant increase was observed among those with less than 10.0 ppm-years of

Table 2. Observed deaths (O) in the entire cohort and the standardized mortality ratios (SMR) with their 95% confidence intervals (95% CI) for selected causes by time since first exposure to styrene.

Cause of death	<10 years			10—19 years			≥20 years			Total		
	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI
Diseases of the nervous system	1	0.10	0.00—0.57	6	0.68	0.25—1.49	7	1.13	0.45—2.33	14	0.56	0.31—0.95
Epilepsy	-	0	.	1	1.00	0.02—5.58	1	2.84	0.07—15.84	2	0.73	0.09—2.66
Nonmalignant respiratory diseases	11	0.65	0.32—1.17	40	1.27	0.90—1.73	46	1.44	1.05—1.92	97	1.21	0.98—1.47
Pneumonia	3	0.44	0.09—1.31	8	0.84	0.36—1.65	12	1.38	0.71—2.41	23	0.92	0.58—1.38
Bronchitis, emphysema and asthma	6	1.26	0.46—2.75	7	0.96	0.38—1.98	10	1.65	0.79—3.03	23	1.27	0.80—1.91
Other nonmalignant respiratory diseases	2	0.39	0.04—1.44	25	1.74	1.13—2.57	24	1.41	0.90—2.10	51	1.40	1.04—1.84
Nonmalignant genitourinary diseases	1	0.23	0.01—1.30	8	1.45	0.62—2.86	4	0.79	0.21—2.03	13	0.87	0.46—1.50
Nephritis	-	0	.	2	1.70	0.20—6.17	-	0	.	2	0.54	0.06—1.96

exposure (SMR 1.64, 95% CI 1.09—2.37), whereas no statistically significant increase was evident among those with more than 100.0 ppm-years of exposure (SMR 1.04, 95% CI 0.66—1.57). The increased mortality from nonmalignant respiratory diseases among those with a cumulative exposure of less than 10.0 ppm-years came from the 2 subcategories "bronchitis, emphysema and asthma" (SMR 2.07, 95% CI 0.89—4.08) and "other nonmalignant respiratory diseases" (SMR 2.15, 95% CI 1.23—3.49). Only the latter was statistically significant. There was a nonsignificant increase in mortality from nonmalignant genitourinary diseases among the workers with ≥100 ppm-years of exposure (5 observed, 3.7 expected, SMR 1.34, 95% CI 0.43—3.13).

Table 5 shows the mortality analysis by average intensity of styrene exposure. The category nonmalignant

Table 3. Observed deaths (O) in the entire cohort and the standardized mortality ratios (SMR) with their 95% confidence intervals (95% CI) for selected causes by duration of exposure to styrene.

Cause of death	<1.0 year			1.0—1.9 years			2.0—4.9 years			5.0—9.9 years			≥10.0 years		
	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI
Diseases of the nervous system	1	0.18	0.01—1.05	4	0.82	0.22—2.11	3	0.47	0.09—1.38	2	0.46	0.05—1.68	4	1.03	0.28—2.64
Epilepsy	1	1.36	0.03—7.60	-	0	.	-	0	.	1	2.59	0.06—14.46	-	0	.
Nonmalignant respiratory diseases	20	1.41	0.86—2.18	17	1.25	0.73—2.01	22	1.18	0.74—1.79	20	1.29	0.79—2.00	18	0.97	0.57—1.54
Pneumonia	3	0.62	0.12—1.83	6	1.36	0.50—2.97	6	0.98	0.36—2.14	3	0.65	0.13—1.92	5	0.98	0.32—2.30
Bronchitis, emphysema and asthma	6	1.95	0.71—4.26	3	0.99	0.20—2.90	4	0.94	0.25—2.41	6	1.68	0.61—3.66	4	0.96	0.26—2.46
Other nonmalignant respiratory diseases	11	1.79	0.89—3.21	8	1.34	0.58—2.65	12	1.49	0.77—2.61	11	1.54	0.77—2.76	9	0.99	0.45—1.88
Nonmalignant genitourinary diseases	1	0.34	0.01—1.93	-	0	.	3	0.82	0.17—2.41	3	1.10	0.22—3.22	6	2.04	0.74—4.44
Nephritis	-	0	.	-	0	.	1	1.02	0.02—5.73	-	0	.	1	1.94	0.04—10.82

Table 4. Observed deaths (O) in the entire cohort and the standardized mortality ratios (SMR) with their 95% confidence intervals (95% CI) for selected causes by cumulative exposure to styrene.

Cause of death	<10.0 ppm-years			10.0—29.9 ppm-years			30.0—99.9 ppm-years			≥100.0 ppm-years		
	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI
Diseases of the nervous system	3	0.49	0.10—1.45	2	0.32	0.04—1.18	6	0.67	0.32—1.80	3	0.53	0.10—1.54
Epilepsy	1	1.42	0.03—7.96	-	0	.	1	1.38	0.03—7.71	-	0	.
Nonmalignant respiratory diseases	28	1.64	1.09—2.37	17	0.94	0.54—1.51	29	1.25	0.84—1.80	23	1.04	0.66—1.57
Pneumonia	4	0.72	0.19—1.84	7	1.18	0.47—2.44	9	1.26	0.57—2.40	3	0.47	0.09—1.38
Bronchitis, emphysema and asthma	8	2.07	0.89—4.08	5	1.24	0.40—2.89	4	0.76	0.20—1.95	6	1.22	0.44—2.65
Other nonmalignant respiratory diseases	16	2.15	1.23—3.49	5	0.63	0.20—1.48	16	1.52	0.87—2.47	14	1.33	0.72—2.23
Nonmalignant genitourinary diseases	1	0.30	0.01—1.67	4	1.13	0.30—2.90	3	0.71	0.14—2.07	5	1.34	0.43—3.13
Nephritis	-	0	.	1	1.05	0.02—5.88	1	0.97	0.02—5.45	-	0	.

Table 5. Observed deaths (O) in the entire cohort and the standardized mortality ratios (SMR) with their 95% confidence intervals (95% CI) for selected causes by average exposure to styrene.

Cause of death	< 5.0 ppm			5.0—9.9 ppm			10.0—19.9 ppm			20.0—59.9 ppm			≥60.0 ppm		
	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI
Diseases of the nervous system	2	0.35	0.04—1.26	4	0.61	0.16—1.57	5	0.79	0.25—1.84	2	0.48	0.05—1.74	1	0.50	0.01—2.81
Epilepsy	-	0	.	-	0	.	2	2.95	0.35—10.68	-	0	.	-	0	.
Nonmalignant respiratory diseases	33	1.69	1.16—2.37	16	0.75	0.43—1.23	26	1.26	0.82—1.85	18	1.17	0.67—1.91	6	1.10	0.40—2.39
Pneumonia	7	1.16	0.46—2.39	4	0.61	0.16—1.51	6	0.95	0.35—2.08	4	0.93	0.25—2.38	2	1.10	0.13—4.00
Bronchitis, emphysema and asthma	10	2.24	1.07—4.12	3	0.62	0.12—1.82	4	0.85	0.23—2.18	6	2.03	0.74—4.41	-	0	.
Other nonmalignant respiratory diseases	16	1.81	1.03—2.94	9	0.94	0.43—1.79	16	1.71	0.98—2.78	6	0.97	0.35—2.11	4	1.64	0.44—4.20
Nonmalignant genitourinary diseases	2	0.57	0.06—2.05	5	1.27	0.41—2.97	4	1.05	0.28—2.70	1	0.39	0.01—2.20	1	0.94	0.02—5.26
Nephritis	-	0	.	2	1.97	0.23—7.14	-	0	.	-	0	.	-	0	.

Table 6. Observed deaths (O) and the standardized mortality ratios (SMR), with their 95% confidence intervals (95% CI) for selected causes by major processing categories.[a]

Cause of death	Open mold process (N=1386)			Mixing and closed-mold process (N=1225)			Finish and assemble (N=2273)			Plant office and support (N=1474)			Maintenance and preparation (N=1192)			Supervisory and professional (N=507)		
	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI	O	SMR	95% CI
Diseases of the nervous system	-	0	.	-	0	.	2	0.50	0.06—1.80	3	1.02	0.21—2.99	4	1.07	0.29—2.75	-	0	.
Epilepsy	-	0	.	-	0	.	-	0	.	1	3.60	0.09—20.05	-	0	.	-	0	.
Nonmalignant respiratory diseases	7	1.21	0.48—2.50	7	1.32	0.53—2.72	15	0.96	0.53—1.58	11	1.06	0.53—1.90	18	1.16	0.69—1.84	5	0.97	0.31—2.26
Pneumonia	-	0	.	1	0.61	0.01—3.44	6	1.33	0.48—2.90	4	1.31	0.35—3.35	4	0.88	0.24—2.27	2	1.23	0.15—4.44
Bronchitis, emphysema and asthma	3	2.38	0.49—6.96	3	2.61	0.54—7.63	4	1.13	0.48—2.90	1	0.41	0.01—2.28	4	1.10	0.30—2.81	1	0.85	0.02—4.72
Other nonmalignant respiratory diseases	4	1.47	0.40—3.76	3	1.22	0.25—3.57	5	0.67	0.21—1.56	6	1.26	0.46—2.75	10	1.40	0.67—2.57	2	0.86	0.10—3.10
Nonmalignant genitourinary diseases	1	0.99	0.02—5.55	1	0.95	0.02—5.30	4	1.47	0.40—3.78	4	2.20	0.60—5.63	2	0.77	0.09—2.81	-	0	.
Nephritis	-	0	.	-	0	.	1	1.82	0.04—10.14	-	0	.	1	1.85	0.04—10.34	-	0	.

[a] Workers could be included in more than one category.

Table 7. Analysis of selected causes of death based on the Cox proportional hazards model, with age, gender, and cumulative exposure as independent variables.

Cause of death (N)	β-coefficient	SD	P-value
Diseases of the nervous system (N=14)			
Age (years)	0.019	0.024	0.41
Cumulative exposure (ppm-years)	-0.001	0.001	0.75
Nonmalignant genitourinary diseases (N=13)			
Age (years)	0.028	0.023	0.24
Gender	-0.193	0.660	0.76
Cumulative exposure (ppm-years)	0.000	0.001	0.94
Nonmalignant respiratory diseases (N=97)			
Age (years)	0.084	0.008	0.00
Gender	-0.954	0.298	0.00
Cumulative exposure (ppm-years)	-0.001	0.001	0.14
Pneumonia (N=23)			
Age (years)	0.078	0.017	0.00
Gender	-0.644	0.551	0.24
Cumulative exposure (ppm-years)	-0.004	0.002	0.14

respiratory diseases was significantly elevated among workers in the lowest average exposure group (<5.0 ppm). The increase came from "bronchitis, emphysema and asthma" (SMR 2.24, 95% CI 1.07—4.12) and from "other nonmalignant respiratory diseases" (SMR 1.81, 95% CI 1.03—2.94). There was no significant increase in mortality from any of the causes of death examined in any higher categories of average intensity of styrene exposure. Altogether 2257 workers were exposed to an average intensity of more than 60 ppm throughout their employment in the reinforced plastics and composites industry. In this group of workers, there was only 1 death from diseases of the nervous system (SMR 0.50), and no death from epilepsy. In the same group, for nonmalignant respiratory diseases, the SMR was 1.10 (95% CI 0.40—2.39), and that for "other nonmalignant respiratory diseases" was 1.64 (95% CI 0.44—4.20). For nonmalignant genitourinary diseases, the SMR was 0.94 (95% CI 0.02—5.26).

The mortality analysis by major industrial processing categories is presented in table 6. A worker was classified into a specific processing category if he or she spent at least 2 years in that category. Therefore, a worker could be classified into more than 1 category. No significant increase in mortality from any cause of death included in table 6 was found for any of the industrial processing categories.

In addition to the indirect method of standardization (SMR values), the data were also analyzed using the Cox proportional hazards model (tables 7 and 8). The following 4 causes of death were selected for this analysis: diseases of the nervous system, nonmalignant genitourinary diseases, nonmalignant respiratory diseases, and pneumonia. The independent variables included in the models in table 7 consisted of age, gender, and cumulative exposure. For diseases of the nervous system, gender was not included in the model because all 14 deaths from this cause occurred among the male workers. As indicated in table 7, cumulative exposure to styrene was not associated with an increased risk of mortality from any of the diseases examined. The models in table 8 included 2 additional exposure indices as independent variables: duration of exposure and average intensity of exposure. No significant increase in mortality from any of the

4 causes of death was seen in relation to any of the 3 styrene exposure indices: duration, average intensity, or cumulative exposure.

Discussion

This report is based on data from a previous study of workers in the reinforced plastics and composites industry in the United States (6, 7). The analysis presented in the report was stimulated by the recent papers of Welp et al (3—5), which reported mortality from diseases of the nervous system, nonmalignant respiratory diseases, and nonmalignant genitourinary diseases, as well as some of the subcategories within these 3 broad categories, in a study of workers exposed to styrene in Europe. Most of these diseases have not been reported previously in the US cohort.

For the broad category of nonmalignant respiratory diseases, Welp and her colleagues (3) found a significant deficit for the IARC study overall (SMR 0.81, 95% CI 0.67—0.96). However, they (3) found a positive trend by average intensity for mortality from pneumonia ($P<0.01$) and a significantly elevated risk of 6.10 (95% CI 1.44—25.8) for an average intensity of >200 ppm.

In the US cohort, we found a nonsignificant increase in mortality from nonmalignant respiratory diseases (SMR 1.21, 95% CI 0.98—1.47) (table 2). The increase was due primarily to a significant increase in "other nonmalignant respiratory diseases" (SMR 1.40, 95% CI 1.04—1.84). When mortality from "other nonmalignant respiratory diseases" was examined by various indices of styrene exposure, it was found that the increase occurred among workers with a short length of exposure, low average exposure, or low cumulative exposure. As discussed in our previous reports (6, 7), the increase was probably not related to exposure to styrene, but more likely to the low socioeconomic class, smoking, or life-style factors characteristic of short-term workers. The observation that most of the deaths (70%) from "other nonmalignant respiratory diseases" were chronic obstructive pulmonary diseases tended to confirm the role of cigarette smoking.

For pneumonia, specifically, in the US cohort we did not find any relationship with any of the exposure indices. The pneumonia SMR values were 0.98 and 0.47 for workers with ≥10.0 years of exposure and ≥100.0 ppm-years of cumulative exposure, respectively. In terms of the average intensity of exposure, the SMR values were 1.16, 0.61, 0.95, 0.93, and 1.10 for <5, 5.0—9.9, 10.0—19.9, 20.0—59.9, and ≥60.0 ppm, respectively. None of the SMR values were significantly elevated, and there was no upward trend. Thus the pneumonia finding from the IARC study could not be confirmed in the US study.

Table 8. Analysis of selected causes of death based on the Cox proportional hazards model, with age, gender, cumulative exposure, duration of exposure, and average intensity of exposure to styrene as independent variables.

Cause of death (N)	β-coefficient	SD	P-value
Diseases of the nervous system (N=14)			
Age (years)	0.015	0.027	0.58
Cumulative exposure (ppm-years)	0.000	0.002	0.91
Duration of exposure (years)	-0.009	0.050	0.86
Average intensity of exposure (ppm)	-0.005	0.014	0.72
Nonmalignant genitourinary diseases (N=13)			
Age (years)	0.012	0.027	0.65
Gender	-0.195	0.662	0.77
Cumulative exposure (ppm-years)	0.000	0.003	0.98
Duration of exposure (years)	0.067	0.050	0.19
Average intensity of exposure (ppm)	-0.011	0.020	0.59
Nonmalignant respiratory diseases (N=97)			
Age (years)	0.088	0.009	0.00
Gender	-0.938	0.300	0.00
Cumulative exposure (ppm-years)	-0.001	0.001	0.66
Duration of exposure (years)	-0.037	0.021	0.08
Average intensity of exposure (ppm)	0.002	0.054	0.77
Pneumonia (N=23)			
Age (years)	0.083	0.018	0.00
Gender	-0.655	0.552	0.24
Cumulative exposure (ppm-years)	-0.005	0.004	0.18
Duration of exposure (years)	0.004	0.047	0.94
Average intensity of exposure (ppm)	0.013	0.010	0.18

Similarly, in the IARC study, Welp and her colleagues (4) reported that mortality from nonmalignant diseases of the genitourinary system increased with increasing average intensity of exposure. In the US cohort, there were only 13 deaths due to nonmalignant genitourinary diseases, slightly fewer than the 14.8 expected. When the data were examined by duration of exposure, cumulative exposure, or average intensity of exposure, no significantly elevated SMR values or upward trends were found. Therefore, the results of nonmalignant genitourinary diseases from the US cohort were not consistent with the findings reported by Welp et al (4) in the IARC study.

Of the 3 categories of diseases reported by Welp et al in 1996, it appears that these authors considered the finding of diseases of the nervous system much more definitive than the other 2 categories. They (5) stated that "Mortality from diseases of the central nervous system (27 deaths) increased with time since first exposure, duration of exposure, average level of exposure, and cumulative exposure to styrene [p 623]". However, the statistical tests conducted by Welp and her co-workers indicated that there was no significant upward trend for time since first exposure ($P_{trend}=0.32$) or average exposure ($P_{trend}=0.37$). Welp and her colleagues (5) also concluded that "Mortality from epilepsy increased monotonically with all styrene exposure indicators [p 623]". Again, the trend tests reported (5) indicated that there were no significant trends for average exposure

(P_{trend}=0.32) or cumulative exposure (P_{trend}=0.07). On the other hand, the trend between mortality from epilepsy and time since first exposure was highly significant (P_{trend}=0.008), due to an extremely high risk ratio of 485.9 (95% CI 1.19—9,999) based on only 1 observed death for the category "20 or more years after first exposure". Thus, except for epilepsy and time since first exposure, there was no significant upward trend in the exposure-response analyses in the IARC study.

In the US cohort there was no increased mortality from diseases of the nervous system in general or from epilepsy in particular. When mortality was analyzed in relation to various indices of exposure to styrene, no pattern was found. For US workers with more than 10 years of styrene exposure, the SMR for diseases of the nervous system was 1.03, and that for epilepsy was 0 (no epilepsy death occurred in the group). Similarly, in the US cohort, among persons with the highest cumulative exposure (≥100.0 ppm-years), the SMR for diseases of the nervous system was 0.53, and that for epilepsy was 0. Thus data from the US cohort did not support the findings on mortality from diseases of the nervous system in the IARC study reported by Welp et al (5).

Thus the results between the IARC study and the US cohort were considerably different for diseases of the nervous system. In fact, the results for diseases of the nervous system were considerably different across countries within the IARC study. An 1994 internal IARC report provided a detailed mortality analysis by country (8). Of the 40 deaths due to central nervous system diseases, 30 were from Denmark (N=13) or the United Kingdom (N=17), with the remaining 10 from Italy (N=3), Finland (N=1), Norway (N=3), and Sweden (N=3). Table 1—22 in part III of the 1994 IARC report (8) shows that the SMR for central nervous system diseases for Denmark was 1.10 (13/11.82) and that for the United Kingdom was 0.52 (17/32.57). The increase in Denmark could not have been explained by higher exposure since exposure levels in the United Kingdom were consistently higher than those in Denmark (figure 1 in reference 5). Furthermore, according to Welp et al (5), for central nervous system diseases "an inconsistently increasing risk by average exposure was observed in Denmark, while there was no increase in the other five countries [p 629]".

Mortality from central nervous system diseases or epilepsy has not been associated with chronic occupational exposures. On the other hand, persons with the following conditions are known to have a high risk of epilepsy: birth trauma (inadequate oxygen supply to the brain), perinatal infection, anoxia (postrespiratory or postcardiac arrest), infectious diseases (meningitis, encephalitis), inherited disorders or degenerative diseases (phenylketonuria or tuberous sclerosis), head injury or trauma, metabolic disorders (hyperglycemia or hypoparathyroidism), and cerebrovascular accident. Unfortunately, none of these potential confounding risk factors were available for analysis in the IARC study. Alcohol consumption is a known risk factor of central nervous system diseases. The Danish cohort consisted of a disproportionally higher percentage of short-term workers. In the IARC study, 20 of the 28 deaths due to cirrhosis of the liver occurred among subjects with less than 1 year of employment (2). Thus alcohol consumption could have been a potential confounding factor among the Danish workers.

In summary, mortality from nonmalignant genitourinary diseases, nonmalignant respiratory diseases, and diseases of the nervous system among 15 826 US workers exposed to styrene in the reinforced plastics and composites industry was examined in our investigation. We found no relationship between styrene exposure and any of these causes of death. The increased risks reported in the IARC study could not be replicated in our study. Apart from some of the reasons already discussed, there are at least 2 additional possible explanations for the observed discrepancies.

First, exposures among the US workers appeared to be lower than those among their European counterparts. More than 40% of the US workers were exposed to average levels below 10 ppm (table 1). Only 14% were exposed to average levels above 60 ppm. A great majority of the US workers were employed in the reinforced plastics and composites industry for a short period of time (70% for <5 years). Half of the US workers (50%) had cumulative exposures of less than 30 ppm-years. Only 24% of the US workers had cumulative exposures of more than 100 ppm-years. On the contrary, exposures in the IARC study appeared to be much higher. Although the number of workers were not reported, judging by the exposure categories used, it appeared that a considerable number of workers in the IARC study were exposed to average levels above 200 ppm (3, 4) and many accumulated more than 750 ppm-years (5). Thus exposure levels among the US workers might not have been high enough to produce the risks reported in the IARC study.

Second, the numbers of deaths in some of the analyses in the US study were small. For example, there were only 2 deaths each from epilepsy and nephritis. Even for the broader categories "diseases of the nervous system" and "nonmalignant genitourinary diseases," there were 14 and 13 deaths, respectively. The numbers were made even smaller in some of the subcohort analyses stratified by exposure category. The statistical power to detect a modest increase in risk was low in some of the analyses.

Therefore, in comparing the results between the US and the IARC studies, differences in exposure levels and statistical power between the 2 studies should be taken into consideration. Hopefully, future updates of the 2 studies and studies from other locations will provide further insights.

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SECTION III

ANNOTATED PAGES FROM THE DRAFT PROFILE DOCUMENT

ANNOTATED PAGES SUBMITTED BY

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What is styrene?

What is the source of this statement? Manufactured styrene is greater than 99.7% pure

Colorless liquid that evaporates easily	In its pure form, styrene has a sweet smell. Manufactured styrene often contains other chemicals that give it a sharp, unpleasant odor
Used in manufacturing and in consumer products	Large amount of styrene are produced in the United States. Small amounts are produced naturally by plants, bacteria, and fungi. Styrene is also present in combustion products such as cigarette smoke and automobile exhaust. Styrene is widely used to make plastics and rubber. Consumer products containing styrene include: • packaging materials • insulation for electrical uses (i.e., wiring and appliances) • insulation for homes and other buildings • fiberglass, plastic pipes, automobile parts • drinking cups and other "food-use" items • carpet backing These products mainly contain styrene linked together in long chains (polystyrene). However, most of these products also contain a small amount of unlinked styrene.

For more information on the physical and chemical properties of styrene, and its production, disposal, and use, see Chapters 4 and 5.

What happens to styrene when it enters the environment?

Most commonly found in air	Styrene can be found in air, soil, and water after release from the manufacture, use, and disposal of styrene-based products.
Rapidly broken down	*Air:* Styrene is quickly broken down in the air, usually within 1–2 days. *Water and soil:* Styrene evaporates from shallow soils and surface water. Styrene that remains in soil or water may be broken down by bacteria or other microorganisms.

For more information on styrene in the environment, see Chapter 6.

How might I be exposed to styrene?

Air	The primary way you can be exposed to styrene is by breathing air containing it. Releases of styrene into the air occur from: • industries using or manufacturing styrene • automobile exhaust • cigarette smoke • use of photocopiers Rural or suburban air generally contains lower concentrations of styrene than urban air. The range levels of styrene in air are: • 0.06–4.6 ppb in outdoor air • 0.023–11.5 ppb in indoor air
Water and soil	Styrene is rarely detected in groundwater, drinking water, or soil samples.
Workplace air	A large number of workers are potentially exposed to styrene. The highest potential exposure occurs in the reinforced-plastics industry, where workers may be exposed to high air concentrations and also have dermal exposure to liquid styrene or resins. Workers involved in styrene polymerization, rubber manufacturing, and styrene-polyester resin facilities and workers at photocopy centers may also be exposed to styrene.
Food	Low levels of styrene occur naturally in a variety of foods, such as fruits, vegetables, nuts, beverages, and meats. Small amounts of styrene can be transferred to food from polystyrene packaging material.

there are other packaging polymers that are not polystyrene
Probably better to say "styrene-based packaging"

For more information on human exposure to styrene, see Chapter 6.

How can styrene enter and leave my body?

Rapidly enters your body	When you breathe air containing styrene, most of the styrene will rapidly enter your body through your lungs. Styrene in food or water may also rapidly enter your body through the digestive tract. A very small amount may enter through your skin when you come into contact with liquids containing styrene.
Styrene is rapidly broken down to other chemicals	Once in your body, styrene is broken down into other chemicals. Most of these other chemicals leave your body in the urine within few days.

For more information on how styrene enters and leaves the body, see Chapter 3.

How can styrene affect my health?

Scientists use many tests to protect the public from harmful effects of toxic chemicals and to find ways for treating persons who have been harmed.

One way to learn whether a chemical will harm people is to determine how the body absorbs, uses, and releases the chemical. For some chemicals, animal testing may be necessary. Animal testing may also help identify health effects such as cancer or birth defects. Without laboratory animals, scientists would lose a basic method for getting information needed to make wise decisions that protect public health. Scientists have the responsibility to treat research animals with care and compassion. Scientists must comply with strict animal care guidelines because laws today protect the welfare of research animals.

Effects in workers exposed by inhalation	The most common health problems in workers exposed to styrene involve the nervous system. These health effects include changes in color vision, tiredness, feeling drunk, slowed reaction time, concentration problems, and balance problems. 1000 times The styrene concentrations that cause these effects are more than 100 times higher than the levels normally found in the environment.
Effects in laboratory animals exposed by inhalation	*Hearing:* Hearing loss has been observed in animals exposed to very high concentrations of styrene *Nose:* Animal studies have shown that inhalation of styrene can result in changes in the lining of the nose. However, animals may be more sensitive than humans to effects in the nose. *Liver:* Exposure to high levels of styrene can also damage the livers of mice, but this effect has not been seen in people, and mice may be more sensitive than humans.
Cancer	The International Agency for Research on Cancer has determined that styrene is possibly carcinogenic to humans.

Further information on the health effects of styrene in humans and animals can be found in Chapters 2 and 3.

Information about tests for detecting styrene in the body is given in Chapters 3 and 7.

What recommendations has the federal government made to protect human health?

The federal government develops regulations and recommendations to protect public health. Regulations *can* be enforced by law. The EPA, the Occupational Safety and Health Administration (OSHA), and the Food and Drug Administration (FDA) are some federal agencies that develop regulations for toxic substances. Recommendations provide valuable guidelines to protect public health, but *cannot* be enforced by law. The Agency for Toxic Substances and Disease Registry (ATSDR) and the National Institute for Occupational Safety and Health (NIOSH) are two federal organizations that develop recommendations for toxic substances.

Regulations and recommendations can be expressed as "not-to-exceed" levels, that is, levels of a toxic substance in air, water, soil, or food that do not exceed a critical value that is usually based on levels that affect animals; they are then adjusted to levels that will help protect humans. Sometimes these not-to-exceed levels differ among federal organizations because they used different exposure times (an 8-hour workday or a 24-hour day), different animal studies, or other factors.

Recommendations and regulations are also updated periodically as more information becomes available. For the most current information, check with the federal agency or organization that provides it.

Some regulations and recommendations for styrene include the following:

Levels in drinking water set by EPA	The EPA has determined that exposure to styrene in drinking water at concentrations of 20 ppm for 1 day or 2 ppm for 10 days is not expected to cause any adverse effects in a child. RfC is 1000 ug/m3 or 0.23 ppm The EPA has determined that lifetime exposure to 0.7 ppm styrene is not expected to cause any adverse effects.
Levels in bottled water set by FDA	The FDA has determined that the styrene concentration in bottled drinking water should not exceed 0.1 ppm.
Levels in workplace air set by OSHA	OSHA set a legal limit of 100 ppm styrene in air averaged over an 8-hour work day.

For more information on regulations and advisories, see Chapter 8.

and long-term exposures to styrene can result in neurological effects. Acute exposure data are limited to the finding of impaired performance on tests of vestibular function in test subjects exposed to 87–376 ppm for 1–3 hours (Odkvist et al. 1982; Stewart et al. 1968). A variety of neurological effects have been observed in chronically exposed styrene workers; these effects include ~~impaired color vision~~ decreased color discrimination (Campagna et al. 1995, 1996; Chia et al. 1994; Eguchi et al. 1995; Fallas et al. 1992; Gobba et al. 1991; not really "impaired" within normal range Gong et al. 2002; Kishi et al. 2001; Mutti et al. 1984a), vestibular effects (Calabrese et al. 1996; Moller et al. 1990), hearing impairment (Morata et al. 2002; Morioka et al. 1999; Muijser et al. 1988; Sliwinska-Kowalska et al. 2003), symptoms of neurotoxicity, particularly "feeling drunk" and tiredness (Checkoway et al. 1992; Cherry et al. 1980; Edling et al. 1993; Viaene et al. 1998, 2001), delays in reaction time (Cherry et al. 1980; Fallas et al. 1992; Gamberale et al. 1976; Jegaden et al. 1993; Mutti et al. 1984a; Tsai and Chen 1996), impaired performance on tests measuring attention and memory (Chia et al. 1994; Jegaden et al. 1993; Mutti et al. 1984a), impaired nerve conduction velocity (Behari et al. 1986; Gobba et al. 1995; Murata et al. 1991; Rosen et al. 1978; Štětkářová et al. 1993; Yuasa et al. 1996), and EEG alterations (Harkonen et al. 1984; Seppalainen and Harkonen 1976). The LOAELs for these effects range from about 10 ppm to 93 ppm. In most of the occupational exposure studies, neurological function tests were conducted in the morning before work, suggesting that the deficits were not acute effects. Results of a meta-analysis suggest that the severity of the some of the neurological symptoms increases with exposure duration (Benignus et al. 2005). See Comment For example, 8, 15, 25, and 35% increases in reaction time were observed in workers exposed to 100 ppm for 2, 4, 6, and 8 work-years, respectively. However, this may also be reflective of higher exposure levels in the past rather than an a duration-related increase in severity. The existing data are inadequate to determine whether chronic styrene exposure results in permanent damage. Mixed results have been found in studies examining workers before and after an extended period without styrene exposure. Animal studies have also reported neurological effects, although most of these studies have focused on effects on hearing and damage to the organ of Corti (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2000, 2001; Loquet et al. 1999, 2000; Makitie et al. 2002; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992)

Other effects that have been observed in animal studies include damage to the nasal olfactory epithelium and liver necrosis; testicular damage and developmental effects have also been reported, but the weight of evidence does not support concluding that these are sensitive targets. Damage to the nasal olfactory epithelium was observed in mice after 3 days of exposure (Cruzan et al. 2001). The severity of the lesion progressed from single cell necrosis to atrophy and respiratory metaplasia (Cruzan et al. 1997, 2001) with increasing exposure duration. The lowest-observed-adverse-effect levels (LOAELs) for these lesions are 80, 50, and 20 ppm for acute, intermediate, and chronic exposure, respectively. Rats do not appear to be

as sensitive as mice to the nasal olfactory epithelial damage; an intermediate-duration study identified a no-observed-adverse-effect level (NOAEL) and LOAEL of 500 and 1,000 ppm for focal hyperplasia and a chronic study identified a LOAEL of 50 ppm for atrophy and degeneration (Cruzan et al.1998). The observed species differences may be due to differences in styrene metabolism in the nasal cavity. In particular, rats have a higher capacity to detoxify styrene oxide with epoxide hydrolases and glutathione S-transferase. It is not likely that humans will be sensitive to the nasal toxicity of styrene because styrene oxide has not been detected and high levels of epoxide hydrolases have not been found in *in vitro* assays of human nasal tissue (Green et al. 2001a). → cyto chromes 2E1 and 2F1 have not been found.

Unlike the nasal lesions, the severity of hepatic lesions decreases with increased exposure durations. Severe hepatocellular necrosis was observed in mice exposed to 250 ppm for 3 days (Morgan et al. 1993b); however, continued exposure at this concentration resulted in focal necrosis and an increase in pigmented macrophages (Morgan et al. 1993a). Centrilobular aggregates of siderphages were observed in mice exposed to 200 ppm for 13 weeks (Cruzan et al. 1997); no liver effects were observed at 160 ppm after 2 years of exposure (Cruzan et al. 2001). Rats are less sensitive than mice to liver toxicity; no liver effects were observed in an intermediate-duration study in which rats were exposed to a styrene concentration 10-fold higher than the concentration eliciting hepatic effects in mice. No alterations in serum markers of liver damage were observed in styrene workers exposed to 40 ppm for approximately 5 years (Harkonen et al. 1984). Liver effects have not been observed in oral exposure studies; however, no studies examined systemic end points following acute exposure.

Occupational exposure studies have not found significant increases in the occurrence of stillbirth, infant death, malformations, or low birth weight (Ahlborg et al. 1987; Lemasters et al. 1989). Most single and multigeneration inhalation and oral exposure studies did not find significant alterations in fetus/pup survival, growth, or incidence of abnormalities in rats, mice, rabbits, and hamsters exposed to styrene (Beliles et al. 1985; Cruzan et al. 2005b; Daston et al. 1991; Kankaanpää et al. 1980; Murray et al. 1978). An increase in fetal deaths were observed in hamsters exposed to very high concentrations (1,000 ppm) (Kankaanpää et al. 1980) and in rats exposed to lower concentrations (Katakura et al. 1999, 2001). Two studies have examined neurodevelopmental effects in rats; one study found some minor effects (slight delays in some developmental landmarks) (Katakura et al. 1999, 2001). The other, higher-quality study did not find any significant alterations in a number of neurodevelopmental end points (Cruzan et al. 2005a). The National Toxicology Program (NTP) Expert Panel examining the developmental potential of styrene (NTP 2006) concluded that the human data are not sufficient to evaluate the potential

developmental toxicity of styrene in humans and that there was no convincing evidence of developmental toxicity in animals.

Although several epidemiology studies have examined potential reproductive effects in male and female styrene workers, adequate analysis of the data is limited by the lack of exposure information and concomitant exposure to other compounds. Mixed results have been found for increased occurrence of spontaneous abortions (Harkonen and Holmberg 1982; Hemminki et al. 1980, 1984; Lindbohm et al. 1985; McDonald et al. 1988) and oligomenorrhea (Cho et al. 2001; Lemasters et al. 1985). In male workers, sperm abnormalities have been reported (Kolstad et al. 1999a), but not alterations in time-to-pregnancy (Kolstad et al. 2000; Sallmén et al. 1998) or fertility rates (Kolstad et al. 1999c). No adverse reproductive effects were observed in inhalation (Cruzan et al. 2005b) and oral (Beliles et al. 1985) multigeneration studies in rats. A series of studies found decreases in spermatozoa counts in rats exposed as adults, as neonates, and through lactation (Srivastava et al. 1989, 1992a, 1992b). However, as noted by the NTP Expert Panel (NTP 2006), this finding is not consistent with the lack of reproductive effects found in the inhalation two-generation study (Cruzan et al. 2005b). The NOAEL identified in the two-generation inhalation study was 500 ppm (6 hours/day), which is roughly equivalent to 230 mg/day using a reference inhalation rate of 0.42 m^3/day. The LOAEL for spermatozoa effects in adult rats was 400 mg/kg (6 days/week), which is roughly equivalent to 158 mg/day using a reference body weight of 0.462 kg.

Note Cruzan et al 2005a,b examined spermatozoa and found no abnormalities in sperm. NOAEL = 500 ppm.

There are several epidemiologic studies of workers at styrene manufacturing and polymerization facilities and reinforced plastics facilities that suggest an association between occupational exposure and an increased incidence of cancer of the lymphatic and hematopoietic tissues in styrene (Hodgson and Jones 1985; Kogevinas et al. 1993, 1994; Kolstad et al. 1993, 1994; Nicholson et al. 1978; Ott et al. 1980). However, the reported studies are inconclusive due to exposure to multiple chemicals (including benzene) and the small size of the cohorts. Other studies have reported negative results (Bond et al. 1992; Coggon et al. 1987; Frentzel-Beyme et al. 1978; Matanoski and Schwartz 1987; Okun et al. 1985; Wong 1990). Ruder et al. 2004 More consistent results for increases in the risk of lymphatic and hematopoietic cancers have been observed among workers at styrene-butadiene manufacturing facilities (Delzell et al. 1996; Macaluso et al. 1996; Matanoski and Schwartz 1987; Matanoski et al. 1990; McMichael et al. 1976; Meinhardt et al. 1982; Sathiakumar et al. 2005). There is suggestive evidence that these increased risks may be due to exposure to 1,3-butadiene rather styrene exposure (Cheng et al. 2007; Delzell et al. 2001; Graff et al. 2005; Macaluso et al. 1996, 1993; Matanoski et al. 1997; Santos-Burgoa et al. 1992); however, it is difficult to separate the risks for styrene and 1,3-butadiene because the exposure is highly correlated.

because it helps the users of the profiles to identify levels of exposure at which major health effects start to appear. LOAELs or NOAELs should also help in determining whether or not the effects vary with dose and/or duration, and place into perspective the possible significance of these effects to human health.

The significance of the exposure levels shown in the Levels of Significant Exposure (LSE) tables and figures may differ depending on the user's perspective. Public health officials and others concerned with appropriate actions to take at hazardous waste sites may want information on levels of exposure associated with more subtle effects in humans or animals (LOAELs) or exposure levels below which no adverse effects (NOAELs) have been observed. Estimates of levels posing minimal risk to humans Minimal Risk Levels ~~or~~ (MRLs) may be of interest to health professionals and citizens alike.

A User's Guide has been provided at the end of this profile see Appendix B). This guide should aid in the interpretation of the tables and figures for Levels of Significant Exposure and the MRLs.

3.2.1 Inhalation Exposure

Most information on the effects of inhalation exposure to styrene in humans comes from studies of workers exposed to styrene vapors in the production and use of plastics and resins, especially ~~polystyrene~~ thermoset polyester resins dissolved in styrene. In most cases, the studies involve workplace exposures such as fiberglass boat building factories where the actual levels of styrene are reported as a range of styrene air concentrations. However, there are a few human clinical studies in which exposures are better quantified. Provided below are descriptions of the known effects of inhalation exposure of humans and animals to styrene.

3.2.1.1 Death

There have been no reports of deaths in humans directly associated with exposure to styrene in the workplace (EPA 1988b; Gosselin et al. 1984; NIOSH 1983).

In animals, inhalation studies indicate that the acute toxicity of styrene is low to moderate. An LC_{50} of 2,770 ppm after 2 hours of exposure was reported in rats, and the LC_{50} for mice after exposure for 4 hours was 4,940 ppm (Shugaev 1969). All rats and guinea pigs survived after exposure to 1,300 ppm styrene for 30 hours and 16 hours, respectively (Spencer et al. 1942). However, all animals died after 40 hours of exposure. Gender differences in mortality were observed in repeated-exposure studies (Cruzan et al.

steatosis, and congestion were observed in rats exposed to 300 ppm for 2 weeks (Vainio et al. 1979); the lack of incidence data limits the interpretation of these results.

Renal Effects. Based on the results of occupational exposure studies and animal toxicity studies, the kidney does not appear to be a sensitive target of styrene toxicity. Occupational exposure studies of workers exposed to 24 ppm (Viau et al. 1987), 53 ppm (Vyskocil et al. 1989), or 26 ppm styrene (Verplanke et al. 1998) did not find significant alterations in urinary levels of β-microglobulin (not examined in Verplanke et al. 1998 study), retinol-binding protein, or albumin. The Vyskocil et al. (1989) study also found no significant alterations in total protein, glucose, lysozyme, lactate dehydrogenase, or β-N-acetyl-D-glucosaminidase levels and Verplanke et al. (1998) did not find alterations in β-galactosidase, N-acetyl-β-D-glucosaminidase, or alanine aminopeptidase. No histological alterations were observed in the kidneys following acute exposure of rats to 300 ppm (Vaino et al. 1979) or mice to 500 ppm (Morgan et al. 1993a), intermediate exposure of rats to 133–1,500 ppm (Cruzan et al. 1997; Spencer et al. 1942; Viau et al. 1987), or chronic exposure of rats to 1,000 ppm (Cruzan et al. 1998) or mice to 160 ppm (Cruzan et al. 2001). Additionally, no alterations in urinary levels of N-acetyl-D-glucosaminidase, γ-glutamyl transpeptidase, protein, or urea were observed in rats exposed to 500 ppm for 4 weeks (Loquet et al. 2000).

Endocrine Effects. Several occupational studies have examined potential endocrine effects in reinforced plastics industry workers exposed to styrene. Significant increases in serum prolactin levels were observed in male and female workers (Bergamaschi et al. 1996, 1997; Luderer et al. 2004; Mutti et al. 1984b). The serum prolactin levels significantly correlated with urinary metabolite (mandelic acid plus phenylglyoxylic acid) levels (Mutti et al. 1984b) and blood styrene levels (Luderer et al. 2004). Based on a logistic regression model, Luderer et al. (2004) estimated that workers exposed to styrene exposures >20 ppm would be more likely to have elevated serum prolactin levels than workers exposed to lower levels; a 10-fold increase in blood styrene concentrations would result in a 2.06-fold increase in serum prolactin levels. Similarly, Arfini et al. (1987) found that female styrene workers had an abnormal response to an intravenous dose of thyrotropin-releasing hormone; the levels of serum prolactin were significantly higher following exposure to thyrotropin-releasing hormone, as compared to referents. Two of these workers were re-examined after a 3-month period without styrene exposure; the serum prolactin response following thyrotrophin-releasing hormone exposure was similar to that in the referent group. No significant alterations in the levels of thyroid stimulating hormone, follicle stimulating hormone, or luteinizing hormone were found in this study (Arfini et al. 1987). However, thyroid stimulating hormone

all these levels are within the normal human range.

In an international cohort of styrene workers, a significant association between mortality from central nervous system disease and cumulative styrene exposure was found (Welp et al. 1996c). The rate ratio was 3.29 (95% confidence interval [CI] of 0.48–22.65) for workers exposed to 25–49 ppm-years and 16.32 (95% CI 3.47–76.73) for those exposed for 200–349 ppm-years. A similar relationship was found for shorter durations of styrene exposure. The rate ratio was 2.33 (95% CI 0.40–13.56) for workers exposed for 6–11 months and 8.80 (95% CI 1.87–41.33) for workers exposed for 7–9 months. A significant association between mortality from epilepsy and duration of styrene exposure was found; the rate ratio in workers exposed for ≥10 years was 28.4 (95% CI 2.11–381.5). Time since first exposure was also significantly associated with mortality from epilepsy. Significant associations between mental disorders and duration of exposure and between suicide and duration of exposure were also found; however, for both of these causes of death, the rate ratio decreased with increasing duration of exposure and the investigators noted that lifestyle factors, rather than a direct effect of styrene, appear to be the most likely cause of the higher mortality. Note: no increase in US cohort (Wong et al, 1994)
Also death from epilepsy seldom recorded as cause of death.

A variety of neurological effects have been reported in workers chronically exposed to styrene including altered vestibular function, impaired hearing, ~~impaired color~~ decreased color discrimination vision, impaired performance on neurobehavioral tests, and increased in clinical symptoms. In general, these occupational exposure studies have several limitations. In most cases, the exposure levels reflect ~~current~~ exposure conditions at the time of testing and do not take into consideration past exposure to higher styrene levels that may have resulted in permanent damage. Some workers, particularly laminators, wore respiratory masks with or without canisters; many investigators estimated exposure based on biomarker levels, particularly urinary mandelic acid levels, while others did not. Additionally, significant differences between workers and referents were reported as LOAELs; however, the magnitude of the alteration may have been subclinical. A summary of the neurological effects observed in styrene workers is presented in Table 3-2.

most of these were not "impaired", only slightly different from controls.

Color vision appears to be one of the more sensitive targets of styrene toxicity, with many studies reporting alterations. Color vision was typically measured using the Lanthony desaturated panel D-15 test in which the subjects were asked to arrange 15 painted caps in a line with definite chromatic sequence; the color confusion index (CCI) quantifies the number of types of mistake. A significant correlation between CCI and urinary mandelic acid concentration (after correction for age) was observed in workers at fiberglass reinforced plastic facilities (Kishi et al. 2001). When workers were divided into three groups based on end-of-shift urinary mandelic acid levels, there were significant differences between CCI in workers with a mean a mandelic acid level of 0.14 or 0.65 g/L and age-matched referents;

no difference was found for the third group with a mean mandelic acid level of 0.05 g/L. The investigators estimated that these urinary mandelic acid levels were equivalent to styrene exposure levels of 4, 10, and 46 ppm. Thus, this study identifies a NOAEL of 4 ppm and a LOAEL of 10 ppm for impaired color vision. Similarly, Gong et al. (2002) found significantly higher CCI values in workers at a fiberglass reinforced plastic boat facility with end-of-shift urinary mandelic acid and phenylglyoxylic acid levels of ≥0.24 g/g creatinine or <0.24 g/g creatinine; a mandelic acid plus phenylglyoxylic acid urine level of 0.24 g/g creatinine is equivalent to a styrene exposure level of 10 ppm. A significant increase in CCI was also observed in workers at fiberglass reinforced plastic facilities exposed to a geometric man concentration of 16 ppm, as compared to age-matched controls (Gobba et al. 1991). In contrast to other studies, Gobba et al. (1991) did not find a significant relationship between end-of-shift urinary mandelic acid levels and CCI; however, urinary styrene levels correlated with CCI values. Significantly higher CCI values were observed in fiberglass reinforced workers with a mean urinary mandelic acid levels of 1.06 g/L, which is roughly equivalent to a styrene exposure level of 93 ppm (Eguchi et al. 1995). This study did not find significant alteration in workers with a mean urinary mandelic acid level of 0.02 g/L, equivalent to 8 ppm. Another study of fiberglass reinforced plastic workers (some of this cohort was examined by Gobba et al. 1991 and Campagna et al. 1995) found a significant association between CCI and styrene exposure levels (Campagna et al. 1996). The investigators concluded that color vision impairment could be detected at styrene levels of 4 ppm with a 95% upper confidence limit of 26 ppm. Two other occupational exposure studies using different measures of color vision impairment also found significant alterations. Chia et al. (1994) found significantly poorer color discrimination, after adjusting for age, education, and alcohol consumption, in 21 workers at a fiber-reinforced plastic boat manufacturing facility; the styrene exposure level of 6 ppm was estimated from a mean end-of-shift urinary mandelic acid level of 84.0 mg/g creatinine. No relationship between the total color difference score and the urinary mandelic acid level was found. In 60 workers in the shipbuilding industry with a mean styrene exposure level of 24.3 ppm, a significantly higher incidence of workers with errors in the blue-yellow or red-green ranges, compared to a referent group, was found (Fallas et al. 1992). Total error score was significantly different in workers, with a lifetime weighted average exposure level of 22 ppm styrene, as compared to workers in a low exposure group (9 ppm) (Iregren et al. 2005). Several studies found improvements in color vision following an extended period of no styrene exposure or lower exposure. Triebig et al. (2001) reported a significant improvement in CCI scores following a 4-week period with no styrene exposure; in contrast, no improvement in CCI scores was found in another group of styrene workers following a 1-month period without styrene exposure (Gobba et al. 1991). Two studies found significant improvements in color vision (age-adjusted color confusion score or CCI score) were observed in styrene workers following a decrease in styrene air level (Castillo et al. 2001; Triebig et

dizziness, and sensory symptoms in the upper and lower extremities were observed in workers with high exposure to styrene compared to those with low styrene exposure (Matikainen et al. 1992a); exposure levels were not reported. A significantly higher incidence of subjective symptoms (nausea, feeling of drunkenness, dizziness, and disturbance) was observed in styrene workers exposed to 4–164 ppm, as compared to controls (Geuskens et al. 1992). No significant difference in the incidence of symptoms related to cognitive motor disturbances (lack of concentration, understanding, trouble with movements) was found. Fiberglass boat manufacturing workers exposed to 92 ppm reported a higher prevalence of physical and mental tiredness at the end of the work shift than controls (Cherry et al. 1980). No alterations in the reporting of clinical symptoms were observed in plastic industry workers exposed to a mean concentration of 8.6 ppm (Edling et al. 1993).

A number of studies have examined styrene-induced damage to hearing and the vestibular system in chronically exposed workers. Several studies have reported significant associations between styrene exposure and hearing impairment; however, interpretation of the findings is limited by confounding exposure to noise or other solvents. Noise studies have found that exposure to >85 dB for over 10 years can result in a 10% hearing loss (Prince et al. 1997). Morioka et al. (1999) found an increased prevalence of workers with a urinary mandelic acid level of >0.3 g/L (approximately 16 ppm) with an upper frequency of hearing below the 75th percentile for normal. However, interpretation of the results is limited by confounding exposure to noise and exposure to other solvents, particularly toluene, which has been shown to be ototoxic. The noise levels ranged from 53.0 to 95.0 dBA with 14% of the measurements exceeding 85 dBA. Another study (Muijser et al. 1988) of styrene workers found a significant difference in hearing threshold only at 8 kHz not at other frequencies between indirectly exposed workers (mean styrene level of 14 ppm) and directly exposed workers (mean styrene level of 32 ppm); however, no differences were found in comparisons of indirectly and directly exposed workers with referent workers. The noise level for both groups of styrene workers was 80–85 dBA for most of the day. Śliwiński-Kowalska et al. (2003) found a significantly elevated risks of hearing loss among styrene workers exposed to a mean styrene concentration of 15.6 ppm (average noise level of 80.3 dBA). The odds ratio (adjusted for noise and gender) in workers only exposed to styrene was 5.2 (95% CI 2.9–8.9). The hearing losses were found within the range of 2–8 kHz. Morata et al. (2002) found significant decreases in hearing thresholds at 2, 3, 4, and 6 kHz in workers exposed to 0.05–22 ppm (mean of 4 ppm); no difference in the prevalence of high frequency hearing loss, as compared to referent workers, was found. The fairly wide range of exposure levels adds a great deal of uncertainty to estimating the LOAEL from this study; although the mean exposure is reported as the LOAEL in Figure 3-1, this value may be an overly conservative estimate of the true LOAEL. Other studies have not found significant alterations in hearing. Möller et al. (1990)

The highest NOAEL values and all reliable LOAEL values for neurological effects in each species and duration category are recorded in Table 3-1 and plotted in Figure 3-1.

3.2.1.5 Reproductive Effects

Information on the reproductive effects of styrene in humans is available from epidemiological studies of the reproductive outcomes of females employed in the various industrial operations in which styrene is used. However, exposures to styrene were not adequately quantified in any of the studies cited. In ~~one~~ a preliminary study, spontaneous abortions among 9,000 Finnish chemical workers from 1973 to 1976 were analyzed (Hemminki et al. 1980). The risk of spontaneous abortion expressed as number of abortions per 100 pregnancies) was significantly higher in women employed in styrene production compared to all women in Finland 15.0 vs. 5.5). However, this increase was not detected in a follow-up study of the same workers (Hemminki et al. 1984). An increase in the occurrence of spontaneous abortions was also observed in a study of 76 women involved in processing polystyrene plastics (McDonald et al. 1988); the ratio of observed to expected abortions was 1.58 (95% CI 1.02–2.35). The possible embryotoxic effects of styrene on 67 female lamination workers compared to 67 age-matched controls were evaluated in a second study (Harkonen and Holmberg 1982). The number of births was significantly lower among the workers exposed to styrene. This result was explained in part by a greater number of induced abortions in the styrene-exposed group. The number of spontaneous abortions was not elevated in the exposed women. No increased risk of spontaneous abortions among workers processing polymerized plastics or heated plastics made of vinyl chloride or styrene was reported (Lindbohm et al. 1985). The authors reported that the statistical power of the study was low due to the small study population. These studies are not conclusive since the workers were exposed to chemicals other than styrene in the workplace and the concentrations of styrene were not adequately reported. Two studies have examined the potential of styrene to induce menstrual disturbances. A significant increase in the incidence of oligomenorrhea was observed in petrochemical industry workers (estimated styrene exposure 1 ppm; only 3 women exposed only to styrene – none had oligomenorrhea.); the adjusted odds ratio was 1.65 (95% CI 1.05–2.55) (Cho et al. 2001). Although the odds ratio includes an adjustment for exposure to other aromatic chemicals, there was potential for exposure to other chemicals. In contrast, no significant alterations were observed in women working at reinforced plastics facilities with a mean styrene exposure level of 52 ppm for women directly exposed to styrene and 13 ppm for those indirectly exposed (Lemasters et al. 1985). Several studies have examined levels of prolactin, follicle stimulating hormone, and luteinizing hormone levels in female styrene workers; the results of these studies are discussed in Section 3.2.1.2, Endocrine Effects.

the esophagus (SMR 191.7; 95% CI 104.8–321.7), bronchus, trachea, or lung (SMR 140.6; 95% CI 119.3–164.0), cervix or uteri (SMR 283.5; 95% CI 135.9–521.3), and female genital organs (SMR 201.6; 95% CI 107.4–344.8) were observed. However, no relationships between styrene exposure (exposure level or duration of exposure) and deaths from these cancer types were found. No significant increases in the incidence of non-Hodgkin's lymphoma, Hodgkin's disease, multiple myeloma, leukemia, or all lymphohematopoietic malignancies were observed in workers at Danish reinforced plastics facilities in which 50–100% of the workers were involved in reinforced plastics production (Kolstad et al. 1993, 1994). However, when workers were divided by first year of employment, there was a significant increase in leukemia (standard incidence ratio [SIR] 1.69, 95% CI 1.09–2.49) among workers with a latency of ≥10 years and first year of employment of 1964–1970; when the data were analyzed by the length of employment, the incidence of leukemia was only significantly elevated among workers employed for <1 year. Significant increases in incidence were also observed for pancreatic cancer in workers with a high probability of styrene exposure (incidence rate ratio of 2.2; 95% CI 1.1–4.5) and urinary bladder cancer in workers with the highest probability of exposure and employed for >1 year (incidence rate ratio of 2.1; 95% CI 1.1–4.1) (Kolstad et al. 1995). No significant alterations in the incidence of leukemia, lymphoma, or other cancers were observed in styrene workers at eight British reinforced plastic manufacturing facilities (Coggon et al. 1987). In a large international cohort of workers employed in the reinforced plastics industry (this cohort included the British cohort examined by Coggon et al. 1987 and the Danish cohort examined by Kolstad et al. 1993, 1994, 1995), no significant alterations in no excess in mortality from all cancer or cancer of the lymphatic and hematopoietic tissues were observed (Kogevinas et al. 1993, 1994). However, significant increases in the incidence of lymphatic and hematopoietic neoplasms were observed in workers with a latency of at least 10 years (relative risk [RR] 2.90; 95% CI 1.29–6.48 in workers with a latency of 10–19 years and RR 3.97; 95% CI 1.30–12.13 for workers with a latency of ≥20 years) and in workers exposed to ≥100 ppm styrene (RR 3.11; 95% CI 1.07–9.06 for workers exposed to 100–119 ppm; RR 3.08; 95% CI 1.04–9.08 for workers exposed to 120–199 ppm; RR 3.59; 95% CI 0.98–13.14 for workers exposed to ≥200 ppm), as compared to unexposed workers. An increase in the number deaths from malignant lymphomas was also observed in workers exposed to 120–199 ppm styrene (RR 7.15; 95% CI 1.21–42.11). When analyzed by sub-cohort, this increase was primarily in the persons from the Kolstad study.

An increase in lymphatic leukemia (4 observed deaths versus 0.5 expected) in workers exposed to polymer extrusion fumes, solvents, and colorants, but was not found to be related to duration or level of exposure (Ott et al. 1980). In a follow-up to this study, which followed the workers for another 11 years (Bond et al. 1992), a nonsignificant increase in the number of deaths from lymphatic and heamtopoietic tissue cancers (SMR 144; 95% CI 95–208) was observed. Statistically significant increases in the number

these studies is the lack of exposure information, including levels of styrene and confounding exposure to other chemicals; thus, it is difficult to ascribe the increased cancer risks to styrene exposure. Loughlin et al. (1999) examined former students who attended a high school adjacent to synthetic styrene-butadiene rubber production facilities between 1963 and 1993 and found no significant alterations in deaths from lymphatic and hematopoietic cancer. Two studies have examined the possible association between styrene exposure and breast cancer. A case-control study by Cantor et al. (1995) found significant elevations in the risk of breast cancer among women possibly exposed to styrene in the workplace. Coyle et al. (2005) found a significant higher incidence of age-adjusted breast cancer rate in men and women, women, and women ≥50 years of age and living in counties with EPA toxics release inventory (TRI) facilities with on-site releases of styrene. As with the other population-based studies, these studies did not monitor styrene levels or exposure to other potentially carcinogenic chemicals and thus provided limited information on the carcinogenic potential of styrene. Burns et al (2006) commented that ~~the~~ breast cancer rate in Texas is lower than US average despite having highest styrene releases + breast cancer has not been associated with styrene in cohort studies with hundreds fold higher exposure.

The carcinogenicity of styrene has been examined in three studies in rats (Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1982) and one study in mice (Cruzan et al. 2001). No significant increases in the incidence neoplastic lesions were observed in rats exposed to styrene concentrations as high as 1,000 ppm 6 hours/day, 5 days/week for 2 years (Cruzan et al. 1998). Similarly, exposure of female rats to 600 or 1,000 ppm styrene 6 hours/day, 5 days/week for 21 months did not result in styrene-related increases in the incidence neoplastic tumors (Jersey et al. 1978); a high incidence of chronic murine pneumonia in the control and 1,000 ppm male rats precludes the use of the male data for assessing the carcinogenic potential of styrene. A non-concentration-related increase in the incidence of malignant mammary tumors were observed in female rats exposed to 100, 200, or 300 ppm styrene 4 hours/day, 5 days/week for 52 weeks (Conti et al. 1988); the incidences were 6/60, 6/30, 4/30, 9/30, 12/30, and 9/30 in the 0, 25, 50, 100, 200, and 300 ppm groups, respectively. No other significant increases in specific tumors were observed in this study (Conti et al. 1988; Maltoni et al. 1982). The findings of the Conti et al. (1988) study conflict with those of Cruzan et al. (1998), who found a concentration-related decrease in mammary tumors in female rats exposed to similar or higher styrene concentrations for a longer duration. In contrast to the results in rat studies, significant increases in the incidence of bronchioalveolar carcinoma were observed in female mice exposed to 160 ppm 6 hours/day, 5 days/week for approximately 2 years (Cruzan et al. 2001). Significant trends for increasing incidences of bronchioalveolar adenoma were also observed for the male and female mice.

As reviewed by IARC (2002) and Cruzan et al. (2002), toxicokinetic differences in the metabolism of styrene in the lungs have been observed in humans, rats, and mice. In rats and mice, Clara cells have the

doses of 150 or 300 mg/kg/day for 78 weeks showed increased mortality; however, the female mice did not.

The highest reliable LOAEL values and LD_{50}s values in each species and duration category are recorded in Table 3-2 and plotted in Figure 3-2.

3.2.2.2 Systemic Effects

No studies were located regarding endocrine, metabolic, musculoskeletal, or dermal/ocular effects in humans or animals after oral exposure to styrene.

For the following systemic effects resulting from oral exposure to styrene, the highest NOAEL values and all reliable LOAEL values for each species and duration category are recorded in Table 3-3 and plotted in Figure 3-2.

Respiratory Effects. No studies were located regarding respiratory effects in humans after oral exposure to styrene.

Severe lung congestion was observed in mice that were the offspring of dams given a single oral dose of styrene at 1,350 mg/kg on the 17th day of gestation and that continued to receive the same dose once per week after weaning (Ponomarkov and Tomatis 1978). The lung congestion was noted following continuous administration of the styrene for 16 weeks. No respiratory effects were observed in rats exposed to 35 mg/kg/day styrene in drinking water for 105 weeks (Beliles et al. 1985).

Cardiovascular Effects. No studies were located regarding cardiovascular effects in humans following oral exposure to styrene.

No cardiovascular effects were observed in rats chronically exposed to 35 mg/kg/day in drinking water (Beliles et al. 1985).

Gastrointestinal Effects. Abdominal pain was reported by 11% of the residents of two apartment buildings exposed to high levels of styrene in drinking water for 3 days (Arnedo-Peno et al. 2003). The concentration of styrene in the water was 900 μg/L and the dose was estimated to be 0.026 mg/kg/day.

Based on other symptoms and the higher prevalence of symptoms among residents living near the contaminated water tank, it is likely that some of the observed effects were due to inhalation exposure. Air concentration would have been very low from 900ug/l Styrene in water.

No gastrointestinal effects were observed in rats chronically exposed to 35 mg/kg/day styrene in drinking water (Beliles et al. 1985).

Hematological Effects. No studies were located regarding hematological effects in humans after oral exposure to styrene.

Intra-erythrocytic Heinz bodies were regularly detected in a dose-related manner in male and female dogs chronically exposed to 400 or 600 mg/kg/day groups and sporadically in females in the 200 mg/kg/day group (Quast et al. 1979). There were occasional decreased red blood cell counts, hemoglobin levels, and erythrocyte sedimentation rates in males and females in the 600 mg/kg/day groups. Increased hemosiderin deposits and intranuclear inclusions in liver were noted in animals dosed with 600 mg/kg/day. This was probably secondary to the effects on the red blood cells. The formation of intra-erythrocytic Heinz bodies was readily reversible upon discontinuing the administration of styrene in the 600 mg/kg/day group after 470 days of exposure. No hematological effects were observed in rats chronically exposed to 35 mg/kg/day (Beliles et al. 1985).

Hepatic Effects. No studies were located regarding hepatic effects in humans after oral exposure to styrene.

Some animal studies have reported hepatic effects; however, the inconsistency of the findings and poor reporting of the data preclude drawing conclusion on the hepatotoxicity of orally administered styrene. Small areas of focal necrosis was observed in the livers of rats administered 400 mg/kg styrene in groundnut oil 6 days/week for 100 days (Srivastava et al. 1982). Because the incidence or statistical analysis data were not reported, it is not possible to determine whether 400 mg/kg is an adverse effect level. This study also found alteration in mitochondrial and microsomal enzymes at 200 and 400 mg/kg; the significance of these alterations in the absence of histological damage is not known. An increase in liver weights was observed in rats administered 400 or 677 mg/kg via gavage 5 days/week for 6 months (Wolf et al. 1956); no histological alterations were observed. Although the alterations in the liver weight were considered slight at 400 mg/kg and moderate at 677 mg/kg, the magnitude of the change and statistical significance is not known; slight and moderate alterations in body weight were also observed at these dose levels. Hepatic glutathione content was reduced in rats orally administered 900 mg/kg styrene

for 7 consecutive days (Das et al. 1981); the toxicological significance of this effect is not known. As noted above, increased numbers of hemosiderin deposits and intranuclear crystalline inclusions were reported in the hepatocytes of dogs orally administered 600 mg/kg/day of styrene by gavage for 316 days (Quast et al. 1979). This was presumably secondary to Heinz body formation, and no other hepatic histological effects were in this study. No hepatic effects were observed in rats exposed to 35 mg/kg/day styrene in drinking water for 105 weeks (Beliles et al. 1985) or in rats administered 500 mg/kg styrene 1 day/week for 120 weeks (Ponomarkov and Tomatis 1978).

Renal Effects. No studies were located regarding renal effects in humans after oral exposure to styrene.

A decrease in renal glutathione content and decreased glutathione-S-transferase activity was noted in rats orally administered 900 mg/kg styrene for 7 days (Das et al. 1983). Growth depression and slightly increased kidney weight were reported in female rats administered 400 and 667 mg/kg, 5 days/week for 6 months (Wolf et al. 1956); the magnitude and statistical significance of the effect were not reported. Histopathological examination of kidney tissue showed no abnormalities; thus, the changes in organ weight were not considered adverse. In another study, female rats and mice were exposed to 1,350 mg/kg/day of styrene on the 17th day of gestation; the offspring were also administered styrene, by gavage, 1 day/week for 120 weeks. No statistically significant increases in the incidence of kidney lesions were observed in rats exposed to 500 mg/kg ~~day/week~~ (Ponomarkov and Tomatis 1978). No histological alterations were observed in the kidneys of rats chronically exposed to 35 mg/kg/day styrene in drinking water (Beliles et al. 1985).

3.2.2.3 Immunological and Lymphoreticular Effects

No studies were located regarding immunological effects in humans after oral exposure to styrene.

The World Health Organization (WHO 1983) reviewed a Russian study (Sinitskij 1969) in which styrene was fed to 36 rabbits at doses of 250 mg/kg for 58 days, 5 mg/kg for 216 days, and 0.5 mg/kg for 202 days. Impairment of the immunological defense system was indicated by a nearly total suppression of leukocyte phagocytic activity. Although no statistical analysis was provided, the data showed a dose-response relationship for both the severity of the effect and the time of onset. Similarly, impaired host resistance was observed in mice exposed to 30 mg/kg/day and infected with encephalomyocarditis or a

rodent strain of malaria and rats exposed to 294 mg/kg/day and infected with a rodent hookworm parasite (Dogra et al. 1992).

3.2.2.4 Neurological Effects

No studies were located regarding neurological effects in humans following oral exposure to styrene.

Neurobehavioral effects and alterations in neurochemicals have been observed in animal studies. Significant learning impairment was observed in an operant behavioral test in rats administered 500 mg/kg styrene in corn oil, 5 days/week for 8 weeks (Bushnell 1994). A reversal of the effect was not observed 1 year after exposure termination. Another study found significantly increased mean percent avoidance response, indicative of impaired learning, in rats administered 100 or 200 mg/kg/day styrene for 14 days (Husain et al. 1985). No alterations in foot shock-induced aggressive behavior or amphetamine-induced motor activity were observed in young rats administered via gavage 250 mg/kg/day styrene for 15 days (Khanna et al. 1994). However, significant alterations were observed in similarly exposed rats maintained on a low protein diet (8% casein versus 20% in normal diet).

Significant increases in serotonin levels in the hypothalamus, hippocampus, and midbrain were observed in rats administered 200 mg/kg/day for 14 days (Husain et al. 1985); no alterations in dopamine or noradrenaline levels were observed. Exposure to a higher dose (906 mg/kg/day) for 15 days resulted in increases in serotonin and noradrenalin in brain tissue (Husain et al. 1980). Neither study found significant alterations in brain dopamine levels. Another study found a significant increase in dopamine receptor binding, as assessed using labeled spiroperidol binding, in rats administered 200 or 400 mg/kg/day for 1 day or 90 days (Agrawal et al. 1982).

The highest NOAEL and LOAEL values for neurological effects in each species and duration category are recorded in Table 3-3 and plotted in Figure 3-2.

3.2.2.5 Reproductive Effects

No studies were located regarding reproductive effects in humans after oral exposure to styrene.

Marked degeneration in the seminiferous tubules and decreased spermatozoa were observed in rats administered 400 mg/kg styrene via gavage 6 days/week for 60 days (Srivastava et al. 1989). No adverse reproductive effects were observed in a three-generation reproduction study in which rats were exposed to

This may have been secondary to liver degeneration.

3.2.2.7 Cancer

No studies were located regarding cancer effects in humans after oral exposure to styrene.

Investigations of the carcinogenic potential of styrene in animals after oral exposure have yielded variable results. No significant alterations in the incidence of neoplastic tumors were observed in rats exposed to gavage doses as high as 250 mg/kg 4–5 days/week for 52 weeks (Conti et al. 1988; Maltoni et al. 1982) or 2,000 mg/kg 5 days/week for 78–103 weeks (NCI 1979b) or in rats exposed to 35 mg/kg/day styrene in drinking water for 2 years (Beliles et al. 1985). In contrast, significant increases in the incidence of lung tumors were observed in mice receiving gavage doses of 300 mg/kg 5 days/week for 78–103 weeks (NCI 1979b). The incidences of bronchiolo-alveolar carcinoma in male mice were 0/20, 3/44, and 5/43 in mice exposed to 0, 150, or 300 mg/kg, respectively, and the respective combined incidences of bronchiolo-alveolar carcinoma and adenoma in male mice were 0/20, 6/44, and 9/43. The incidence of bronchiolo-alveolar carcinoma in the 300 mg/kg group was similar to the incidence in untreated historical controls (12%), but lower than the incidence in historical vehicle controls 0/40; however, the National Cancer Institute (NCI 1979b) noted that the incidence in historical vehicle controls is based on too small a number of animals for meaningful use of historical control data. Two studies conducted by Ponomarkov and Tomatis (1978) examined the carcinogenicity of styrene following gestation and postnatal exposure. In mice, offspring exposed on gestation day 17 for 16 weeks starting after weaning (1 day/week) and observed for 100 weeks, a significant increase in lung tumors was observed ~~at 1,350 mg/kg~~; this dose was ~~also associated with treatment-related toxicity and mortality~~. In the second study, the mice were exposed on gestation day 17 ~~followed by exposure~~ for 120 weeks (1 day/week) beginning at weaning. No significant alterations in tumor incidence were observed.

3.2.3 Dermal Exposure

No studies were located regarding health effects in humans after dermal exposure to styrene.

3.2.3.1 Death

No studies were located regarding lethality in humans or animals after dermal exposure to styrene.

3.2.3.2 Systemic Effects

No studies were located regarding respiratory, cardiovascular, gastrointestinal, hematological, musculoskeletal, hepatic, endocrine, or renal effects in humans or animals after dermal exposure to styrene.

Dermal Effects. Marked irritation with denaturation of the skin was noted when styrene was applied in small amounts over a 4-week period to the shaved abdomen of rabbits at 20,000 mg/kg total dose) (Spencer et al. 1942).

Ocular Effects. Moderate conjunctival irritation and transient corneal injury of the eyes were observed when undiluted styrene was tested in rabbit eyes (Wolf et al. 1956). The effects were produced immediately (within 3 minutes) by a single administration of two drops (about 0.1 mL) and persisted throughout the 7-day observation period.

No studies were located regarding the following health effects in humans or animals after dermal exposure to styrene:

3.2.3.3 Immunological and Lymphoreticular Effects

3.2.3.4 Neurological Effects

3.2.3.5 Reproductive Effects

3.2.3.6 Developmental Effects

3.2.3.7 Cancer

3.3 GENOTOXICITY

The genotoxicity of styrene has been examined in numerous *in vivo* studies of workers and laboratory animals; these data are summarized in Table 3-4. Chromosomal damage, DNA strand breaks, and mutagenic effects have frequently been studied in workers exposed to styrene in the production of reinforced plastic products and styrene/polystyrene production. In general, these studies are limited by the fact that workers in these industries are often exposed to chemicals other than styrene, such as methylene chloride and epoxide resins, and many studies did not control for potential confounding factors such as age, sex, and smoking status. Chromosomal aberrations have been reported in numerous studies of workers exposed to styrene for 1– 25 years in reinforced plastic operations (Anwar and Shamy 1995; Artuso et al. 1995; Hogstedt et al. 1979; Mäki-Paakkanen et al. 1991; Meretoja et al. 1977, 1978;

3.4 TOXICOKINETICS

3.4.1 Absorption

3.4.1.1 Inhalation Exposure

The uptake of styrene following inhalation exposure in humans and animals is rapid (Ramsey and Andersen 1984; Ramsey and Young 1978; Ramsey et al. 1980; Withey and Collins 1979; Withey and Karpinski 1985). Pulmonary retention of inhaled styrene in humans is approximately 2/3 of the administered concentrations (Engstrom et al. 1978a, 1978b). For example, male human subjects were exposed to styrene in inspired air during 30-minute rest and three 30-minute work periods on a bicycle ergometer. The mean uptake was approximately 63% (range was 59–70%) of the amount of inspired styrene. In exercising volunteers exposed to 50 ppm styrene for 2 hours, an average of 66.5% of the inhaled styrene was absorbed (Johanson et al. 2000). Another study in volunteers exposed to 50 ppm styrene for 2 hours during exercise calculated that 64% of the styrene was absorbed (Norstöm et al. 1992). Exposures of rats to styrene concentrations of 50–2,000 ppm for 5 hours yielded blood uptakes that showed a continued and increasing rapid absorption, proportional to the styrene air level (Withey and Collins 1979). Plateau levels of styrene in rats' blood were reached within 6–8 hours during exposures ranging from 80 to 1,200 ppm styrene for up to 24 hours (Ramsey and Young 1978). Physiologically-based inhalation pharmacokinetic models indicate that styrene metabolism becomes saturated at inhaled levels above 200 ppm in mice, rats, and humans (Ramsey and Andersen 1984). When inhaled concentrations are below 200 ppm, the ratio of styrene concentration in the blood to inhaled air is moderated by perfusion-limited metabolism rather than blood:air partition coefficients.

Morris

3.4.1.2 Oral Exposure

No studies were located regarding absorption in humans after oral exposure to styrene.

The absorption of styrene from the gastrointestinal tract was rapid and complete in rats deprived of food overnight and given styrene by gavage at a total dose of 3.147 mg styrene in 10 mL aqueous solution. A peak blood level of 6 μg/mL was reached in a few minutes. There was a much slower uptake of the styrene administered in vegetable oil (Withey 1976). Styrene administered in vegetable oil at a total dose of 32.61 mg produced a peak level of 12 μg/mL. This was reached at about 100 minutes (Withey 1976).

An oral dose of 20 mg/kg of ^{14}C styrene was administered to male and female rats (Plotnick and Weigel 1979). Tissue levels peaked at 4 hours or earlier after dosing. Less than 10% of the administered dose was found in the stomach, small intestine, and large intestine 8 hours after dosing. The kidney had the highest concentration of radioactivity at all time intervals, with decreasing amounts in the liver and pancreas. Fat tissue showed increased levels after 2 hours. All tissue levels were below 1 μg/g at 24 hours and at 48 and 72 hours were below the limit of detection. Excretion data from the Plotnick and Weigel (1979) study are presented in Section 3.4.4.2.

3.4.2.3 Dermal Exposure

No studies were located regarding distribution in humans after dermal exposure to styrene.

Immersion of rats' tails in pure liquid styrene for 1 hour resulted in styrene levels in the liver and brain that were estimated to be between 50 and 70% of the concentrations found in the same organs after 4-hour inhalation exposure to a vapor concentration of 11.8 g/m^3 (Shugaev 1969). A skin:air partition coefficient of 91.9 was calculated using rat skin (Mattie et al. 1994).

the metabolism of styrene is organ + species specific. General statement refers to liver.

outdated - 1 groups last 15 years of research.

3.4.3 Metabolism

~~See Sumner et al 2001~~ Cruzan et al. 2002.

There have been numerous studies, conducted primarily via inhalation, that address the metabolism of styrene in humans and animals (Drummond et al. 1989; Engstrom et al. 1976; Korn et al. 1984, 1987; Leibman 1975; Lof et al. 1983; Withey and Collins 1979; Young et al. 1979). The proposed pathways of styrene metabolism are shown in Figure 3-3. Styrene is metabolized by the microsomal NADPH-cytochrome P-450 dependent mono-oxygenase to styrene oxide. The styrene oxide is then hydrated to phenylethylene glycol (styrene glycol). This transformation is catalyzed by microsomal epoxide hydratase. The styrene glycol is then metabolized directly to MA or to benzoic acid and then hippuric acid. Mandelic acid is also metabolized to PGA. The MA, hippuric acid, and PGA are excreted in the urine. In another pathway, styrene oxide is metabolized by cystolic glutathione-S-transferase to mercapturic acids appearing in the urine as hydroxyphenylethyl mercapturic acid. A minor metabolic pathway of styrene in rats involves the formation of 1- and 2-phenylethanol and ring hydroxlation to form vinyl phenol as urinary metabolites. The presence of 4-vinylphenol has been reported in the urine of workers exposed to styrene, but this may have been due to the contamination of the styrene to which the subjects were exposed (Pfaffli et al. 1981). The urinary metabolites that predominate in humans are MA and PGA. In rats, the predominant urinary metabolites are MA, PGA, hippuric acid, and glucuronide. Metabolic conversion to styrene-7,8-epoxide (styrene oxide) by the microsomal mixed function oxidase

and epoxide hydratase from the liver and spleen of several rodent species has been demonstrated (Belvedere and Tursi 1981; Cantoni et al. 1978; Leibman 1975; Lof et al. 1984; Vainio et al. 1979). However, styrene oxide has only been found at low concentration, close to detection levels (0.02 μmol/L), in the blood of workers exposed to styrene (Lof et al. 1986a; Mendrala et al. 1991) investigated the species differences in the *in vitro* hepatic metabolism of styrene. The results indicated that mice had the greatest capacity to produce styrene oxide (highest styrene epoxidase activity), followed by rats and then humans. In addition, humans may have the highest capacity to metabolize styrene oxide to styrene glycol, since the human form of styrene oxide hydratase had the highest affinity (lowest Km) for styrene oxide. Assuming that styrene oxide is the metabolite responsible for styrene-induced toxicity (see below), the results of this study indicate that care must be taken in extrapolation of data from animal studies to humans for risk assessment.

Need section on nasal metabolism and lung metabolism – See Cruzan et al. 2002.

3.4.4 Elimination and Excretion

3.4.4.1 Inhalation Exposure

Several studies have demonstrated that styrene is almost totally excreted as urinary metabolites in humans, and at higher doses, the elimination profile indicates saturation of metabolic excretion or processes (Ramsey and Young 1978; Ramsey et al. 1980). Most of the inhaled styrene is excreted in urine as MA and PGA. In a study of the excretion of styrene and its metabolites resulting from a 100-ppm/8-hour inhalation exposure, 2.6% of the total uptake was excreted as unchanged styrene in exhaled air (Guillemin and Berode 1988). The metabolites MA, PGA, and hippuric acid were excreted in the urine at 56.9, 33, and 7.5% of the absorbed dose, respectively. In exercising volunteers exposed to 50 ppm styrene for 2 hours, 0.7–2.2% of the retained dose was exhaled as unchanged styrene (Johanson et al. 2000). Peak levels of styrene in the urine were measured immediately after exposure termination, whereas urinary excretion of MA and PGA peaked at 2 hours after exposure termination. MA excretion accounted for 6–29% of the estimated retained dose and PGA excretion accounted for 4–6%; the halftime excretion rates of MA and PGA were 2.2–4.2 and 3.5–13.9 hours, respectively. Phenylaceturic acid and hippuric acid was also detected in the urine samples collected 2 hours after exposure termination. At this time point, MA account for 73% of the total excreted metabolites, PGA 18%, phenylaceturic acid 4.5%, and hippuric acid 5.7%. In styrene workers exposed to 29–42 ppm styrene, both R-mandelic acid and S-mandelic acid were detected in the urine (Hallier et al. 1995). The ratio of R- to S-mandelic acid ranged from 0.7 to 1.2 in 19 of the 20 workers; in the last worker, the ratio was 2.2.

2,000 ppm styrene by inhalation for 5 hours exhibited a dose dependent biphasic pattern of elimination (Withey and Collins 1979).

3.4.4.2 Oral Exposure

No studies were located regarding excretion in humans after oral exposure to styrene.

Excretion of styrene was studied in the same rats for which there was good distribution data (Plotnick and Weigel 1979). Styrene was rapidly excreted in the urine with 90% of the dose detected in the urine within 24 hours of administration. Less than 2% of the dose was found in the feces. Detectable tissue levels were not found 48 and 72 hours after administration.

3.4.4.3 Dermal Exposure

In a study of the absorption of liquid styrene applied to the forearms of male volunteers, about 13% of the absorbed dose was excreted as MA (Dutkiewicz and Tyras 1968).

No studies were located regarding excretion in animals after dermal exposure to styrene.

3.4.5 Physiologically Based Pharmacokinetic PBPK)/Pharmacodynamic PD) Models

Physiologically based pharmacokinetic (PBPK) models use mathematical descriptions of the uptake and disposition of chemical substances to quantitatively describe the relationships among critical biological processes Krishnan et al. 1994). PBPK models are also called biologically based tissue dosimetry models. PBPK models are increasingly used in risk assessments, primarily to predict the concentration of potentially toxic moieties of a chemical that will be delivered to any given target tissue following various combinations of route, dose level, and test species Clewell and Andersen 1985). Physiologically based pharmacodynamic PBPD) models use mathematical descriptions of the dose-response function to quantitatively describe the relationship between target tissue dose and toxic end points.

PBPK/PD models refine our understanding of complex quantitative dose behaviors by helping to delineate and characterize the relationships between: 1) the external/exposure concentration and target tissue dose of the toxic moiety, and 2) the target tissue dose and observed responses (Andersen and Krishnan 1994; Andersen et al. 1987a). These models are biologically and mechanistically

based and can be used to extrapolate the pharmacokinetic behavior of chemical substances from high to low dose, from route to route, between species, and between subpopulations within a species. The biological basis of PBPK models results in more meaningful extrapolations than those generated with the more conventional use of uncertainty factors.

The PBPK model for a chemical substance is developed in four interconnected steps: 1) model representation, 2) model parameterization, 3) model simulation, and 4) model validation Krishnan and Andersen 1994). In the early 1990s, validated PBPK models were developed for a number of toxicologically important chemical substances, both volatile and nonvolatile Krishnan and Andersen 1994; Leung 1993). PBPK models for a particular substance require estimates of the chemical substance-specific physicochemical parameters, and species-specific physiological and biological parameters. The numerical estimates of these model parameters are incorporated within a set of differential and algebraic equations that describe the pharmacokinetic processes. Solving these differential and algebraic equations provides the predictions of tissue dose. Computers then provide process simulations based on these solutions.

The structure and mathematical expressions used in PBPK models significantly simplify the true complexities of biological systems. If the uptake and disposition of the chemical substances) are adequately described, however, this simplification is desirable because data are often unavailable for many biological processes. A simplified scheme reduces the magnitude of cumulative uncertainty. The adequacy of the model is, therefore, of great importance, and model validation is essential to the use of PBPK models in risk assessment.

PBPK models improve the pharmacokinetic extrapolations used in risk assessments that identify the maximal i.e., the safe) levels for human exposure to chemical substances Andersen and Krishnan 1994). PBPK models provide a scientifically sound means to predict the target tissue dose of chemicals in humans who are exposed to environmental levels for example, levels that might occur at hazardous waste sites) based on the results of studies where doses were higher or were administered in different species. Figure 3-4 shows a conceptualized representation of a PBPK model.

If PBPK models for styrene exist, the overall results and individual models are discussed in this section in terms of their use in risk assessment, tissue dosimetry, and dose, route, and species extrapolations.

Sarangapani et al 2002 most complete

Several investigators have developed toxicokinetic models for styrene (Csanády et al. 1994, 2003; Jonsson and Johanson 2002; Leavens and Bond 1996). The Csanády et al. (1994, 2003) model is useful for evaluating the carcinogenic risk associated with inhalation exposure to styrene. As discussed in Section 3.2.1.7, species differences exist in the metabolism of styrene in the lungs of rats, mice, and humans; these differences result in increased sensitivity of mice. Jonsson and Johanson (2002) developed a population-based PBPK model for styrene, which decreased the intraindividual variability for estimating the metabolic capacity for styrene in humans. Leavens and Bond (1996) described initial work on developing a model for co-exposure to 1,3-butadiene and styrene in mice. Some of these models provide strong support for the observed differences in styrene toxicity between rats, mice, and humans. As discussed further in Section 3.5.3, some have primarily focused on the species differences in the metabolism of styrene and metabolic differences between rats, mice, and humans

3.5 MECHANISMS OF ACTION

3.5.1 Pharmacokinetic Mechanisms

Styrene is rapidly absorbed through the respiratory tract (Ramsey and Andersen 1984; Ramsey and Young 1978; Ramsey et al. 1980; Withey and Collins 1979; Withey and Karpinski 1985) with a mean uptake of approximately 60–70% in humans (Johanson et al. 2000; Norström et al. 1992). A concentration-dependent uptake efficiency was found in the upper respiratory tract of rats and mice (Morris 2000). In rats, the uptake efficiency was 23.7% at 5 ppm and 10.1% at 200 ppm; in mice, uptake efficiency decreased from 41.7% at 5 ppm to 9.6% at 200 ppm. Based on the decreased uptake efficiency observed in rats and mice following exposure to the cytochrome P450 inhibitor, metyrapone, Morris (2000) suggested that styrene was metabolized *in situ* and this metabolism enhanced styrene uptake. In humans, blood styrene levels reached steady state after 75 minutes of exposure to 70 ppm (Wigaeus et al. 1983). The elimination of styrene from blood was biphasic, with a half-time of 1 minute for the rapid distribution phase and 40.8 minutes for the elimination phase. Styrene is rapidly distributed throughout the body with the highest concentrations found in adipose tissue. In rats, the styrene concentration in the adipose tissue was approximately 50-fold higher than in muscle; the biological half-time was 6.3 hours in adipose tissue and 2.4–2.0 hours in the blood, liver, kidney, spleen, muscle, and brain (Teramoto and Horiguchi 1979). Wigaeus et al. (1983) estimated a human adipose tissue:blood partition coefficient of 74. In humans, styrene is primarily excreted in the urine as mandelic acid and phenyoxyglylic acid. The half-times of mandelic acid and phenylglyoxylic acid in the urine were 3.6 and 8.8 hours, respectively, in humans exposed to 70 ppm for 2 hours (Wigaeus et al. 1983); another study reported

elimination half-times of 2.2–4.2 hours for mandelic acid and 3.5–13.9 hours for phenylglyoxylic acid following a 2-hour exposure to 50 ppm styrene (Johanson et al. 2000).

3.5.2 Mechanisms of Toxicity

— This is quite incomplete - some comments in Section 3.5.3 should be included here. See separate comments.

A large number of studies have investigated the mechanism of styrene carcinogenic activity, particularly the increased susceptibility of mice. Increases in lung tumors have been observed in mice exposed to 160 ppm 6 hours/day, 5 days/week for approximately 2 years (Cruzan et al. 2001) and following gavage exposure to 300 mg/kg/day administered 5 days/week (NCI 1979b); however, neoplastic tumors have not been observed in rats exposed to concentrations as high as 1,000 ppm 6 hours/day, 5 days/week for 2 years (Cruzan et al. 1998) or 2,000 mg/kg/day 5 days/week for 2 years (NCI 1979b). As reviewed by IARC (2002), Cohen et al. (2002), and Cruzan et al. (2002), genotoxic and nongenotoxic modes of action have been proposed. Although styrene itself does not appear to be DNA reactive, styrene 7,8-oxide is DNA reactive and has been shown to form stable N_2 and O_6 adducts of deoxyguanosine. Styrene oxide, DNA adducts, and genotoxic effects have been detected in humans, rats, and mice. Styrene (styrene 7,8-oxide is the likely causative agent) has been shown to be mutagenic in bacteria, and exposure can result in increased frequency of sister chromatid exchange, chromosomal aberrations, micronucleated cells, and DNA strand breaks. However, elevated levels of blood styrene oxide do not explain the species differences in tumor formation. In humans, styrene 7,8-oxide is rapidly hydrolyzed by epoxide hydrolase as evidenced by the high levels of mandelic acid, phenylglyoxylic acid, and hippuric acid detected in the urine. Styrene 7,8-oxide is relatively stable in rats and mice, and elevated levels have been detected in blood. The blood levels of styrene oxide in rats exposed to 1,000 ppm is 100-fold higher than the levels in mouse exposed to 20–40 ppm; however, tumors have not been detected in rats.

SO is presumed to be, but evidence does not support.

Although the nongenotoxic potential mode of action also implicates styrene 7,8-oxide as the causative agent, it involves cytotoxic damage at the target tissue, the lung. In the lung, the cytotoxic effects of repeated exposure to styrene 7,8-oxide results in bronchiolar epithelial hyperplasia, which eventually results in the formation of neoplastic tumors. In the mouse lung, styrene is primarily metabolized by cytochrome P450, particularly the CYP2F2 isoform, in the Clara cell. Humans appear to have a lower capacity to metabolize styrene in the lung compared to rats and a much lower capacity compared to mice. Mouse Clara cells metabolize higher levels of styrene than rat Clara cells and produce a higher ratio of R-enantiomer styrene oxide-to S entantiomer styrene oxide, as compared to rats. It has been estimated that mice produce 15 times more R-enantiomers styrene oxide than rats. This is particularly important since R-styrene oxide is a more potent pneumotoxicant than S-styrene oxide. In mice and rats, a portion

of the styrene oxide generated is metabolized via glutathione conjugation. Mice appear to be more susceptible to glutathione depletion than rats, and glutathione depletion has been observed in mouse lung tissue at exposure concentrations of 80–300 ppm.

IARC (2002) concluded that the proposed mechanism involving the metabolism of styrene to styrene 7,8-oxide is the mouse Clara cell is the likely mode of action resulting in lung tumors in mice. This mode of action is not likely to be relevant to humans to a biologically significant extent. However, this mechanism and the genotoxic mode of action has not been excluded for humans, and styrene is considered a possible human carcinogen.

3.5.3 Animal-to-Human Extrapolations

R vs S-SO

Species differences in exist in the metabolism of styrene in humans, rats, and mice. In all three species, styrene in predominantly metabolized to styrene 7,8-oxide. Species differences in the subsequent metabolism of styrene 7,8-oxide exist. In humans, styrene 7,8-oxide is primarily hydrolyzed to mandelic acid via epoxide hydrolase. Conjugation with glutathione also appears to be an important pathway in rats and mice. In rats, 68–72% of the styrene metabolites in urine are generated from the epoxide hydrolase pathway and 26–35% are from the glutathione transferase pathway; in mice, 48–59 and 20–35% arise from the epoxide hydrolase and glutathione transferase pathways, respectively (Cruzan et al. 2002). In contrast, 95–100% of the styrene 7,8-oxide is metabolized via the epoxide hydrolase pathway; only trace amounts of mercapturic acids (from the glutathione transferase pathway) have been detected in human urine. The difference in metabolism could result in significant increases in styrene 7,8-oxide levels in the body following exposure to high levels of styrene which may result in depletion of gluthatione. Additionally, a small percentage of styrene can under ring oxidation resulting in the formation of 4-vinylphenol. Ring-opened compounds account for 4–8% of the urinary metabolites in mice, less than 1% in rats, and were not detected in humans. The production of 4-vinylphenol is potentially significant mode of action because it is considered to be more toxic to the liver and lung than styrene or styrene oxide (Cruzan et al. 2005b). (this sentence should be included on P62)

As discussed in above, differences in the metabolism of styrene between humans, rats, and mice have resulted in toxicity differences. Species differences in toxicity have been detected in the nasal epithelium, lungs, and liver. In the respiratory tract, the species differences between rats and mice are due to local metabolize of styrene to R-styrene oxide. The higher rate of metabolism in mice and higher production of the more reactive enantiomer likely results in increased susceptibility. The fact that humans have a more

and/or ring oxidized metabolites (Cruzan et al, 2002, 2005)

No *in vitro* studies were located regarding endocrine disruption of styrene.

There is some evidence in styrene workers and in female rats that inhalation exposure to styrene may disrupt the tuberoinfundibular dopaminergic system. Significant alterations in serum prolactin levels have been observed in male and female workers exposed to air concentrations as low as 50 ppm (Bergamaschi et al. 1996, 1997; Luderer et al. 2004; Mutti et al. 1984b); a regression model predicts that exposure to ≥20 ppm would result in significant elevations in serum prolactin levels (Luderer et al. 2004). Elevated serum prolactin levels have also been observed in female rats acutely exposed to 150 ppm (Umemura et al. 2005); alterations have not been observed in male rats exposed to concentrations as high as 1,500 ppm (Jarry et al. 2002; Umemura et al. 2005). As noted by NTP (2006), the clinical significance of the increased serum prolactin levels, in the absence of other reproductive effects, is not known. Styrene exposure does not appear to adversely affect thyroid stimulating hormone levels in humans (Arfini et al. 1987) or rats (Umemura et al. 2005) or follicle stimulating hormone or luteinizing hormone in humans (Arfini et al. 1987).

All these values are within the normal range for prolactin in humans.

No significant alterations were observed in a gonadal sex differentiation assay using genetic male frogs exposed to styrene (Ohtani et al. 2001).

3.7 CHILDREN'S SUSCEPTIBILITY

This section discusses potential health effects from exposures during the period from conception to maturity at 18 years of age in humans, when all biological systems will have fully developed. Potential effects on offspring resulting from exposures of parental germ cells are considered, as well as any indirect effects on the fetus and neonate resulting from maternal exposure during gestation and lactation. Relevant animal and *in vitro* models are also discussed.

Children are not small adults. They differ from adults in their exposures and may differ in their susceptibility to hazardous chemicals. Children's unique physiology and behavior can influence the extent of their exposure. Exposures of children are discussed in Section 6.6, Exposures of Children.

Children sometimes differ from adults in their susceptibility to hazardous chemicals, but whether there is a difference depends on the chemical (Guzelian et al. 1992; NRC 1993). Children may be more or less susceptible than adults to health effects, and the relationship may change with

developmental age (Guzelian et al. 1992; NRC 1993). Vulnerability often depends on developmental stage. There are critical periods of structural and functional development during both prenatal and postnatal life, and a particular structure or function will be most sensitive to disruption during its critical periods). Damage may not be evident until a later stage of development. There are often differences in pharmacokinetics and metabolism between children and adults. For example, absorption may be different in neonates because of the immaturity of their gastrointestinal tract and their larger skin surface area in proportion to body weight (Morselli et al. 1980; NRC 1993); the gastrointestinal absorption of lead is greatest in infants and young children (Ziegler et al. 1978). Distribution of xenobiotics may be different; for example, infants have a larger proportion of their bodies as extracellular water, and their brains and livers are proportionately larger (Altman and Dittmer 1974; Fomon 1966; Fomon et al. 1982; Owen and Brozek 1966; Widdowson and Dickerson 1964). The infant also has an immature blood-brain barrier Adinolfi 1985; Johanson 1980) and probably an immature blood-testis barrier (Setchell and Waites 1975). Many xenobiotic metabolizing enzymes have distinctive developmental patterns. At various stages of growth and development, levels of particular enzymes may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (Komori et al. 1990; Leeder and Kearns 1997; NRC 1993; Vieira et al. 1996). Whether differences in xenobiotic metabolism make the child more or less susceptible also depends on whether the relevant enzymes are involved in activation of the parent compound to its toxic form or in detoxification. There may also be differences in excretion, particularly in newborns who all have a low glomerular filtration rate and have not developed efficient tubular secretion and resorption capacities (Altman and Dittmer 1974; NRC 1993; West et al. 1948). Children and adults may differ in their capacity to repair damage from chemical insults. Children also have a longer remaining lifetime in which to express damage from chemicals; this potential is particularly relevant to cancer.

Certain characteristics of the developing human may increase exposure or susceptibility, whereas others may decrease susceptibility to the same chemical. For example, although infants breathe more air per kilogram of body weight than adults breathe, this difference might be somewhat counterbalanced by their alveoli being less developed, which results in a disproportionately smaller surface area for alveolar absorption (NRC 1993).

No studies were identified that examined the toxicity of styrene in children or young laboratory animals. Several occupational exposure studies have examined the developmental toxicity of styrene; these studies did not find statistically significant alterations in the occurrence of stillbirths, infant deaths,

malformations, or birth weight (Ahlborg et al. 1987; Härkönen et al. 1984; Lemasters et al. 1989). However, an expert panel (NTP 2006) evaluating these data concluded that the human studies were not sufficient to evaluate developmental toxicity due to the low statistical power of the studies and the lack of adequate information on exposure.

In general, animal studies have not found styrene-related developmental effects following inhalation exposure in rats (Murray et al. 1978), mice (Kankaanpää et al. 1980), rabbits (Murray et al. 1978), or hamsters (Kankaanpää et al. 1980) or oral exposure in rats (Daston et al. 1991; Murray et al. 1978); additionally, no developmental effects were observed in a rat two-generation study (Cruzan et al. 2005b). An expert panel determined that there was sufficient animal data to conclude that styrene does not cause developmental toxicity in rats following inhalation or oral exposure or in rabbits following inhalation exposure.

Studies in adults, particularly reinforced plastics industry workers, have identified the nervous system as the most sensitive target of styrene toxicity. Inconsistent results have been found in animal neurodevelopmental toxicity studies. Minor alterations in forelimb grip strength and performance in the swimming maze test were observed in F2 offspring of rats exposed to 500 ppm styrene; however, the investigators (Cruzan et al. 2005a) attributed these alterations to a lower body weight rather than a neurodevelopmental effect of styrene. Another inhalation study found impaired righting reflex in the offspring of rats exposed to 300 ppm during gestation (Katakura et al. 2001). Similarly, impaired amphetamine-induced locomotor activity and apomorphine-induced stereotypy were observed in the offspring of rats orally administered 200 mg/kg/day styrene during gestation and lactation (Zaidi et al. 1985). there was no difference in maze performance - only in straight-line swimming on day 22.

No human or animal data were located on the toxicokinetic properties of styrene in children or immature animals or possible age-related differences in the toxicokinetics of styrene. A lactational toxicokinetic model predicted that styrene can by transferred via maternal milk (Fisher et al. 1997). Subsequent sections of this chapter (Sections 3.8, 3.10, and 3.11) discuss the available information on biomarkers, interactions, and methods for reducing toxic effects. The available information is from adults and mature animals; no child-specific information was identified. It is likely that this information will also be applicable to children.

studies have examined the toxicity of styrene following exposure to an acute oral dose. Abdominal discomfort was observed in residents exposed to ~~high~~ low levels of styrene in drinking water (Arnedo-Pena et al. 2003); concomitant inhalation exposure to styrene limits the utilization of this study for MRL derivation. A study in rats identified a LOAEL for neurotoxicity (Husain et al. 1985) and another rat study examined potential developmental effects, but found no adverse effects (Daston et al. 1991). The animal data are not considered sufficient to derive an oral acute-duration MRL. Thus, additional single-dose oral and inhalation studies are needed to better define toxicity thresholds. However, the potential carcinogenicity of styrene prevents the design of controlled laboratory exposures in humans. Dermal exposure to styrene at significant levels is unlikely except in the case of workplace spills and dermal absorption is probably low based on limited human studies. However, the almost complete lack of dermal toxicity data in animals and humans creates a degree of uncertainty on this issue. Therefore, single-dose dermal studies would be useful in determining target organs and thresholds for dermal exposure. In designing these types of studies, precautions should be taken to avoid concomitant inhalation exposure.

Intermediate-Duration Exposure. Intermediate-duration inhalation exposure studies in humans is limited to a study examining potential reproductive effects in workers (Lindbolm et al. 1985); exposure information was not provided. Inhalation studies in animals have reported damage to the nasal olfactory epithelium in rats (Cruzan et al. 1997, 2005a, 2005b; Ohashi et al. 1986) and mice (Cruzan et al. 1997, 2001), liver damage in mice (Cruzan et al. 1997), eye irritation in rats (Cruzan et al. 1997) and guinea pigs (Spencer et al. 1942), ototoxicity in rats (Campo et al. 2001; Lataye et al. 2000; Loquet et al. 1999, 2000; Makitie et al. 2002; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992), and impaired nerve conduction velocity (Yamamoto et al. 1997). A two-generation study in rats did not find reproductive, developmental, or neurodevelopmental effects (Cruzan et al. 2005a, 2005b), but another study did find neurodevelopmental effects (Katakura et al. 1999, 2001). However, additional studies are needed, as the data are not considered sufficient to derive an intermediate-duration inhalation MRL. Oral exposure studies of intermediate-duration are limited. Animal studies indicate that respiratory, hepatic, neurological, and neurodevelopmental end points need further evaluation. There are limited animal data and no human data on the toxicity of styrene following intermediate-duration oral exposure. Studies by Srivastava et al. (1982), Ponmarkov and Tomatis (1978), and Wolf et al. (1956) have examined a limited number of systemic end points. Other studies have identified adverse effect levels for neurological effects (Agrawal et al. 1982; Bushnell 1994; Husain et al. 1980), impaired development of the reproductive system (Srivastava et al. 1992a, 1992b), or neurodevelopmental effects (Zaidi et al. 1985); no reproductive effects were observed in a three-generation reproduction study (Beliles et al. 1985). The

current information is not sufficient to develop an oral MRL. One study examined the dermal toxicity of styrene in rabbits (Spencer et al. 1942); basic information on the adverse effects of intermediate-duration dermal exposure to styrene in animals is also needed due to the sparsity of available data.

Chronic-Duration Exposure and Cancer. A large number of occupational exposure studies have examined the chronic toxicity of styrene. Systemic toxicity studies have examined endocrine (Bergamaschi et al. 1997; Mutti et al. 1984b), hematological (Checkoway and Williams 1982; Thiess and Friedheim 1978), hepatic (Hotz et al. 1980; Lorimer et al. 1978), or renal (Verplanke et al. 1998; Viau et al. 1987; Vyskocil et al. 1989) end points; most studies relied on biomarkers of toxicity. The most widely examined end point is neurotoxicity and the available data suggest that this is the most sensitive end point. Examined neurological end points included color vision (Campagna et al. 1995, 1996; Chia et al. 1994; Eguchi et al. 1995; Fallas et al. 1992; Gobba et al. 1991; Gong et al. 2002; Kishi et al. 2001; Mutti et al. 1984a), vestibular effects (Calabrese et al. 1996; Moller et al. 1990), hearing impairment (Morata et al. 2002; Morioka et al. 1999; Muijser et al. 1988; Sliwinska-Kowalska et al. 2003), symptoms of neurotoxicity (Checkoway et al. 1992; Cherry et al. 1980; Edling et al. 1993; Viaene et al. 1998, 2001), performance on neurobehavioral tests (Cherry et al. 1980; Edling et al. 1993; Gamberale et al. 1976; Jegaden et al. 1993; Lindstrom et al. 1976; Mutti et al. 1984a; Tsai and Chen 1996; Viaene et al. 1998, 2001), nerve conduction velocity (Behari et al. 1986; Murata et al. 1991; Rosen et al. 1978), olfactory alterations (Dalton et al. 2003), and EEG alterations (Harkonen et al. 1984; Seppalainen and Harkonen 1976). Other human studies have examined reproductive (Harkonen and Holmberg 1982; Hemminki et al. 1980) and developmental (Ahlborg et al. 1987; Lemasters et al. 1989) end points. The chronic toxicity of styrene has also been examined in rat (Cruzan et al. 1998; Jersey et al. 1978) and mouse (Cruzan et al. 2001) studies. The occupational exposure studies were considered adequate for derivation of a chronic-duration inhalation MRL for styrene. Further research to define the dose-response curve more fully and to identify a chronic inhalation NOAEL for neurological effects would be valuable and would help to reduce uncertainty in the MRL. Data on chronic oral exposure to styrene is only available through animal studies (Beliles et al. 1985; Conti et al. 1988; NCI 1979b; Quast et al. 1979). In these studies, the most sensitive indicator of toxicity appears to be Heinz body formation in red blood cells in dogs only. (Quast et al. 1979) and the EPA has calculated a chronic oral RfD based on this study (IRIS 2007). However, there is some doubt regarding the chronic oral NOAEL, and whether hematological effects are really more sensitive than neurological effects. Moreover, decreased survival has been noted in rats at exposure levels only slightly higher than the no-effect level for hematological effects (Conti et al. 1988). Therefore, no chronic oral MRL has been derived. Further studies on the effects of oral exposure, with special emphasis on neurological or neurobehavioral effects, would be valuable. Although chronic dermal

note that NCI, 1979 NOAEL for survival was 1000 mg/kg/day

exposure by the general public is not likely, there may be some potential for dermal contact with soil at hazardous waste sites. Therefore, data on long-term effects of dermal contact with styrene would be useful.

Taken together, the animal and human data indicate that styrene may possibly be a weak human carcinogen. Although data from epidemiological studies are limited due to concurrent chemical exposures and small cohorts, the data are suggestive of some carcinogenic potential in humans (Antilla et al. 1998; Bond et al. 1992; Cheng et al. 2007; Coggon et al. 1987; Delzell et al. 1996, 2001; Frentzel-Beyme et al. 1978; Gerin et al. 1998; Graff et al. 2005; Hodgson and Jones 1985; Kogevinas et al. 1993, 1994; Kolstad et al. 1993, 1994, 1995; Macaluso et al. 1996; Matanoski and Schwartz 1987; Matanoski et al. 1990; McMichael et al. 1976; Meinhardt et al. 1982; Nicholson et al. 1978; Okun et al. 1985; Ott et al. 1980; Sathiakumar et al. 2005; Wong 1990; Wong et al. 1994). Inhalation and oral exposure studies in rats have not found significant increases in the incidence of neoplastic tumors (Beliles et al. 1985; Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1992; NCI 1979b). However, inhalation and oral studies in mice have found significant increases in the incidence of neoplastic lung tumors (Cruzan et al. 2001; NCI 1979b). The available data suggest that toxicokinetic differences between rats, mice, and humans result in an increased sensitivity of mice. Clarification of the data is needed in several areas. Almost all of the available epidemiological studies involve concurrent exposures to other chemicals. The role of the metabolism of styrene ~~to styrene oxide~~ in humans and animals needs to be clarified ~~and the carcinogenic mechanisms~~ needed to be further elucidated. Additional studies that account for these issues would be valuable. Much of published data not included

Genotoxicity. The results of genotoxicity tests for styrene both *in vivo* and *in vitro* are frequently conflicting, and the genotoxic potential of styrene is not clear (Andersson et al. 1980; Beliles et al. 1985; Hogstedt et al. 1979; Meretoja et al. 1977, 1978; Watanabe et al. 1981). The reasons for the mixed or conflicting genotoxicity results may be differences in the metabolism or detoxification of styrene in the various test systems employed. The role of the metabolite styrene oxide in genotoxicity assays on styrene should be fully evaluated, preferably in mammalian *in vivo* systems. Toxicokinetic studies evaluating the presence, level, and activity of styrene oxide in humans will influence the interpretation of genotoxicity studies on styrene and their relevance to public health.

Reproductive Toxicity. Occupational exposure studies have examined male and female styrene workers to evaluate potential reproductive effects; however, most of these studies did not quantify styrene exposure or exposure to other compounds, thus, interpretation of results is difficult. Inconsistent results

have been reported for spontaneous abortions with some studies reporting significant increases (Harkonen and Holmberg 1982; Hemminki et al. 1980; McDonald et al. 1988) and others reporting no effect (Harkonen and Holmberg 1982; Hemminki et al. 1980, 1984; Lindbohm et al. 1985). Oligomenorhhea was observed in one study of workers (Cho et al. 2001), but not in another study (Lemasters et al. 1985). Studies in male workers have found alterations in sperm parameters (Kolstad et al. 1999a), but no alterations in time-to-pregnancy (Kolstad et al. 2000; Sallmén et al. 1998) or fertility rates (Kolstad et al. 1999c). A two-generation inhalation study (Cruzan et al. 2005b) and three-generation oral study (Beliles et al. 1985) in rats showed no styrene-related reproductive effects. However, testicular effects have been observed in an oral exposure study (Srivastava et al. 1989), but not in ~~an~~ two inhalation study i.e. (Salomaa et al. 1985) (Cruzan et al 2005). Additional reproductive data on occupationally-exposed males would be useful in evaluating the existing animal data that indicates altered testicular function and studies in females would be useful in evaluating the inconsistent findings in the existing studies.

Developmental Toxicity. Data on the developmental effects of inhalation exposure to styrene are available in humans and animals. Occupational exposure studies (Ahlborg et al. 1987; Härkönen et al. 1984; Lemasters et al. 1989) have not found increases in the occurrence of birth defects or birth weight. However, interpretation of the results are complicated by exposure to other chemicals and lack of information on exposure levels. Additional occupational studies are needed to adequately assess this end point. Developmental studies in animals via inhalation (Cruzan et al. 2005b; Kankaapää et al. 1980; Murray et al. 1978) or oral (Beliles et al. 1985) exposure have not found effects on fetal outcome, birth weight, or incidence of abnormalities. However, several studies have reported neurodevelopmental (Katakura et al. 1999, 2001; Zaidi et al. 1985) or reproductive (Srivastava et al. 1992a, 1992b) effects. Additional studies are needed to examine the potential effects on the nervous and reproductive systems of developing organisms. No studies examined the developmental toxicity of styrene following dermal exposure.

Immunotoxicity. Occupational exposure studies have found alterations in lymphocyte subsets (Bergamaschi et al. 1995b; Biró et al. 2002), which may be indicative of reduced cell-mediated immunity and an impaired immune response to concanavalin (Tulinska et al. 2000); another study found no alterations in immunoglobulin levels (Chmielewski et al. 1977). Limited data in animals indicate that inhalation (Ban et al. 2006) and oral (Sinitskij 1969) exposure can also result in impaired immune response. No dermal exposure studies examining immunotoxicity were identified. Human and animal studies provide suggestive evidence that the immune system is a target; additional studies would be useful to further investigate the effect of styrene on immune function.

How is "impaired" defined – color discrimination in normal range, leaving questionable

Neurotoxicity. The neurotoxicity of styrene in workers in the reinforced plastic industry has been extensively examined (Behari et al. 1986; Calabrese et al. 1996; Campagna et al. 1995, 1996; Castillo et al. 2001; Checkoway et al. 1992; Cherry et al. 1980; Chia et al. 1994; Dalton et al. 2003; Edling et al. 1993; Eguchi et al. 1995; Fallas et al. 1992; Fung and Clark 1999; Gamberale et al. 1976; Gobba et al. 1991, 1995; Gong et al. 2002; Harkonen et al. 1984; Iregren et al. 2005; Jegaden et al. 1993; Kishi et al. 2001; Lindstrom et al. 1976; Matikainen et al. 1992a; Moller et al. 1990; Morata et al. 2002; Morioka et al. 1999; Muijser et al. 1988; Murata et al. 1991; Mutti et al. 1984a; Niklasson et al. 1993; Rosen et al. 1978; Seppalainen and Harkonen 1976; Sliwinska-Kowalska et al. 2003; Štětkářová et al. 1993; Triebig et al. 1985, 2001; Tsai and Chen 1996; Viaene et al. 1998, 2001; Yuasa et al. 1996). A variety of neurological effects have been observed in these studies including ~~impaired~~ color vision, slowed reaction time, impaired performance on neurobehavioral tests of memory and learning, altered vestibular function, impaired hearing, reduced nerve conduction velocity, and increased clinical symptoms such as dizziness, tiredness, memory loss, and feeling drunk. Additionally, several experimental studies have examined the effects of acute exposure on vestibular function (Odvist et al. 1982; Stewart et al. 1968), clinical symptoms (Seeber et al. 2004; Stewart et al. 1968), and performance on neurobehavioral tests (Seeber et al. 2004). Animal studies have primarily focused on the damage to the organ of Corti and hearing loss (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003; Loquet et al. 1999, 2000; Makitie et al. 2002; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992), although nerve conduction velocity has also been examined (Kulig et al. 1988; Yamamoto et al. 1997). The potential for neurotoxicity has not been examined in humans orally exposed to styrene and a limited number of end points have been examined in animals (Agrawal et al. 1982; Bushnell 1994; Husain et al. 1980, 1985; Khanna et al. 1994). The neurological effects observed in styrene workers were used as the basis of a chronic-duration inhalation MRL. Since this is based on a LOAEL, further studies which define the chronic NOAEL, as well as acute- and intermediate-duration NOAELs, would be valuable especially at levels of styrene causing problems with coordination and psychological function. These and other neurological effects may play a role in the rate of workplace accidents and the level of performance. Additional studies in mammalian animal models are needed to determine if styrene causes chronic damage to the central and/or peripheral nervous systems and to determine the associated mechanism of toxicity. Also, information is needed to determine if neurotoxicity is a sensitive end point from exposure to styrene via the oral route.

Epidemiological and Human Dosimetry Studies. Numerous studies have examined the toxicity of styrene in workers, as discussed in other sections, most of these studies have focused on neurotoxicity and potential carcinogenicity of styrene. A common limitation of these studies is the poor

characterization of exposure levels and possible exposure to other chemicals. Some studies provided no data on styrene exposure levels and other studies provide current exposure levels with limited or no data on past exposure levels. Occupational exposure and experimental studies also provide suggestive evidence of acute upper respiratory tract irritation and eye irritation (Carpenter et al. 1944; NIOSH 1983) Stewart et al. 1968) and possible endocrine effects (elevated levels of serum prolactin) (Arfini et al. 1987; Bergamaschi et al. 1996, 1997; Luderer et al. 2004; Mutti et al. 1984b); additional studies are needed to confirm the results of these studies and to establish dose-response relationships. Additionally, there are suggestive findings that styrene has the potential to induce reproductive effects (Cho et al. 2001; Harkonen and Holmberg 1982; Hemminki et al. 1980; Kolstad et al. 1999c; McDonald et al. 1988); however, poor characterization of styrene exposure and the low statistical power of the studies limit the usefulness of the studies; studies of males and female styrene workers that examined a variety of reproductive end points and adequately characterized exposure would be useful..

Follow up studies by these investigators determined there were no styrene-effects. CHO is very questionable - styrene exposures are less than 1 ppm.

Biomarkers of Exposure and Effect.

Exposure. Available studies indicate that there are good quantitative relationships between styrene metabolites (MA and PGA) in the urine and styrene exposure levels in humans (Bartolucci et al. 1986; Chua et al. 1992; Elia et al. 1980; Engstrom et al. 1976; Harkonen et al. 1978; Ong et al. 1994; Sedivec et al. 1984; Sollenberg et al. 1988; Symanski et al. 2001). Levels of styrene in blood have also been used as a biomarker of exposure (Antoine et al. 1986; Baselt et al. 1988a; Ramsey et al. 1980).

Effect. There are currently no biomarkers specific for the effects of styrene that are not also typical of other central nervous system depressants. Further research is needed to evaluate potential biomarkers of effect in the areas of chromosome aberrations, psychomotor decrement, hepatic glutathione depletion, and adipose tissue retention of styrene. These potential biomarkers should be evaluated in terms of long-term or chronic exposure periods, and their specificity for exposure to styrene.

Absorption, Distribution, Metabolism, and Excretion. Styrene oxide (styrene epoxide) has been identified as an intermediate metabolite of styrene (Drummond et al. 1989; Engstrom et al. 1976; Korn et al. 1984, 1987; Leibman 1975; Lof et al. 1983; Withey and Collins 1979; Young et al. 1979). However, styrene oxide has only been found in minute amounts in human studies (Lof et al. 1986a). The presence of styrene oxide, a mutagen and carcinogen, may account for some conflicting results and/or interspecies variation in mutagenicity tests and cancer bioassays. The role, if any, of styrene oxide in the overall toxicity of styrene needs to be evaluated by additional metabolism studies to confirm its presence, level,

5.2 IMPORT/EXPORT

Imports of styrene have generally been <1% of U.S. domestic production volumes, with imported styrene amounts decreasing over the last decades, and exported amounts increasing during the same time period. Styrene imports were reported to be 26.4 million pounds for 1976, 320 million pounds in 1986, (Dickson et al. 1983; IARC 1979), but only 1 million pounds in 1999 (HSDB 2007). These trends indicate a higher capacity for domestic producers to meet industry needs. Styrene exports were <1 billion pounds in 1978, but had exceeded 1 billion pounds by 1983. Exports have slowly increased such that recent export data (2000) indicate that the U.S. exports >2 billion pounds of styrene annually (HSDB 2007), also indicating that domestic production is more than capable to serve domestic needs.

Fiberglass products are made from polyester resin dissolved in styrene. During curing some of the styrene cross-reacts with the polyester and the remainder evaporates.

5.3 USE

Styrene is used predominantly (65% of total product) in the production of polystyrene plastics and resins (James and Castor 2005). Some of these resins are used for construction purposes such as in insulation or in the fabrication of fiberglass boats. Styrene is also used as an intermediate in the synthesis of materials used for ion exchange resins and to produce copolymers such as styrene-acrylonitrile (SAN) and acrylonitrile-butadiene-styrene (ABS), both representing approximately 9% of styrene use, and styrene-butadiene rubber (SBR), representing approximately 6% of styrene use. SBR is used for such products as car tires, hoses used for industrial applications, and shoes. A related polymer, styrene-butadiene latex (approximately 7%), is used in making carpet, coatings for paper, and as part of latex paints. Approximately 9% goes into SAN copolymer and polymers of ABS. SAN and ABS are used for materials such as piping, automotive components, refrigerator liners, plastic drinking glasses, and car battery enclosures. An additional 7% is formulated with unsaturated polyester resins in such things as boat hulls (fiberglass reinforcement materials). The remaining amounts of styrene produced are used for several types of applications, including less common thermoplastics and even for laboratory and water purification uses (ion-exchange resins) and glues and adhesives (James and Castor 2005). Styrene copolymers are also frequently used in liquid toner for photocopiers and printers (HSDB 2007).

The Food and Drug Administration (FDA) permits styrene to be used as a direct additive for synthetic flavoring and an indirect additive in polyester resins, ion-exchange membranes, and in rubber articles (5% by weight maximum) intended for use with foods (HSDB 2007; IARC 1979; NIOSH 1983).

6. POTENTIAL FOR HUMAN EXPOSURE

6.1 OVERVIEW

Styrene has been identified in at least 31 of the 1,689 hazardous waste sites that have been proposed for inclusion on the EPA National Priorities List (NPL) (HazDat 2007). However, the number of sites evaluated for styrene is not known. The frequency of these sites can be seen in Figure 6-1. Of these sites, 30 are located within the United States and one is located in the Commonwealth of Puerto Rico (not shown).

Styrene is a widely used industrial chemical with reported atmospheric emissions of >54 million pounds annually in the United States (TRI05 2007). Styrene photodegrades in the atmosphere, with a half-life ranging between 7 and 16 hours (which are the degradation half-lives catalyzed by reactions with hydroxyl radical and ozone, respectively). Styrene is moderately mobile in soil and volatilizes from water to the atmosphere. Styrene will undergo biodegradation in most top soils and aquatic environments, but degradation will be much slower in environments that are anaerobic. Bioconcentration does not appear to be significant.

The principal route of styrene exposure for the general population is probably by inhalation of contaminated indoor air. Mean indoor air levels of styrene have been reported in the range of 0.1–50 $\mu g/m^3$, and can be attributed to emissions from building materials, consumer products, and tobacco smoke. It should be pointed out that the workplace or home office may have substantially higher levels of airborne styrene, due to emissions from laser printers and photocopiers. General workplace styrene concentrations ranged from 89 to 1.5×10^6 $\mu g/m^3$. The most significant exposure route in these settings is also likely by inhalation. The industries with the highest potential exposure are probably the reinforced plastics factories, boatbuilding facilities, and polystyrene factories. very low compared to RP. Exposure may also be high in areas near major spills. Exposure to styrene from hazardous waste sites is potentially important, but the magnitude of the problem is unknown. The potential for outdoor exposure to styrene is lower than indoor exposure, with reported mean air levels ranging from 0.28 to 20 $\mu g/m^3$.

6.2 RELEASES TO THE ENVIRONMENT

The Toxics Release Inventory (TRI) data should be used with caution because only certain types of facilities are required to report (EPA 2005). This is not an exhaustive list. Manufacturing and processing facilities are required to report information to the TRI only if they employ 10 or more

concentration of 0.4 μg/L (Antoine et al. 1986), and in exhaled breath at mean concentrations of 0.7–1.6 μg/m^3 (EPA 1987e).

This data is 24 years old! what is current?

A large number of workers are potentially exposed to styrene. NIOSH estimates that approximately 300,000 workers at 22,000 facilities may be exposed to styrene (NOES 1989); about 30,000 of these on a full-time basis (NIOSH 1983) and about 86,000 are females. The highest potential exposure occurs in the reinforced-plastics industry, where workers may be exposed to high air concentrations and also have dermal exposure to liquid styrene or resins (Lemasters et al. 1985; NIOSH 1983). Hemminki and Vianio (1984) estimated that heavily exposed workers in this industry in Finland might be exposed to up to 3 g of styrene per day. Significant occupational exposures may also occur in other industrial settings, including styrene polymerization, rubber manufacturing, and styrene-polyester resin facilities (Engstrom et al. 1978b; NIOSH 1983; Rappaport and Fraser 1977) as well as in photocopy centers or facilities (Leovic et al. 1996, 1998; Stefaniak et al. 2000).

A current assessment seems to be in order!

6.6 EXPOSURES OF CHILDREN

This section focuses on exposures from conception to maturity at 18 years in humans. Differences from adults in susceptibility to hazardous substances are discussed in Section 3.7, Children's Susceptibility.

Children are not small adults. A child's exposure may differ from an adult's exposure in many ways. Children drink more fluids, eat more food, breathe more air per kilogram of body weight, and have a larger skin surface in proportion to their body volume. A child's diet often differs from that of adults. The developing human's source of nutrition changes with age: from placental nourishment to breast milk or formula to the diet of older children who eat more of certain types of foods than adults. A child's behavior and lifestyle also influence exposure. Children crawl on the floor, put things in their mouths, sometimes eat inappropriate things (such as dirt or paint chips), and spend more time outdoors. Children also are closer to the ground, and they do not use the judgment of adults to avoid hazards (NRC 1993).

Children can be exposed to styrene at home by inhalation of contaminated air and by food consumption. Inhalation-based exposures may occur in both urban and rural home environments, both of which may be contaminated by vehicular and industrial emissions. In addition, exposure to tobacco smoke may provide another route of styrene exposure, especially in homes where one or both parents, any siblings, or other

Table 2-1. Results of Selected Human Neurotoxicity Studies

Result	Reference	NOAEL ppm	LOAEL ppm
~~Impaired color vision~~	Chia et al. 1994		6
Decreased color discrimination	Kishi et al. 2001	4	10
	Gong et al. 2002		10
	Gobba et al. 1991		16
	Iregren et al. 2005		22
	Fallas et al. 1992		24.3
	Campagna et al. 1996		26
	Eguchi et al. 1995	8	93
Neurological symptoms	Flodin et al. 1989	6	
	Edling et al. 1993	8.6	
	Checkoway et al. 1992	10.8	18.9
	Cherry et al. 1980		92
Vestibular effects	Moller et al. 1990		18
	Toppila et al. 2006		24.8
	Calabrese et al. 1996		36
Reaction time	Edling et al. 1993	8.6	
	Tsai and Chen 1996		21.9
	Jegaden et al. 1993		22.68
	Fallas et al. 1992		24.3
	Mutti et al. 1984a		25
	Gamberale et al. 1976		47
	Cherry et al. 1980		92
Hearing	Morata et al. 2002		3.68
	Śliwiński-Kowalska et al. 2003		15.6
	Morioka et al. 1999		16
	Möller et al. 1990	18	
	Calabrese et al. 1996	36	
Nerve conduction velocity	Seppalainen and Harkonen 1976	30	
	Štětkářová et al. 1993		50
	Triebig et al. 1985	100	

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Write out National Priorities List

1. PUBLIC HEALTH STATEMENT

This public health statement tells you about styrene and the effects of exposure to it.

The Environmental Protection Agency (EPA) identifies the most serious hazardous waste sites in the nation. These sites are then placed on the National Priorities List (NPL) and are targeted for long-term federal clean-up activities. Styrene has been found in at least 31 of the 1,689 current or former NPL sites. Although the total number of NPL sites evaluated for this substance is not known, the possibility exists that the number of sites at which styrene is found may increase in the future as more sites are evaluated. This information is important because these sites may be sources of exposure, and exposure to this substance may harm you.

When a substance is released either from a large area, such as an industrial plant, or from a container, such as a drum or bottle, it enters the environment. Such a release does not always lead to exposure. You can be exposed to a substance only when you come in contact with it. You may be exposed by breathing, eating, or drinking the substance, or by skin contact.

If you are exposed to styrene, many factors will determine whether you will be harmed. These factors include the dose (how much), the duration (how long), and how you come in contact with it. You must also consider any other chemicals you are exposed to and your age, sex, diet, family traits, lifestyle, and state of health.

What is styrene?

Colorless liquid that evaporates easily	In its pure form, styrene has a sweet smell. Manufactured styrene often contains other chemicals that give it a sharp, unpleasant odor
Used in manufacturing and in consumer products	Large amounts of styrene are produced in the United States. Small amounts are produced naturally by plants, bacteria, and fungi. Styrene is also present in combustion products such as cigarette smoke and automobile exhaust. Styrene is widely used to make plastics and rubber. Consumer products containing styrene include: • packaging materials • insulation for electrical uses (i.e., wiring and appliances) • insulation for homes and other buildings • fiberglass, plastic pipes, automobile parts • drinking cups and other "food-use" items • carpet backing These products mainly contain styrene linked together in long chains (polystyrene). However, most of these products also contain a small amount of unlinked styrene.

For more information on the physical and chemical properties of styrene, and its production, disposal, and use, see Chapters 4 and 5.

What happens to styrene when it enters the environment?

Most commonly found in air	Styrene can be found in air, soil, and water after release from the manufacture, use, and disposal of styrene-based products.
Rapidly broken down	*Air:* Styrene is quickly broken down in the air, usually within 1–2 days. *Water and soil:* Styrene evaporates from shallow soils and surface water. Styrene that remains in soil or water may be broken down by bacteria or other microorganisms.

For more information on styrene in the environment, see Chapter 6.

Information about tests for detecting styrene in the body is given in Chapters 3 and 7.

What recommendations has the federal government made to protect human health?

The federal government develops regulations and recommendations to protect public health. Regulations *can* be enforced by law. The EPA, the Occupational Safety and Health Administration (OSHA), and the Food and Drug Administration (FDA) are some federal agencies that develop regulations for toxic substances. Recommendations provide valuable guidelines to protect public health, but *cannot* be enforced by law. The Agency for Toxic Substances and Disease Registry (ATSDR) and the National Institute for Occupational Safety and Health (NIOSH) are two federal organizations that develop recommendations for toxic substances.

Regulations and recommendations can be expressed as "not-to-exceed" levels, that is, levels of a toxic substance in air, water, soil, or food that do not exceed a critical value that is usually based on levels that affect animals; they are then adjusted to levels that will help protect humans. Sometimes these not-to-exceed levels differ among federal organizations because they used different exposure times (an 8-hour workday or a 24-hour day), different animal studies, or other factors.

Sentence needs to be broken down to make it more simple to understand.

Recommendations and regulations are also updated periodically as more information becomes available. For the most current information, check with the federal agency or organization that provides it.

Some regulations and recommendations for styrene include the following:

Levels in drinking water set by EPA	The EPA has determined that exposure to styrene in drinking water at concentrations of 20 ppm for 1 day or 2 ppm for 10 days is not expected to cause any adverse effects in a child. The EPA has determined that lifetime exposure to 0.7 ppm styrene is not expected to cause any adverse effects.
Levels in bottled water set by FDA	The FDA has determined that the styrene concentration in bottled drinking water should not exceed 0.1 ppm.
Levels in workplace air set by OSHA	OSHA set a legal limit of 100 ppm styrene in air averaged over an 8-hour work day.

For more information on regulations and advisories, see Chapter 8.

2. RELEVANCE TO PUBLIC HEALTH

2.1 BACKGROUND AND ENVIRONMENTAL EXPOSURES TO STYRENE IN THE UNITED STATES

Styrene is a high production chemical; over 13 billion pounds of styrene was produced in the United States in 2006 (SRI 2006). Small amounts of styrene are naturally present in foods such as legumes, beef, clams, eggs, nectarines, and spices. It can also be present in packaged foods by migration from polystyrene food containers and packaging materials. Styrene is a combustion product of cigarette smoke (EPA 1984b) and automobile exhaust. Manufactured styrene is primarily used in the production of polystyrene plastics and resins (James and Castor 2005) used principally for insulation or in the fabrication of fiberglass boats; production of copolymers such as styrene-acrylonitrile and acrylonitrile-butadiene-styrene, which are used to manufacture piping, automotive components, and plastic drinking glasses; production of styrene-butadiene rubber used to manufacture car tires, hoses for industrial purposes, and shoes; or formulated with unsaturated polyester resins used as fiberglass reinforcement materials. Styrene copolymers are also frequently used in liquid toner for photocopiers and printers (HSDB 2007).

Median styrene concentrations in urban and rural/suburban air samples range from 0.29 to 3.8 μg/m^3 (0.07–0.9 ppb) (EPA 1986e, 1987e, 1988c; Fishbein 1992; Grosjean and Fung 1984; Grosjean et al. 1998; Harkov et al. 1985; Wallace et al. 1986b) and 0.28–0.34 μg/m^3 (0.06–0.08 ppb) (EPA 1988c; Graedel 1978; Islam and Stoncheva 1999; Kinney et al. 2002). The median styrene concentration in indoor air samples ranged from 0.4 to 8.9 μg/m^3 (0.09–2 ppb); the primary sources of styrene in indoor air are cigarette smoke (EPA 1987e; Wallace et al. 1986a) and photocopiers (Stefaniak et al. 2000; Leovic et al. 1996, 1998). Styrene is rarely detected in drinking water samples (EPA 1988b) and is rarely detected in soil samples.

General population exposure to styrene in air and food has been estimated to be 18–54 and 0.2–1.2 μg/person/day, respectively, with a total daily exposure of 18.2–55.2 μg/day (Tang et al. 2000) or 0.0003–0.0008 mg/kg/day (assuming a 70-kg reference body weight).

2.2 SUMMARY OF HEALTH EFFECTS

Styrene-induced neurotoxicity has been reported in workers since the 1970s. Studies over the last 15 years have firmly established the central nervous system as the critical target of toxicity. Both short-

and long-term exposures to styrene can result in neurological effects. Acute exposure data are limited to the finding of impaired performance on tests of vestibular function in test subjects exposed to 87–376 ppm for 1–3 hours (Odkvist et al. 1982; Stewart et al. 1968). A variety of neurological effects have been observed in chronically exposed styrene workers; these effects include impaired color vision (Campagna et al. 1995, 1996; Chia et al. 1994; Eguchi et al. 1995; Fallas et al. 1992; Gobba et al. 1991; Gong et al. 2002; Kishi et al. 2001; Mutti et al. 1984a), vestibular effects (Calabrese et al. 1996; Moller et al. 1990), hearing impairment (Morata et al. 2002; Morioka et al. 1999; Muijser et al. 1988; Sliwinska-Kowalska et al. 2003), symptoms of neurotoxicity, particularly "feeling drunk" and tiredness (Checkoway et al. 1992; Cherry et al. 1980; Edling et al. 1993; Viaene et al. 1998, 2001), delays in reaction time (Cherry et al. 1980; Fallas et al. 1992; Gamberale et al. 1976; Jegaden et al. 1993; Mutti et al. 1984a; Tsai and Chen 1996), impaired performance on tests measuring attention and memory (Chia et al. 1994; Jegaden et al. 1993; Mutti et al. 1984a), impaired nerve conduction velocity (Behari et al. 1986; Gobba et al. 1995; Murata et al. 1991; Rosen et al. 1978; Štětkářová et al. 1993; Yuasa et al. 1996), and EEG alterations (Harkonen et al. 1984; Seppalainen and Harkonen 1976). The LOAELs for these effects range from about 10 ppm to 93 ppm. In most of the occupational exposure studies, neurological function tests were conducted in the morning before work, suggesting that the deficits were not acute effects. Results of a meta-analysis suggest that the severity of the some of the neurological symptoms increases with exposure duration (Benignus et al. 2005). For example, 8, 15, 25, and 35% increases in reaction time were observed in workers exposed to 100 ppm for 2, 4, 6, and 8 work-years, respectively. However, this may also be reflective of higher exposure levels in the past rather than an a duration-related increase in severity. The existing data are inadequate to determine whether chronic styrene exposure results in permanent damage. Mixed results have been found in studies examining workers before and after an extended period without styrene exposure. Animal studies have also reported neurological effects, although most of these studies have focused on effects on hearing and damage to the organ of Corti (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2000, 2001; Loquet et al. 1999, 2000; Makitie et al. 2002; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992)

Should be broken out separately + expanded upon

Other effects that have been observed in animal studies include damage to the nasal olfactory epithelium and liver necrosis; testicular damage and developmental effects have also been reported, but the weight of evidence does not support concluding that these are sensitive targets. Damage to the nasal olfactory epithelium was observed in mice after 3 days of exposure (Cruzan et al. 2001). The severity of the lesion progressed from single cell necrosis to atrophy and respiratory metaplasia (Cruzan et al. 1997, 2001) with increasing exposure duration. The lowest-observed-adverse-effect levels (LOAELs) for these lesions are 80, 50, and 20 ppm for acute, intermediate, and chronic exposure, respectively. Rats do not appear to be

as sensitive as mice to the nasal olfactory epithelial damage; an intermediate-duration study identified a no-observed-adverse-effect level (NOAEL) and LOAEL of 500 and 1,000 ppm for focal hyperplasia and a chronic study identified a LOAEL of 50 ppm for atrophy and degeneration (Cruzan et al.1998). The observed species differences may be due to differences in styrene metabolism in the nasal cavity. In particular, rats have a higher capacity to detoxify styrene oxide with epoxide hydrolases and glutathione S-transferase. ~~It is not likely that~~ humans ~~will be~~ are not likely sensitive to the nasal toxicity of styrene because styrene oxide has not been detected, and high levels of epoxide hydrolases have not been found in *in vitro* assays of human nasal tissue (Green et al. 2001a).

Unlike the nasal lesions, the severity of hepatic lesions decreases with increased exposure durations. Severe hepatocellular necrosis was observed in mice exposed to 250 ppm for 3 days (Morgan et al. 1993b); however, continued exposure at this concentration resulted in focal necrosis and an increase in pigmented macrophages (Morgan et al. 1993a). Centrilobular aggregates of siderophages were observed in mice exposed to 200 ppm for 13 weeks (Cruzan et al. 1997); no liver effects were observed at 160 ppm after 2 years of exposure (Cruzan et al. 2001). Rats are less sensitive than mice to liver toxicity; no liver effects were observed in an intermediate-duration study in which rats were exposed to a styrene concentration 10-fold higher than the concentration eliciting hepatic effects in mice. No alterations in serum markers of liver damage were observed in styrene workers exposed to 40 ppm for approximately 5 years (Harkonen et al. 1984). Liver effects have not been observed in oral exposure studies; however, no studies examined systemic end points following acute exposure.

Occupational exposure studies have not found significant increases in the occurrence of stillbirth, infant death, malformations, or low birth weight (Ahlborg et al. 1987; Lemasters et al. 1989). Most single and multigeneration inhalation and oral exposure studies did not find significant alterations in fetus/pup survival, growth, or incidence of abnormalities in rats, mice, rabbits, and hamsters exposed to styrene (Beliles et al. 1985; Cruzan et al. 2005b; Daston et al. 1991; Kankaanpää et al. 1980; Murray et al. 1978). An increase in fetal deaths were observed in hamsters exposed to very high concentrations (1,000 ppm) (Kankaanpää et al. 1980) and in rats exposed to lower concentrations (Katakura et al. 1999, 2001). Two studies have examined neurodevelopmental effects in rats; one study found some minor effects (slight delays in some developmental landmarks) (Katakura et al. 1999, 2001). The other, higher-quality study did not find any significant alterations in a number of neurodevelopmental end points (Cruzan et al. 2005a). The National Toxicology Program (NTP) Expert Panel examining the developmental potential of styrene (NTP 2006) concluded that the human data are not sufficient to evaluate the potential

Again separate human + animal to make more coherent

these lines seem to contradict lines 24-26

developmental toxicity of styrene in humans and that there was no convincing evidence of developmental toxicity in animals.

Although several epidemiology studies have examined potential reproductive effects in male and female styrene workers, adequate analysis of the data is limited by the lack of exposure information and concomitant exposure to other compounds. Mixed results have been found for increased occurrence of spontaneous abortions (Harkonen and Holmberg 1982; Hemminki et al. 1980, 1984; Lindbohm et al. 1985; McDonald et al. 1988) and oligomenorrhea (Cho et al. 2001; Lemasters et al. 1985). In male workers, sperm abnormalities have been reported (Kolstad et al. 1999a), but not alterations in time-to-pregnancy (Kolstad et al. 2000; Sallmén et al. 1998) or fertility rates (Kolstad et al. 1999c). No adverse reproductive effects were observed in inhalation (Cruzan et al. 2005b) and oral (Beliles et al. 1985) multigeneration studies in rats. A series of studies found decreases in spermatozoa counts in rats exposed as adults, as neonates, and through lactation (Srivastava et al. 1989, 1992a, 1992b). However, as noted by the NTP Expert Panel (NTP 2006), this finding is not consistent with the lack of reproductive effects found in the inhalation two-generation study (Cruzan et al. 2005b). The NOAEL identified in the two-generation inhalation study was 500 ppm (6 hours/day), which is roughly equivalent to 230 mg/day using a reference inhalation rate of 0.42 m^3/day. The LOAEL for spermatozoa effects in adult rats was 400 mg/kg (6 days/week), which is roughly equivalent to 158 mg/day using a reference body weight of 0.462 kg.

There are several epidemiologic studies of workers at styrene manufacturing and polymerization facilities and reinforced plastics facilities that suggest an association between occupational exposure and an increased incidence of cancer of the lymphatic and hematopoietic tissues in styrene (Hodgson and Jones 1985; Kogevinas et al. 1993, 1994; Kolstad et al. 1993, 1994; Nicholson et al. 1978; Ott et al. 1980). However, the reported studies are inconclusive due to exposure to multiple chemicals (including benzene) and the small size of the cohorts. Other studies have reported negative results (Bond et al. 1992; Coggon et al. 1987; Frentzel-Beyme et al. 1978; Matanoski and Schwartz 1987; Okun et al. 1985; Wong 1990). More consistent results for increases in the risk of lymphatic and hematopoietic cancers have been observed among workers at styrene-butadiene manufacturing facilities (Delzell et al. 1996; Macaluso et al. 1996; Matanoski and Schwartz 1987; Matanoski et al. 1990; McMichael et al. 1976; Meinhardt et al. 1982; Sathiakumar et al. 2005). There is suggestive evidence that these increased risks may be due to exposure to 1,3-butadiene rather styrene exposure (Cheng et al. 2007; Delzell et al. 2001; Graff et al. 2005; Macaluso et al. 1996, 1993; Matanoski et al. 1997; Santos-Burgoa et al. 1992); however, it is difficult to separate the risks for styrene and 1,3-butadiene because the exposure is highly correlated.

There are no reports of cancer resulting from styrene exposure by the oral or dermal routes in humans. Species differences in styrene carcinogenicity have been detected in animal studies. Inhalation (Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1982) and oral exposure (Beliles et al. 1985; Conti et al. 1988; Maltoni et al. 1982; NCI 1979b) studies in rats have not found significant increases in neoplastic lesions. However, increases in lung tumors have been found in mice following inhalation (Cruzan et al. 2001) and oral exposure (NCI 1979b). The increased production of styrene 7,8-oxide in lung Clara cells and the higher ratio of styrene oxide R- to S-enantiomers likely resulted in the increased sensitivity of mice. Overall, human and animal studies suggest that styrene may be a weak human carcinogen. The International Agency for Research on Cancer (IARC) has assigned styrene to Group 2B, possibly carcinogenic to humans (IARC 2006).

2.3 MINIMAL RISK LEVELS (MRLs)

Estimates of exposure levels posing minimal risk to humans (MRLs) have been made for styrene. An MRL is defined as an estimate of daily human exposure to a substance that is likely to be without an appreciable risk of adverse effects (noncarcinogenic) over a specified duration of exposure. MRLs are derived when reliable and sufficient data exist to identify the target organ(s) of effect or the most sensitive health effect(s) for a specific duration within a given route of exposure. MRLs are based on noncancerous health effects only and do not consider carcinogenic effects. MRLs can be derived for acute, intermediate, and chronic duration exposures for inhalation and oral routes. Appropriate methodology does not exist to develop MRLs for dermal exposure.

Although methods have been established to derive these levels (Barnes and Dourson 1988; EPA 1990), uncertainties are associated with these techniques. Furthermore, ATSDR acknowledges additional uncertainties inherent in the application of the procedures to derive less than lifetime MRLs. As an example, acute inhalation MRLs may not be protective for health effects that are delayed in development or are acquired following repeated acute insults, such as hypersensitivity reactions, asthma, or chronic bronchitis. As these kinds of health effects data become available and methods to assess levels of significant human exposure improve, these MRLs will be revised.

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Repeat of above paragraph. Delete

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Inhalation MRLs

- An MRL of 2 ppm has been derived for acute-duration inhalation exposure (14 days or less) to styrene.

The acute-duration inhalation toxicity database for styrene consists of several human experimental studies primarily examining neurotoxicity (Odkvist et al. 1982; Seeber et al. 2004; Stewart et al. 1968), systemic toxicity studies in mice (Cruzan et al. 1997, 2001; Morgan et al. 1993a, 1993b, 1993c), neurotoxicity studies in rats (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003), mice (DeCeaurriz et al. 1983), and guinea pigs (Lataye et al. 2003), a reproductive toxicity study in mice (Salomaa et al. 1985), and developmental toxicity studies in rats (Murray et al. 1978), mice (Kankaanpää et al. 1980), hamsters (Kankaanpää et al. 1980), and rabbits (Murray et al. 1978). Exposure to 99 ppm for 7 hours or 376 ppm for 1 hour (Stewart et al. 1968) resulted in eye irritation; nasal irritation was also reported at 376 ppm. A significant inhibition of the vestibular-oculomotor system was observed in subjects exposed to 87 ppm for 1 hour (Odkvist et al. 1982). Studies by (Stewart et al. 1968) found alterations in tests of balance or coordination in subjects exposed to 376 ppm for 1 hour, but not after exposure to 99 ppm for 7 hours or 216 ppm for 1 hour; the test used in the Stewart et al. (1968) studies is probably less sensitive than those used by Odkvist et al. (1982). No significant alterations in performance on tests of reaction time were observed in subjects exposed to 20 ppm for 3 hours (Seeber et al. 2004).

What species?

In mice, the most sensitive target of styrene toxicity appears to be the nasal olfactory epithelium; single cell necrosis was observed following exposure to 80 ppm 6 hours/day for 3 days (Cruzan et al. 2001). At 250 ppm, hepatocellular necrosis and degeneration have been observed (Cruzan et al. 1997; Morgan et al. 1993a, 1993b, 1993c). The severity of this lesion appears to be inversely related to the duration of exposure, with more severe damage observed in mice killed within 3 days of exposure (Morgan et al. 1993a, 1993b, 1993c) compared to animals killed after 2 weeks of exposure (Cruzan et al. 1997; Morgan et al. 1993a). Exposure to 250 ppm 6 hours/day, 5 days/week for 2 weeks also resulted in lethargy and unsteady gait in mice (Cruzan et al. 1997). Impaired performance on a swimming test was observed in mice exposed to 610 ppm for 4 hours, but not in animals exposed to 413 ppm (DeCeaurriz et al. 1983). Exposure of rats to high concentrations (1,000 or 1,600 ppm) 6–8 hours/day for 5–14 days resulted in

auditory threshold shifts (indicative of hearing loss) and loss of outer hair cells (OHC) in the organ of Corti (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003). No alterations in sperm morphology were observed in mice exposed to 300 ppm styrene 5 hours/day for 5 days (Salomaa et al. 1995), and no developmental effects were observed in rats or rabbits exposed to 600 ppm 7 hours/day on gestational days 6–15 or 6–18, respectively, (Murray et al. 1978) or mice exposed to 250 ppm 6 hours/day on gestational days 6–16 (Kankaanpää et al. 1980). An increase in fetal deaths or resorptions was observed in hamsters exposed to 1,000 ppm 6 hours/day on gestational days 6–18 (Kankaanpää et al. 1980).

These data suggest that the nervous system is the most sensitive target of styrene toxicity following acute-duration inhalation exposure. The lowest LOAEL for a relevant end point in humans is 87 ppm for vestibular impairment in subjects exposed to styrene for 1 hour (Odkvist et al. 1982). A similar LOAEL (80 ppm) was identified for nasal effects in mice exposed to styrene for 3 days (Cruzan et al. 2001); this effect was not considered suitable as the basis of an MRL. As stated previously, mice appear to have a greater capacity than humans to generate the reactive metabolite, styrene oxide, in the nasal cavity and a lower capacity to detoxify styrene oxide (Green et al. 2001a). The identification of the nervous system as the critical target of toxicity for styrene is supported by a large number of occupational exposure studies. Delays in reaction time have been observed in workers exposed to 21.9–92 ppm (Cherry et al. 1980; Fallas et al. 1992; Gamberale et al. 1976; Jegaden et al. 1993; Mutti et al. 1984a; Tsai and Chen 1996) and vestibular effects have been observed at 18–36 ppm (Calabrese et al. 1996; Möller et al. 1990; Toppila et al. 2006).

The Odkvist et al. (1982) study did not identify a NOAEL for vestibular effects; however, a NOAEL of 20 ppm for performance on several tests of reaction time and attention was identified by Seeber et al. (2004) in subjects exposed to styrene for 3 hours. Although there is some uncertainty whether deriving an MRL based on a 3-hour exposure study would be protective of continuous exposure to styrene for 2 weeks, the Seeber et al. (2004) study was selected as the basis ~~of~~ because of? an acute duration inhalation MRL for styrene.

Groups of eight volunteers (gender not reported) were exposed to 0.5 or 20 ppm styrene for 3 hours (Seeber et al. 2004). The subjects were tested for simple reaction time, choice reaction time, and attention prior to exposure initiation, during the third hour of exposure, and 1.5 hours after exposure termination. The subjects were also asked to complete a symptom questionnaire before, during, and after exposure. The mean concentration of styrene in blood was 2.2 and 80 μg/L after 3 hours of exposure to 0.5 and 20 ppm, respectively. The blood levels of styrene were correlated with styrene levels in air (r_{xy}=0.98).

No significant alterations in performance on neurobehavioral tests were found. An increase in the reporting of breathing problems was found; however, it was not statistically significant, and the ranking of the severity of the breathing problem was very low (0.5 on a scale of 5).

The NOAEL of 20 ppm from the Seeber et al. (2004) study was divided by an uncertainty factor of 10 to account for human variability resulting in an acute-duration inhalation MRL of 2 ppm.

• Needs subtitle for intermediate duration MRL (same as acute + chronic)

No human intermediate-duration studies were identified. For intermediate duration? Animal studies examining systemic, neurological, reproductive, and developmental toxicity have identified the respiratory tract as the most sensitive target of toxicity. Atrophy of the olfactory epithelium, hypertrophy/hyperplasia of Bowman's gland has been observed in mice exposed to 50 ppm 6 hours/day, 5 days/week for 13 weeks (Cruzan et al. 1997), decreased nasal cilia activity has been observed in rats exposed to 150 ppm 4 hours/day, 5 days/week for 21 days (Ohashi et al. 1986), and focal hyperplasia has been observed in rats exposed to 1,000 ppm 6 hours/day, 5 days/week for 13 weeks (Cruzan et al. 1997). As discussed previously, the mouse does not appear to be a good model for nasal effects in humans due metabolic differences. Other systemic effects that have been observed include eye irritation in rats exposed to 200 ppm 6 hours/day, 5 days/week for 13 weeks (Cruzan et al. 1997) and centrilobular aggregates of siderophages in the livers of mice exposed to 200 ppm 6 hours/day, 5 days/week for 13 weeks (Cruzan et al. 1997).

A number of studies in rats have reported outer hair cell loss in the organ of Corti in rats exposed to 600–650 ppm for 4 weeks (Loquet et al. 2000; Makitie et al. 2002; Pouyatos et al. 2002) and hearing loss at 750–1,000 ppm for 3–4 weeks (Campo et al. 2001; Lataye et al. 2000, 2001; Loquet et al. 1999, 2000; Pouyatos et al. 2002). A NOAEL of hearing effects of 300 ppm was identified in rats exposed for 12 hours/day, 5 days/week for 4 weeks (Makitie et al. 2002). Other neurological effects include alterations in astroglial cells in rats continuously exposed to 320 ppm for 3 months (Rosengren and Haglid 1989) and decreased sensory nerve conduction velocity in rats exposed to 2,000 ppm 8 hours/day, 5 days/week for 32 weeks (Yamamoto et al. 1997). No reproductive, developmental, or neurodevelopmental effects were observed in a two-generation study (Cruzan et al. 2005a, 2005b); the NOAEL was 500 ppm. In contrast, an increase in neonatal deaths, developmental landmark delays, and alterations in neurochemical levels were observed in the offspring of rats exposed 6 hours/day on gestational days 6–20 (Katakura et al. 1999, 2001).

out of place - move to chronic exposure

Chronic-duration studies suggest that the most sensitive target of styrene toxicity is the nervous system. ~~It is likely that~~ this would also be the most sensitive effect following intermediate-duration exposure. In

the absence of human neurotoxicity data, an intermediate-duration inhalation MRL is not recommended at this time.

- An MRL of 0.05 ppm has been derived for chronic-duration inhalation exposure (greater than 365 days) to styrene.

Line 33 from p 15 should be here

A large number of studies have examined the neurotoxicity of styrene in workers at reinforced plastic manufacturing facilities. These studies reported a variety of neurological effects, including impaired color vision, slowed reaction time, permanent hearing threshold shifts, vestibular effects, and increases in subjective symptoms. A summary of the results of studies for some of these neurological effects is presented in Table 2-1. The LOAELs for these effects range from 6 to 93 ppm.

Chronic-duration studies in animals identify the nasal olfactory epithelium as the most sensitive end point. Atrophic and/or degenerative changes were observed in rats exposed to 50 ppm styrene 6 hours/day, 5 days/week for 104 weeks (Cruzan et al. 1998) and respiratory metaplasia in the nasal olfactory epithelium has been observed in mice exposed to 20 ppm 6 hours/day, 5 days/week for 98–104 weeks (Cruzan et al. 2001). As noted previously, mice do not appear to be a good model for potential respiratory effects in humans.

Neurotoxicity observed in styrene workers was selected as the basis of the chronic-duration inhalation MRL for styrene. Two approaches to deriving the MRL were considered. In the first approach, the MRL is based on a single study identifying a sensitive LOAEL. In the study by Kishi et al. (2001), styrene workers were divided into three groups based on urinary mandelic acid excretion levels. A significant increase in color confusion index (CCI) was found in the workers exposed to equivalent styrene concentrations of 10 or 46 ppm, as compared to age-matched controls. No significant alterations were observed in workers exposed to 4 ppm. The second approach involves the use of a LOAEL estimated from a meta-analysis of occupational exposure studies finding effects on color vision and reaction time (Benignus et al. 2005). Benignus et al. (2005) used data color vision impairment data from the Campagna et al. (1996), Eguchi et al. (1995), Gobba et al. (1991), Gong et al. (2002), and Kishi et al. (2001) studies and choice reaction time data from the Jegaden et al. (1993), Mutti et al. (1984a), Triebig et al. (1989), and Tsai and Chen (1996) studies. Average styrene exposure concentrations were estimated from individual data reported in the papers; for studies reporting individual data as urinary mandelic acid levels, standardized methods for converting to styrene exposure levels were used. Cite source for methods Cumulative styrene exposure was estimated by multiplying exposure level by length of employment. A common metric of effect magnitude (percentage of baseline) was calculated for the different neurological effects. The

analysis found a significant linear relationship between choice reaction time and cumulative styrene exposure; cumulative exposure accounted for 91% of the variance in reaction time. Similarly, a significant relationship between CCI and cumulative styrene exposure was found, with cumulative exposure accounting for 35% of the variance in CCI. Using the regression equations for these two effects, Benignus et al. (2005) estimated that exposure to 150 ppm for 8 work-years would result in a 50% increase in choice reaction time and a 17% increase in CCI score; exposure to 20 ppm for 8 work-years would result in a 6.5% increase in choice reaction time and a 2.23% increase in CCI score. As discussed in Benignus et al. (2005), a 7% decrease in reaction time would prevent 58,000–70,000 injuries per year from automobile accidents. The investigators also noted that CCI increases with age, the rate of increase is about 10% per 13 years of age; thus, a 2.23% decrease in color perception would be roughly equivalent to 1.7 additional years of age. Based on this analysis, 20 ppm is considered a LOAEL for neurological effects.

? Should this be 2.9 yrs = $\frac{13\text{yrs}}{10\%} \times 2.23\%$] Benignus et al. 2005 has the 1.7 yrs but this seems incorrect. They also incorrectly state that 115 ppm @ 8 work yrs or 156 @ 6 work yrs leads to 10% ↑ above baseline.

The LOAEL of 20 ppm is consistent with the LOAEL values identified in many of the individual studies. However, using the LOAEL identified from the Benignus et al. (2005) meta-analysis has several advantages over selecting a single study as the basis of the MRL. Because data were pooled from several studies, the relationships between styrene exposure and effects were examined in a large number of subjects (302 subjects for choice reaction time and 383 subjects for color vision). The use of standardized methods for estimating styrene exposure levels from urinary biomarker levels is also an advantage. Additionally, the biological relevance of the observed deficits in reaction time and color vision was estimated. The LOAEL of 20 ppm was adjusted for intermittent exposure (8 hours/~~day~~ 24hrs, 5 days/~~week~~ 7days) resulting in an adjusted LOAEL of 5 ppm, which was divided by an uncertainty factor of 100 (10 for use of a LOAEL and 10 for human variability) resulting in a chronic-duration inhalation MRL of 0.05 ppm.

Oral MRLs

- An MRL of 0.1 mg/kg/day has been derived for acute-duration oral exposure (14 days or less) to styrene.

A limited number of studies have examined the acute toxicity of orally administered styrene; these studies have examined potential neurotoxicity and developmental toxicity. No developmental effects were observed in rats administered a single dose of 300 mg/kg on gestational day 11 (Daston et al. 1991) or administered 300 mg/kg/day (administered as two daily doses of 150 mg/kg) on gestational days 6–15 (Murray et al. 1978). Impaired learning was observed in rats administered via gavage 100 or 200 mg/kg/day for 14 days; increases in serotonin levels were observed in the hypothalamus,

hippocampus, and midbrain (Husain et al. 1985). In another study, increases in dopamine receptor binding was observed in rats administered a single gavage dose of 200 mg/kg (Agrawal et al. 1982).

Although there is a limited acute toxicity database, longer-term oral studies support the selection of neurotoxicity as the principal effect. The lowest LOAEL identified for a systemic effect is 400 mg/kg/day for Heinz body formation in dogs administered styrene by gavage for 561 days (Quast et al. 1979); the identified NOAEL was 200 mg/kg/day. Decreased spermatozoa counts were observed in adult rats administered 400 mg/kg 6 days/week for 60 days (Srivastava et al. 1989), young rats exposed via lactation on postnatal days 1–21 (maternal dose of 400 mg/kg/day) (Srivastava et al. 1992a), and young rats administered 200 mg/kg 6 days/week on postnatal days 1–61 (Srivastava et al. 1992b); the NOAELs identified in these three studies were 200, 200, and 100 mg/kg, respectively. Marked degeneration of the seminiferous tubules was also observed in the adult rats administered 400 mg/kg (Srivastava et al. 1989). Impaired learning was observed in rats administered 500 mg/kg 5 days/week for 8 weeks (Bushnell 1994); a NOAEL was not identified in this study. The extensive inhalation toxicity database for styrene also supports the selection of neurotoxicity as the most sensitive target of toxicity; both the acute- and chronic-duration inhalation MRLs are based on neurological effects in humans. Neurological effects observed in chronically exposed styrene workers include impaired color vision, slowed reaction time, increased prevalence of neurological symptoms, and ototoxicity (hearing and vestibular effects).

What do these studies have to do with selection of neurotoxicity as principal effect?

Does this mean the inhalation studies support selection of neurotoxicity as endpoint for oral exposure?

The Husain et al. (1985) study was selected as the basis of the acute-duration oral MRL. In this study, groups of 15 male Wistar rats were administered by gavage 0, 100, or 200 mg/kg/day styrene in ground nut oil for 14 consecutive days. Spontaneous motor activity with or without amphetamine induction was observed 1 day after the last dose. Two days after exposure termination, the rats underwent acquisition training for 4 days. Learning was assessed by measuring the number of times the rat climbed the pole after the conditioned stimulus to avoid the foot-shock unconditioned stimulus. Noradrenaline, dopamine, and serotonin levels were measured in seven regions of the brain in six rats/group sacrificed after the acquisition training. No overt signs of toxicity were observed. No significant alterations in locomotor activity were observed with or with amphetamine induction. Significantly greater increases in percent avoidance response in the conditioned avoidance response test (indicative of impaired learning) were observed at 100 and 200 mg/kg/day; no difference was found between the two styrene groups. The effects were observed on test day 3 and 4. Significant increases in the level of serotonin in the hypothalamus (70%), hippocampus (51%), and midbrain (29%) were observed at 200 mg/kg/day. Styrene exposure did not affect brain noradrenaline and dopamine levels.

The LOAEL of 100 mg/kg/day was divided by an uncertainty factor of 1,000 (10 for use of a LOAEL, 10 for extrapolation from animals to humans, and 10 for human variability).

- Subtitle for intermediate oral MRL

The systemic toxicity of styrene has not been investigated in intermediate-duration oral exposure studies. Neurotoxicity studies have identified a LOAEL of 200 mg/kg/day for increased dopamine receptor binding in rats (Agrawal et al. 1982), a LOAEL of 500 mg/kg (5 days/week) for impaired learning in rats (Bushnell 1994), and a LOAEL of 906 mg/kg/day for alterations in serotonin and noradrenaline levels in rats (Husain et al. 1980); none of these studies identified a NOAEL for neurological effects. An increase in dopamine receptor binding was also observed in the offspring of rats administered 200 mg/kg/day during gestation, lactation, or both (Zaidi et al. 1985). The remaining intermediate-duration studies reported decreases in spermatozoa counts in rats exposed as 400 mg/kg (6 days/week) as adults, 200 mg/kg (6 days/week) as neonates, or during lactation (maternal dose of 400 mg/kg/day) (Srivatava et al. 1989, 1992a, 1992b).

The LOAELs identified in these intermediate-duration studies are higher than the lowest LOAEL for neurotoxicity identified in an acute-duration study (Husain et al. 1985); thus, an intermediate-duration MRL is not recommended at this time.

Subtitle for chronic oral MRL

The available data on the chronic toxicity of styrene comes from three systemic toxicity studies. No adverse effects were observed in rats exposed to 35 mg/kg/day styrene in drinking water for 2 years (Beliles et al. 1985), and no liver or kidney alterations were observed in rats administered 500 mg/kg 1 day/week for 120 weeks (Ponomarkov and Tomatis 1978). Increase in Heinz body formation was observed in dogs administered 400 mg/kg/day for 561 days (Quast et al. 1979); the NOAEL for this effect is 200 mg/kg/day.

The chronic-duration inhalation database provides strong evidence that neurotoxicity is the most sensitive target of styrene toxicity. It is not known if this would also be true for chronic-duration oral exposure; the acute-toxicity oral database provides suggestive evidence that it would be a sensitive target. In the absence of a long-term oral study examining neurological end points, a chronic-duration oral MRL is not recommended.

because it helps the users of the profiles to identify levels of exposure at which major health effects start to appear. LOAELs or NOAELs should also help in determining whether or not the effects vary with dose and/or duration, and place into perspective the possible significance of these effects to human health.

The significance of the exposure levels shown in the Levels of Significant Exposure LSE) tables and figures may differ depending on the user's perspective. Public health officials and others concerned with appropriate actions to take at hazardous waste sites may want information on levels of exposure associated with more subtle effects in humans or animals LOAELs) or exposure levels below which no adverse effects NOAELs) have been observed. Estimates of levels posing minimal risk to humans Minimal Risk Levels or MRLs) may be of interest to health professionals and citizens alike.

A User's Guide has been provided at the end of this profile see Appendix B). This guide should aid in the interpretation of the tables and figures for Levels of Significant Exposure and the MRLs.

3.2.1 Inhalation Exposure

Most information on the effects of inhalation exposure to styrene in humans comes from studies of workers exposed to styrene vapors in the production and use of plastics and resins, especially polystyrene resins. In most cases, the studies involve workplace exposures such as fiberglass boat building factories where the actual levels of styrene are reported as a range of styrene air concentrations. However, there are a few human clinical studies in which exposures are better quantified. Provided below are descriptions of the known effects of inhalation exposure of humans and animals to styrene.

3.2.1.1 Death

There have been no reports of deaths in humans directly associated with exposure to styrene in the workplace (EPA 1988b; Gosselin et al. 1984; NIOSH 1983).

In animals, inhalation studies indicate that the acute toxicity of styrene is low to moderate. An LC_{50} of 2,770 ppm after 2 hours of exposure was reported in rats, and the LC_{50} for mice after exposure for 4 hours was 4,940 ppm (Shugaev 1969). All rats and guinea pigs survived after exposure to 1,300 ppm styrene for 30 hours and 16 hours, respectively (Spencer et al. 1942). However, all animals died after 40 hours of exposure. Gender differences in mortality were observed in repeated-exposure studies (Cruzan et al.

specifics of exposure

observed in the trachea at 150 and 1,000 ppm. After a 12-week recovery period, nasal and tracheal cilia activity in the 150 ppm group was similar to controls; decreases in nasal cilia activity was still lower than controls but was increased compared to rats killed at the end of the exposure period (Ohashi et al. 1986). In a longer-term study, focal hyperplasia was observed in the nasal olfactory epithelium of rats exposed to 1,000 ppm for 13 weeks (Cruzan et al. 1997); at 500 ppm, no histological alterations were observed in the respiratory tract. Chronic exposure to 50 ppm resulted in atrophic and/or degenerative changes in the nasal olfactory epithelium (Cruzan et al. 1998).

Doesn't make since. Does this need to refer to the 1000 ppm group in which the nasal cilia hadn't completely recovered at 12 wks.

Mice appear to be more sensitive than rats to the respiratory toxicity of styrene. Exposure to 50 ppm styrene for 13 weeks resulted in atrophy of the nasal olfactory epithelium and dilatation, hypertrophy and hyperplasia of Bowman's gland (Cruzan et al. 1997). At 100 ppm, atrophy of the nasal olfactory nerve fibers was observed; focal crowding of nonciliated epithelial cells in the bronchioles were observed at 150 ppm. Chronic exposure resulted in respiratory metaplasia of the nasal olfactory epithelium and dilatation, respiratory metaplasia, epithelial hyperplasia of the Bowman's gland in mice exposed to 20 ppm and higher for 2 years (Cruzan et al. 2001). Decreased eosinohilia of epithelial cells and bronchiolar epithelial hyperplasia were observed in the lungs of mice exposed to 20 ppm and higher.

A study by Spencer et al. (1942) also provides some information on species differences. Rats and guinea pigs exposed 1,300 ppm for 7–8 hours/day, 5 days/week for 6 months showed nasal irritation, but rabbits and monkeys did not (Spencer et al. 1942). Histopathological examinations revealed no changes between test and control rats, but pronounced lung irritation was seen in guinea pigs that died after a few exposures. The irritation, which included congestion, hemorrhages, edema, exudation, and a general acute inflammatory reaction, was not seen in the guinea pigs, rabbits, and monkeys that survived the 6-month exposure period (Spencer et al. 1942).

Green et al. (2001a) suggest that the observed species differences between mice and rats are due to differences in styrene metabolism in the nasal epithelium. The rates of metabolism of styrene by cytochromes P-450 CYP2E1 and CYP2F2 to styrene oxide was similar for the two species. However, styrene oxide is more efficiently metabolized by epoxide hydrolases and glutathione S-transferases in rats than in mice. Thus, the higher levels of the reactive epoxide styrene oxide in mice is the likely cause of the increased sensitivity in this species. In *in vitro* assays in fresh human nasal tissues, styrene oxide was not detected and high levels of epoxide hydrolases were detected. Suggesting that humans have limited capacity to metabolize styrene in the nasal cavity and a high potential to detoxify styrene oxide. These data suggest that rodents may not be a good model for nasal toxicity in humans.

Reference

These well-conducted human and animal studies demonstrate the characteristic irritant properties of styrene on the upper respiratory tract.

Cardiovascular Effects. No studies were located regarding cardiovascular effects in humans after inhalation exposure to styrene.

No cardiovascular effects were observed in rats or mice exposed to concentrations as high as 1,000 ppm or 160 ppm, respectively, for 2 years (Cruzan et al. 1998, 2001).

Gastrointestinal Effects. Nausea was observed in humans exposed to 376 ppm styrene after 1 hour of exposure (Stewart et al. 1968). This effect is probably secondary to effects on the central nervous system, although mucociliary transport of styrene aerosol droplets from the upper respiratory tract to the gastrointestinal tract might also contribute to gastrointestinal irritation. A Russian study (Basirov 1975) reviewed by the World Health Organization (WHO 1983) investigated the effects of styrene on digestive function by testing the secretory, excretory, motor, and pepsinogen-generating functions of the stomach in 20 unexposed and 80 exposed workers. The authors reported that some workers in the styrene-butadiene synthetic rubber manufacture exposed to 60–130 mg/m^3 (14–31 ppm) styrene ~~for~~ from <5 to 10 years had decreased digestive function and decreased stomach acidity.

No histological alterations were observed in the stomach or intestines of rats exposed to 1,000 ppm (Cruzan et al. 1998) or mice exposed to 160 ppm (Cruzan et al. 2001) styrene for 2 years.

Hematological Effects. Several studies indicate that inhalation exposure of humans to styrene cause mild or no effects on the blood. In one study, the incidence of abnormal values for hematological parameters including erythrocyte, leukocyte, and platelet counts, and hemoglobin levels for 84 styrene workers generally exposed to <1 ppm styrene for 1–36 years was investigated. However, these workers were also exposed to intermittent high levels of styrene as well as to other chemicals. The percentages of the exposed group with abnormally low hemoglobin and erythrocyte values or abnormally high leukocyte values were less than those percentages in the 62-person control group. There were no abnormal thrombocyte values reported in either the exposed or control groups (Thiess and Friedheim 1978). Findings from a group of 93 workers engaged in the manufacture of styrene polymers and exposed to generally <1 ppm styrene for 1–38 years were also presented in this study; only the incidence of abnormally low erythrocyte counts (in the group exposed to styrene) was found to be statistically

1978). No significant alterations in alanine aminotransferase, aspartate aminotransferase, or γ-glutamyl transferase levels were observed in workers exposed to generally <1 ppm for 1–36 years (Thiess and Friedheim 1978) or 50–120 ppm for 5.1 years (Harkonen et al. 1984). A significant increase in γ-glutamyl transferase levels was observed in workers exposed to 5–20 ppm for up to 20 years; however, no alterations in alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase levels were observed (Lorimer et al. 1978). Another study of workers (Hotz et al. 1980) found significant correlations between the exposure level (as measured by styrene metabolite concentrations in morning urine) and ornithine carbamoyl transferase, alanine aminotransferase, and γ-glutamyl transferase levels. Among workers exposed to 50–100 ppm, the increases in these enzymes were modest, 67.8, 55, and 64.9% of reference levels.

Animal studies provide evidence that the liver is a target tissue for styrene; however, the hepatotoxicity of styrene in mice is inversely related to the duration of exposure. Hepatic effects have been observed following acute- and intermediate-duration exposure, but not after chronic exposure and the severity of the effects decreases with continuing exposure. A 1–4-day exposure, resulted in marked to severe hepatocellular necrosis and degeneration in mice exposed to 250 or 500 ppm (Morgan et al. 1993a, 1993b, 1993c). The necrosis was characterized as centrilobular coagulative necrosis and was accompanied by pooling of erythrocytes in dilated sinusoids (Morgan et al. 1993a). The necrosis was often observed after a single exposure to 500 ppm or a 2-day exposure to 250 ppm, and the severity did not increase with increasing duration (Morgan et al. 1993a). However, continued exposure resulted in regeneration and repair of the initial hepatic damage. After 14 days of exposure, minimal to mild focal necrosis was observed in female mice exposed to 250 ppm, and no hepatic effects were observed in male mice exposed to 250 ppm or male and female mice exposed to 500 ppm (Morgan et al. 1993a). Similarly, a 13-week exposure to 200 ppm resulted in focal loss of hepatocytes with siderosis and centrilobular aggregates of siderophages in female mice (Cruzan et al. 1997). No histological alterations were observed in the livers of mice exposed to 160 ppm for 2 years (Cruzan et al. 2001). Strain differences have also been detected in mice. Morgan et al. (1993c) found that B6C3F1 and C57BL/6 mice were more sensitive than DBA/2 mice, which were more sensitive than Swiss mice. The severity scores for hepatocellular degeneration/necrosis following a 4-day exposure to 250 ppm were 3.2–3.5 in B6C3F1 mice, 3.6 in C57BL/6 mice, 2.4–2.9 in DBA/2 mice, and 2.0 in Swiss mice.

Rats appear to be less sensitive than mice to styrene-induced hepatoxicity. No histological alterations were observed in the livers of Sprague Dawley rats exposed to 1,000 ppm styrene for 13 weeks (Cruzan et al. 1997) or 2 years (Cruzan et al. 1998; Jersey et al. 1978). Parenchymal hydropic degeneration,

subsets in styrene workers exposed to an 8-hour time-weighted average (TWA) of 10–50 ppm. Some of these alterations, particularly the reduction in total T-lymphocytes (CD3+) and T-helper cells (CD4+), may be indicative of reduced cell-mediated immunity. This is supported by the finding of an impaired response to concanavalin in styrene workers exposed to a median styrene concentration of 26 ppm (Tulinska et al. 2000), 187–256 ppm (Somorovská et al. 1999) or 54–56 ppm (Somorovská et al. 1999). No alterations in the response to pokeweed mitogen were observed (Somorovská et al. 1999; Tulinska et al. 2000).

In patch-testing studies of cross-reactors to styrene, styrene epoxide was more sensitizing than styrene itself (Sjoborg et al. 1984). The authors interpreted this as evidence that styrene requires metabolism by skin aryl hydrocarbon hydroxylase to styrene epoxide for its sensitizing activity.

In animals, styrene exacerbated the inflammatory reaction in mice challenged with ovalbumin (Ban et al. 2006). Styrene-only exposure resulted in slight increases in Th2 cytokine (IL-4, IL-5, IL-13) and Th1 cytokine (interferon-γ) levels; however, the statistical significance of these alterations were not reported.

3.2.1.4 Neurological Effects

The available human data suggest that the nervous system is the most sensitive target following chronic-duration inhalation exposure. It is likely the most sensitive target following shorter-term durations, but this has not been as extensively investigated. In studies examining the acute neurotoxicity of styrene, impairment of the vestibular-oculomotor system was observed in experimental subjects exposed to 87 ppm for 1 hour (Odkvist et al. 1982) or 376 ppm for 1 hour (Stewart et al. 1968). No alterations in the performance of balance tests were observed at 216 ppm for 1 hour (Stewart et al. 1968), 117 ppm for 2 hours (Stewart et al. 1968), or 99 ppm for 7 hours (Stewart et al. 1968). Although these NOAELs are higher than the LOAEL identified in the Odkvist et al. (1982) study, the studies are not comparable. The Odkvist et al. (1982) study used sensitive tests of vestibular-oculomotor function compared to the modified Romberg test (subjects stand on one foot with eyes closed, walk heel to toe, touch finger to nose) used in the Stewart et al. (1968) studies. An increase in the reporting of "feeling inebriated" was found in subjects exposed to 376 ppm for 1 hour (Stewart et al. 1968); no increases in subjective symptoms were observed in subjects exposed to 20 ppm for 3–4 hours (Seeber et al. 2004) or 216 ppm for 1 hour (Stewart et al. 1968). Additionally, no alterations in reaction time were observed in subjects exposed to 20 ppm for 3 or 4 hours (Seeber et al. 2004). No human studies examined neurotoxicity following intermediate-duration exposure.

In an international cohort of styrene workers, a significant association between mortality from central nervous system disease and cumulative styrene exposure was found (Welp et al. 1996c). The rate ratio was 3.29 (95% confidence interval [CI] of 0.48–22.65) for workers exposed to 25–49 ppm-years and 16.32 (95% CI 3.47–76.73) for those exposed for 200–349 ppm-years. A similar relationship was found for shorter durations of styrene exposure. The rate ratio was 2.33 (95% CI 0.40–13.56) for workers exposed for 6–11 months and 8.80 (95% CI 1.87–41.33) for workers exposed for 7–9 months. A significant association between mortality from epilepsy and duration of styrene exposure was found; the rate ratio in workers exposed for ≥10 years was 28.4 (95% CI 2.11–381.5). Time since first exposure was also significantly associated with mortality from epilepsy. Significant associations between mental disorders and duration of exposure and between suicide and duration of exposure were also found; however, for both of these causes of death, the rate ratio decreased with increasing duration of exposure and the investigators noted that lifestyle factors, rather than a direct effect of styrene, appear to be the most likely cause of the higher mortality.

A variety of neurological effects have been reported in workers chronically exposed to styrene including altered vestibular function, impaired hearing, impaired color vision, impaired performance on neurobehavioral tests, and increased ~~in~~clinical symptoms. In general, these occupational exposure studies have several limitations. In most cases, the exposure levels reflect current exposure conditions and do not take into consideration past exposure to higher styrene levels that may have resulted in permanent damage. Some workers, particularly laminators, wore respiratory masks with or without canisters; many investigators estimated exposure based on biomarker levels, particularly urinary mandelic acid levels, while others did not. Additionally, significant differences between workers and referents were reported as LOAELs; however, the magnitude of the alteration may have been subclinical. A summary of the neurological effects observed in styrene workers is presented in Table 3-2.

Color vision appears to be one of the more sensitive targets of styrene toxicity, with many studies reporting alterations. Color vision was typically measured using the Lanthony desaturated panel D-15 test in which the subjects were asked to arrange 15 painted caps in a line with definite chromatic sequence; the color confusion index (CCI) quantifies the number of types of mistake. A significant correlation between CCI and urinary mandelic acid concentration (after correction for age) was observed in workers at fiberglass reinforced plastic facilities (Kishi et al. 2001). When workers were divided into three groups based on end-of-shift urinary mandelic acid levels, there were significant differences between CCI in workers with a mean a mandelic acid level of 0.14 or 0.65 g/L and age-matched referents;

LOAEL's here are ~ 1/2 LOAEL from Benignus used for setting chronic inhalation MRL

no difference was found for the third group with a mean mandelic acid level of 0.05 g/L. The investigators estimated that these urinary mandelic acid levels were equivalent to styrene exposure levels of 4, 10, and 46 ppm. Thus, this study identifies a NOAEL of 4 ppm and a LOAEL of 10 ppm for impaired color vision. Similarly, Gong et al. (2002) found significantly higher CCI values in workers at a fiberglass reinforced plastic boat facility with end-of-shift urinary mandelic acid and phenylglyoxylic acid levels of ≥0.24 g/g creatinine or <0.24 g/g creatinine; a mandelic acid plus phenylglyoxylic acid urine level of 0.24 g/g creatinine is equivalent to a styrene exposure level of 10 ppm. A significant increase in CCI was also observed in workers at fiberglass reinforced plastic facilities exposed to a geometric man concentration of 16 ppm, as compared to age-matched controls (Gobba et al. 1991). In contrast to other studies, Gobba et al. (1991) did not find a significant relationship between end-of-shift urinary mandelic acid levels and CCI; however, urinary styrene levels correlated with CCI values. Significantly higher CCI values were observed in fiberglass reinforced workers with a mean urinary mandelic acid levels of 1.06 g/L, which is roughly equivalent to a styrene exposure level of 93 ppm (Eguchi et al. 1995). This study did not find significant alteration in workers with a mean urinary mandelic acid level of 0.02 g/L, equivalent to 8 ppm. Another study of fiberglass reinforced plastic workers (some of this cohort was examined by Gobba et al. 1991 and Campagna et al. 1995) found a significant association between CCI and styrene exposure levels (Campagna et al. 1996). The investigators concluded that color vision impairment could be detected at styrene levels of 4 ppm with a 95% upper confidence limit of 26 ppm. Two other occupational exposure studies using different measures of color vision impairment also found significant alterations. Chia et al. (1994) found significantly poorer color discrimination, after adjusting for age, education, and alcohol consumption, in 21 workers at a fiber-reinforced plastic boat manufacturing facility; the styrene exposure level of 6 ppm was estimated from a mean end-of-shift urinary mandelic acid level of 84.0 mg/g creatinine. No relationship between the total color difference score and the urinary mandelic acid level was found. In 60 workers in the shipbuilding industry with a mean styrene exposure level of 24.3 ppm, a significantly higher incidence of workers with errors in the blue-yellow or red-green ranges, compared to a referent group, was found (Fallas et al. 1992). Total error score was significantly different in workers, with a lifetime weighted average exposure level of 22 ppm styrene, as compared to workers in a low exposure group (9 ppm) (Iregren et al. 2005). Several studies found improvements in color vision following an extended period of no styrene exposure or lower exposure. Triebig et al. (2001) reported a significant improvement in CCI scores following a 4-week period with no styrene exposure; in contrast, no improvement in CCI scores was found in another group of styrene workers following a 1-month period without styrene exposure (Gobba et al. 1991). Two studies found significant improvements in color vision (age-adjusted color confusion score or CCI score) were observed in styrene workers following a decrease in styrene air level (Castillo et al. 2001; Triebig et

found no indications of hearing loss in workers exposed to 18 ppm styrene. Sass-Korstak et al. (1995) did not find significant relationships between lifetime styrene exposure and hearing loss in workers at fiber-reinforced plastics manufacturing facilities. The cumulative styrene exposure level was calculated using data for current exposure (25 ppm for directly exposed workers and 8 ppm for indirectly exposed workers), length of time in each job category, and a downward adjustment for self-reported respirator use. The average noise levels (L_{eq}) were 88.1 and 89.2 dBA for the directly and indirectly exposed workers, for nonexposed workers, a sound level of 80 dBA was assumed. In another study of fiberglass workers (Calabrese et al. 1996), no significant alterations in audiometric tests or auditory brainstem response were observed in workers exposed to a mean styrene level of 36 ppm. Additionally, a 3-week recovery period did not result in any significant changes in auditory brainstem responses (pre- and post-recovery) in nine of the workers.

Other studies have examined workers for styrene-induced vestibular effects. Significant alteration in tests of central vestibulocular and optocular motor movements (i.e., static posturography, smooth eye pursuit, saccade, and vestibulocular reflex tests) were observed in workers at a plastic boat manufacturing facility exposed to a TWA styrene concentration of 18 ppm (Möller et al. 1990). No indications of labyrinthine or peripheral vestibular lesions were observed. Toppila et al. (2006) also found significant alterations in postural stability in workers at fiberglass-reinforced plastic boat manufacturing facilities exposed to 25 ppm styrene. Concentration-related alterations in nystagmus elicited by optokinetic, vestibular, simultaneous optokinetic-vestibular, and saccadic stimulation were observed in rats exposed to 830–4,000 ppm styrene for at least 60 minutes (actual duration of exposure was not reported) (Niklasson et al. 1993). In contrast, Calabrese et al. (1996) did not find significant alterations in visual suppression tests or postural stability in fiberglass plant workers exposed to 36 ppm styrene. Significant alterations in vestibulocular reflex were found. A 3-week recovery period did not result in significant changes in the test results.

Out of place because animal study

Workers exposed to styrene in several industries at mean concentrations of 5–125 ppm had mild sensory neuropathy characterized by decreased sensory conduction amplitude and increased duration, but there were too few people to define a NOAEL ~~no-adverse-effect levels~~ (Rosen et al. 1978). Peripheral neuropathy and reduced nerve conduction velocity was also reported in an individual following a 2-day exposure to an unknown amount of styrene (and other chemicals) (Fung and Clark 1999). Leg weakness, leg muscle cramps, and paresthesia was also reported in two styrene workers (Gobba et al. 1995). Moderate sensorimotor neuropathy of the demyelinating type was diagnosed in both cases based on the clinical symptoms and the decreased motor nerve conduction velocity in the peroneal nerve and decreased

sensory nerve conduction velocity in the sural and median nerves. Several studies have examined nerve conduction velocity in styrene workers. Decreased peripheral conduction velocities in the median and tibial nerves and prolonged latencies of peripheral and cortical somatosensory evoked potentials were observed in female styrene workers exposed to 30–130 ppm (midpoint of the range is 50 ppm) (Štětkářová et al. 1993). Significant decreases in ulnar and peroneal maximum conduction velocities and increased peroneal motor distal latencies were observed in fiber reinforced workers exposed with urinary mandelic acid levels (end of shift) of ≥250 mg/L, as compared to referent workers. Motor distal latencies in the workers with urinary mandelic acid levels ≥250 mg/L were also significantly lower than in workers with urinary mandelic acid levels <250 mg/L (Yuasa et al. 1996). In contrast, no alterations in motor or sensory nerve conduction velocity in the ulnar, median, deep peroneal, or posterior tibial nerve were observed in workers exposed to a TWA styrene concentration of 30 ppm (based on urinary mandelic acid excretion) (Seppalainen and Harkonen 1976), and no alteration in motor or sensory nerve conduction velocity was observed in workers exposed to approximately 100 ppm for a median of 4 years (Triebig et al. 1985). Although Seppalainen and Harkonen (1976) did not find alterations in nerve conduction velocity, they found abnormal EEGs in 24% of the styrene workers, as compared to reported values for the normal population. The mean urinary mandelic acid level (975 mg/dm^3) was higher in workers with abnormal EEGs compared to those with normal readings (750 mg/dm^3). Similarly, a significantly higher absolute EEG power in alpha band in the fronto-temporal region of the brain was found in workers with high styrene exposures, as compared to workers with low-level exposure (Matikainen et al. 1992a).

Seems out of places a couple of sentences previously we're also noting ↓ nerve conduction velocity

The majority of the available animal neurotoxicity studies have focused on hearing impairment. Hearing loss and a loss of outer hair cells (OHC) in the organ of Corti were observed in rats acutely exposed to 1,000 ppm (Campo et al. 2001; Lataye et al. 2003) or 1,600 ppm (Crofton et al. 1994). In contrast, acute exposure of guinea pigs to 1,000 ppm did not result in hearing loss or OHC damage (Lataye et al. 2003). Intermediate-duration exposure studies have consistently found hearing loss and loss of OHC in rats exposed to ≥750 ppm styrene (Campo et al. 2001; Lataye et al. 2000, 2001; Loquet et al. 2000; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992). Exposure to 600–650 ppm resulted in OHC losses but no alterations in hearing threshold (Loquet et al. 1999; Makitie et al. 2002; Pouyatos et al. 2002). A NOAEL of 300 ppm was identified by Makitie et al. (2002).

Other neurological effects that have been observed in animal studies include an increase in astroglial alterations at 320 ppm (Rosengren and Haglid 1989) and a decrease in nerve conduction velocity in rats exposed to 2,000 ppm, but not 200 ppm, for 32 weeks (Yamamoto et al. 1997).

The highest NOAEL values and all reliable LOAEL values for neurological effects in each species and duration category are recorded in Table 3-1 and plotted in Figure 3-1.

3.2.1.5 Reproductive Effects

Information on the reproductive effects of styrene in humans is available from epidemiological studies of the reproductive outcomes of females employed in the various industrial operations in which styrene is used. However, exposures to styrene were not adequately quantified in any of the studies cited. In one study, spontaneous abortions among 9,000 Finnish chemical workers from 1973 to 1976 were analyzed (Hemminki et al. 1980). The risk of spontaneous abortion expressed as number of abortions per 100 pregnancies) was significantly higher in women employed in styrene production compared to all women in Finland 15.0 vs. 5.5). However, this increase was not detected in a follow-up study of the same workers (Hemminki et al. 1984). An increase in the occurrence of spontaneous abortions was also observed in a study of 76 women involved in processing polystyrene plastics (McDonald et al. 1988); the ratio of observed to expected abortions was 1.58 (95% CI 1.02–2.35). The possible embryotoxic effects of styrene on 67 female lamination workers compared to 67 age-matched controls were evaluated in a second study (Harkonen and Holmberg 1982). The number of births was significantly lower among the workers exposed to styrene. This result was explained in part by a greater number of induced abortions in the styrene-exposed group. The number of spontaneous abortions was not elevated in the exposed women. No increased risk of spontaneous abortions among workers processing polymerized plastics or heated plastics made of vinyl chloride or styrene was reported (Lindbohm et al. 1985). The authors reported that the statistical power of the study was low due to the small study population. These studies are not conclusive since the workers were exposed to chemicals other than styrene in the workplace and the concentrations of styrene were not adequately reported. Two studies have examined the potential of styrene to induce menstrual disturbances. A significant increase in the incidence of oligomenorrhea was observed in petrochemical industry workers; the adjusted odds ratio was 1.65 (95% CI 1.05–2.55) (Cho et al. 2001). Although the odds ratio includes an adjustment for exposure to other aromatic chemicals, there was potential for exposure to other chemicals. In contrast, no significant alterations were observed in women working at reinforced plastics facilities with a mean styrene exposure level of 52 ppm for women directly exposed to styrene and 13 ppm for those indirectly exposed (Lemasters et al. 1985). Several studies have examined levels of prolactin, follicle stimulating hormone, and luteinizing hormone levels in female styrene workers; the results of these studies are discussed in Section 3.2.1.2, Endocrine Effects.

neurodevelopmental effect of styrene (Cruzan et al. 2005a). No alterations in locomotor activity, acoustic startle response, or brain morphology and weights were observed in this study. Another study found delays in righting reflex and incisor eruption in the offspring of rats exposed to 300 ppm on gestational days 6–20 (Katakura et al. 2001). This study (Katakura et al. 1999, 2001) also found alterations in homovanillic acid levels in the cerebrum and 5-hydroxyindoleacetic acid levels in the hippocampus of the offspring in the 300 ppm group.

The highest NOAEL values and all reliable LOAEL values for developmental effects in each species and duration category are recorded in Table 3-1 and plotted in Figure 3-1.

3.2.1.7 Cancer

A number of studies have examined the carcinogenic potential of styrene in workers at styrene manufacturing and polymerization facilities, reinforced plastics facilities, and styrene-butadiene manufacturing facilities and among community members exposed to elevated styrene workers. Although there are several epidemiologic studies which suggest there may be an association between styrene exposure and an increased risk of leukemia and lymphoma, the evidence is generally inconclusive due to multiple chemical exposures and inadequate documentation of the levels and durations of exposure to styrene.

Of the industries examined, workers employed at glass-reinforced plastics manufacturing facilities are likely to be exposed to higher levels of styrene and lower to have a potential for exposure to other carcinogenic agents. Some studies of glass-reinforced plastic workers have found suggestive evidence of increased cancer risks, particularly in workers with longer exposures to higher levels of styrene. No alterations in the number of deaths from cancer were observed in workers with high styrene exposure (mean levels at two facilities were 42.5 and 71.5 ppm) (Okun et al. 1985). In a follow-up study of these workers (Ruder et al. 2004), a significant increase in the number of deaths from urinary tract cancer (standardized mortality ratio [SMR] 3.44; 95% CI 1.26–7.50) was observed among workers with high styrene exposure; a trend for increasing SMRs for urinary tract cancer with increasing duration of exposure was also observed. The SMRs were not significantly elevated for other cancer types. In a very large epidemiological study of nearly 16,000 workers in the styrene plastic industry, the death rate from leukemia was twice as high in areas of high exposure as in areas of low exposure (Wong 1990); however, there were no statistically significant differences. In a follow-up study conducted 12 years later (Wong et al. 1994), significant increases in deaths from all cancers (SMR 115.5, 95% CI 104.8–127.1), cancer of

of deaths from lymphatic and hematopoietic cancer were observed in workers exposed to 1–4 ppm styrene and a 15-year minimum latency (SMR 160; 95% CI 102–238); however, significant alterations were not found in workers exposed to ≥5 ppm or with longer latency periods. In another study of workers involved in the styrene production, polymerization, or processing, a statistically significant excess of lymphoma deaths (3 deaths observed versus 0.56 expected) was reported; 2 of the 3 deaths occurred in men <40 years of age who had been exposed for at least a year (Hodgson and Jones 1985). However, the lack of association with actual exposure levels or specific durations and the small number of observed deaths requires cautious interpretation. No significant alterations in the number of deaths from cancer were observed in workers (1960 subjects) exposed to styrene in a production and polymerization facility (Frentzel-Beyme et al. 1978). In a study of workers at a styrene-polystyrene manufacturing facility who had at least 5 years of exposure, there were no significant increases in cause-specific mortality (Nicholson et al. 1978). However, when workers employed for <5 years were included in the analysis, there was an apparent increase in the number of deaths from lymphoma or leukemia (statistical analysis not conducted).

Why are these older studies when studies that have been discussed up to this point were for workers in the 60s 70s.

A number of older studies provide suggestive evidence of increased risk of lymphatic and hematopoietic cancers in workers at styrene-butadiene rubber manufacturing facilities (Matanoski and Schwartz 1987; Matanoski et al. 1990; McMichael et al. 1976; Meinhardt et al. 1982). A case-control study (Matanoski et al. 1993, 1997; Santos-Burgoa et al. 1992) provides suggestive evidence that the increase in leukemia was due to exposure to 1,3-butadiene rather than to styrene exposure. However, increases in the risk of lymphosarcoma and myeloma were associated with styrene exposure (Matanoski et al. 1997). A cohort mortality study conducted by Delzell and associates (Delzell et al. 1996; Macaluso et al. 1996) examined workers at many of the same styrene-butadiene rubber manufacturing facilities examined by Matanoski and associates and Meinhardt and associates. In this examination of 15,649 male synthetic rubber workers employed for at least 1 year at one of eight styrene-butadiene rubber manufacturing facilities in the United States or Canada, significant increases in deaths from leukemia were observed among hourly employees (SMR 143; 95% CI 104–191), particularly among workers employed for ≥10 and ≥20 years since hire (SMR 224; 95% CI 149–323) (Delzell et al. 1996). When workers were divided by year of hire and age at death, leukemia deaths were elevated in workers who were hired between 1950 and 1959 (SMR 200; 95% CI 122–310) and who were <55 years of age at the time of death (SMR 179; 95% CI 104–287). Using calculated estimates of exposure levels to 1,3-butadiene, styrene, and benzene, Macaluso et al. (1996) found that 75% of the cohort was exposed to 1,3-butadiene with a median cumulative exposure of 11.2 ppm-years, 83% of the cohort was exposed to styrene with a median cumulative exposure of 7.4 ppm-years, and 25% of the cohort was exposed to benzene with a cumulative

exposure of 2.9 ppm-years. Among workers with leukemia, 86% had 1,3-butadiene exposure and 90% had styrene exposure; median cumulative exposure levels of 1,3-butadiene and styrene were about 3 times higher than the rest of the cohort. Workers with a cumulative exposure of 20–79 ppm 1,3-butadiene had a relative risk of leukemia mortality (after adjustment by race, age, and cumulative styrene exposure) that was 50% higher than workers with a cumulative exposure of 0.1–19 ppm and workers with a cumulative exposure of >80 ppm had a 70% higher relative risk than the low exposure group; the progressive of relative risk with increasing cumulative exposure was statistically significant. Although a similar progression was observed for cumulative styrene exposure, the trend was not statistically significant. A follow-up to the Delzell et al. (1996) study, which tracked deaths for an additional 7 years (Sathiakumar et al. 2005), found similar results. A significant increase in deaths from leukemia was observed among hourly workers employed for >10 years and hired 20–29 years earlier (SMR 258; 95% CI 156–403). Increases in deaths for colorectical cancer among workers employed for >10 years and hired 20–29 years earlier (SMR 147; 95% CI 103–205) and deaths from prostate cancer among workers employed for <10 years and hired >30 years earlier (SMR 155; 95% CI 113–206). Significant increases deaths from leukemia were observed in workers involved in polymerization, coagulation, and finishing processes, maintenance workers, and laboratory workers; these workers had the highest potential exposure to 1,3-butadiene, styrene, and possibly dimethyldithiocarbamate. Subsequent analysis of these data using updated exposure assessments (Cheng et al. 2007; Delzell et al. 2001; Graff et al. 2005) found that the increased risk of leukemia was positively associated with 1,3-butadiene exposure. Positive associations between cumulative 1,3-butadiene exposure (ppm-years) and leukemia and between cumulative styrene exposure and leukemia were observed; the associations were only statistically significant at the highest cumulative exposure levels for 1,3-butadiene (≥362.2 ppm-years) or styrene (≥60.4 ppm) (Delzell et al. 2001). However, when the relative risks were adjusted for 1,3-butadiene and dimethyldithiocarbamate cumulative exposure, cumulative styrene exposure was no longer significantly associated with leukemia (Delzell et al. 2001; Graff et al. 2005). Because styrene, 1,3-butadiene, and dimethyldithiocarbamate exposure were correlated, it is difficult to separate the risks for each individual compound.

*As noted earlier these should not be listed under paragraph that has sentence stating the studies were older. These are some of the most recent publications looking at cancer risk to workers in the styrene-butadiene industry.

Several population-based studies have examined the possible carcinogenicity of styrene. A case-control study found a significant increase in prostate cancer (odds ratio of 5.5; 95% CI 1.4–21.8) and rectal cancer (odds ratio of 5.1; 95% CI 1.4–19.4) among workers with medium to high exposure to styrene (Gerin et al. 1998). Workers in the following professions were considered to have medium to high styrene exposure: motor vehicle painters, motor vehicle repairers, firemen, and plastic mouldmakers. Another study found a significant increase in the incidence of rectal cancer (SIR 3.11; 95% CI 1.14–6.77) among individuals with occupational exposure to styrene (Antilla et al. 1998). A limitation of both of

these studies is the lack of exposure information, including levels of styrene and confounding exposure to other chemicals; thus, it is difficult to ascribe the increased cancer risks to styrene exposure. Loughlin et al. (1999) examined former students who attended a high school adjacent to synthetic styrene-butadiene rubber production facilities between 1963 and 1993 and found no significant alterations in deaths from lymphatic and hematopoietic cancer. Two studies have examined the possible association between styrene exposure and breast cancer. A case-control study by Cantor et al. (1995) found significant elevations in the risk of breast cancer among women possibly exposed to styrene in the workplace. Coyle et al. (2005) found a significant higher incidence of age-adjusted breast cancer rate in men and women, women, and women ≥50 years of age and living in counties with EPA toxics release inventory (TRI) facilities with on-site releases of styrene. As with the other population-based studies, these studies did not monitor styrene levels or exposure to other potentially carcinogenic chemicals and thus provided limited information on the carcinogenic potential of styrene.

The carcinogenicity of styrene has been examined in three studies in rats (Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1982) and one study in mice (Cruzan et al. 2001). No significant increases in the incidence neoplastic lesions were observed in rats exposed to styrene concentrations as high as 1,000 ppm 6 hours/day, 5 days/week for 2 years (Cruzan et al. 1998). Similarly, exposure of female rats to 600 or 1,000 ppm styrene 6 hours/day, 5 days/week for 21 months did not result in styrene-related increases in the incidence neoplastic tumors (Jersey et al. 1978); a high incidence of chronic murine pneumonia in the control and 1,000 ppm male rats precludes the use of the male data for assessing the carcinogenic potential of styrene. A non-concentration-related increase in the incidence of malignant mammary tumors were observed in female rats exposed to 100, 200, or 300 ppm styrene 4 hours/day, 5 days/week for 52 weeks (Conti et al. 1988); the incidences were 6/60, 6/30, 4/30, 9/30, 12/30, and 9/30 in the 0, 25, 50, 100, 200, and 300 ppm groups, respectively. No other significant increases in specific tumors were observed in this study (Conti et al. 1988; Maltoni et al. 1982). The findings of the Conti et al. (1988) study conflict with those of Cruzan et al. (1998), who found a concentration-related decrease in mammary tumors in female rats exposed to similar or higher styrene concentrations for a longer duration. In contrast to the results in rat studies, significant increases in the incidence of bronchioalveolar carcinoma were observed in female mice exposed to 160 ppm 6 hours/day, 5 days/week for approximately 2 years (Cruzan et al. 2001). Significant trends for increasing incidences of bronchioalveolar ademona were also observed for the male and female mice.

As reviewed by IARC (2002) and Cruzan et al. (2002), toxicokinetic differences in the metabolism of styrene in the lungs have been observed in humans, rats, and mice. In rats and mice, Clara cells have the

ability to metabolize styrene to styrene 7,8-oxide in the lung; humans have limited ability to metabolize styrene to styrene 7,8-oxide in the lung. A physiologically based pharmacokinetic (PBPK) model predicts that the total amount of styrene oxide in the terminal bronchioles in mice is 10 times higher than in rats and 100-fold higher than in humans. In addition to these quantitative differences in the generation of styrene 7,8-oxide between rats and mice, there are qualitative differences in styrene metabolism. Mice produce a higher levels of the R-enantiomer of styrene oxide, as compared to rats; the R-enantiomer has been shown to be more potent pneumotoxic than the S-enantiomer. The ratio of R- to S-enantiomers ranges from 2.2 to 2.87 in mice exposed to 20–160 ppm styrene and from 0.7 to 0.73 in rats exposed to 50–1,000 ppm. Thus, mice appear to be very sensitive to the induction of lung tumors and the mechanism of inducing lung tumors is not likely to be relevant to humans.

This information needs to be in PK section on metabolism

3.2.2 Oral Exposure

No studies were located regarding health effects in humans after oral ingestion of styrene. Based on the animal data that follow, the oral toxicity of styrene in humans would be expected to be low to moderate.

3.2.2.1 Death

No deaths in humans from ingesting styrene have been reported in the evaluations of case studies (EPA 1989c; Gosselin et al. 1984; NIOSH 1983).

The approximate reported oral LD_{50} for male and female rats was 5,000 mg/kg (Wolf et al. 1956). A 100% survival rate and 100% mortality rate were reported in rats exposed to single oral doses of styrene (observation period 2 weeks) at 1,600 and 8,000 mg/kg, respectively (Spencer et al. 1942). Death in this study was mainly due to pronounced irritation of the esophagus and stomach. In another study, female mice were given a single oral dose of 1,350 mg/kg styrene on the 17th day of pregnancy (Ponomarkov and Tomatis 1978). After weaning, the progeny received the same dose once per week. The treatment was suspended after 16 weeks due to high mortality among the progeny (including both males and females). Fifty percent of the males and 20% of the females had died after 20 weeks, despite the suspension of treatment at week 16. The cause of death was liver necrosis and lung congestion. A high mortality rate was reported in 40 female rats exposed to 250 mg/kg/day styrene for 52 weeks (Conti et al. 1988). Mortality was significantly elevated in male and female rats administered styrene by gavage at a dosage level of 2,000 mg/kg/day for 78 weeks (NCI 1979b). In this study, mortality was unaffected at dosage levels of 500 and 1,000 mg/kg/day in male and female rats. Male mice administered styrene at

3.4 TOXICOKINETICS

3.4.1 Absorption

3.4.1.1 Inhalation Exposure

The uptake of styrene following inhalation exposure in humans and animals is rapid (Ramsey and Andersen 1984; Ramsey and Young 1978; Ramsey et al. 1980; Withey and Collins 1979; Withey and Karpinski 1985). Pulmonary retention of inhaled styrene in humans is approximately 2/3 of the administered concentrations (Engstrom et al. 1978a, 1978b). For example, male human subjects were exposed to styrene in inspired air during 30-minute rest and three 30-minute work periods on a bicycle ergometer. The mean uptake was approximately 63% (range was 59–70%) of the amount of inspired styrene. In exercising volunteers exposed to 50 ppm styrene for 2 hours, an average of 66.5% of the inhaled styrene was absorbed (Johanson et al. 2000). Another study in volunteers exposed to 50 ppm styrene for 2 hours during exercise calculated that 64% of the styrene was absorbed (Norstöm et al. 1992). Exposures of rats to styrene concentrations of 50–2,000 ppm for 5 hours yielded blood uptakes that showed a continued and increasing rapid absorption, proportional to the styrene air level (Withey and Collins 1979). Plateau levels of styrene in rats' blood were reached within 6–8 hours during exposures ranging from 80 to 1,200 ppm styrene for up to 24 hours (Ramsey and Young 1978). Physiologically based inhalation pharmacokinetic (PBPK) models indicate that styrene metabolism becomes saturated at inhaled levels above 200 ppm in mice, rats, and humans (Ramsey and Andersen 1984). When inhaled concentrations are below 200 ppm, the ratio of styrene concentration in the blood to inhaled air is moderated by perfusion-limited metabolism rather than blood:air partition coefficients.

Seems out of place since this isn't absorption

3.4.1.2 Oral Exposure

No studies were located regarding absorption in humans after oral exposure to styrene.

The absorption of styrene from the gastrointestinal tract was rapid and complete in rats deprived of food overnight and given styrene by gavage at a total dose of 3.147 mg styrene in 10 mL aqueous solution. A peak blood level of 6 μg/mL was reached in a few minutes. There was a much slower uptake of the styrene administered in vegetable oil (Withey 1976). Styrene administered in vegetable oil at a total dose of 32.61 mg produced a peak level of 12 μg/mL. This was reached at about 100 minutes (Withey 1976).

What was dose per BW?

3.4.1.3 Dermal Exposure

Limited data indicate that absorption of styrene via the dermal route is probably low compared to absorption via other routes. When liquid styrene was applied to the forearms of male subjects, the absorption rate was estimated to be 9–15 mg/cm^2/hour (Dutkiewicz and Tyras 1968). By contrast, the rate of absorption through human skin was very low (1±0.5 μg/cm^2/minute) in subjects who dipped one hand into liquid styrene (Berode et al. 1985). ~~It is believed that~~ the higher absorption rate reported by Dutkiewicz and Tyras (1968) ~~also~~ likely included the disappearance rate of the solvent from the surface of the skin (Guillemin and Berode 1988). Riihimaki and Pfaffli (1978) demonstrated that in humans, dermal exposure to moderate concentrations of styrene vapor (300 and 600 ppm) resulted in percutaneous penetration corresponding to approximately 0.1–2% of the amount estimated to be absorbed from the respiratory tract. Similarly, Limasset et al. (1999) did not find significant differences in the levels of urinary metabolites in workers wearing total protective equipment (insulating suit and respiratory mask) and those wearing a respiratory mask only.

Although absorption of styrene applied to the abdomen of rabbits was reported, there was no information on absorption rates (Spencer et al. 1942). Dermal exposure to styrene resulted in peak blood levels of 5.3 μg/mL within 1 hour of exposure (Morgan et al. 1991). ? What dose, what species, duration

3.4.2 Distribution

- Since styrene fat soluble may be worthwhile to give either fat:air or fat:blood partition coefficient

A blood:air partition coefficient of 40.2 was calculated for rats (Gargas et al. 1989). Fisher et al. (1997) calculated a human blood:air partition coefficient of 69.74 and a breast milk:blood partition coefficient of 2.17.

Change to μg/L to emphasize magnitude relative to workers

In a study of 81 adults without occupational exposure to styrene, average blood styrene levels were 221 ng/L; in comparison, blood styrene levels in reinforced plastics industry workers were 1,068–1,590 μg/L at the end of workshift and 60–119 μg/L in the morning after exposure (Brugnone et al. 1993).

3.4.2.1 Inhalation Exposure

Inhalation studies in both humans and animals resulted in the widespread distribution of styrene with the highest concentration in adipose tissue.

Three humans were exposed to 8–20 ppm styrene which resulted in a mean daily uptake of 193–558 mg styrene (Engstrom et al. 1978b). The concentration of styrene in adipose tissue was 2.8-8.1 mg/kg at the beginning of the week and 4.7–11.6 mg/kg at the end of the week. The authors estimated the half-life of styrene in the subcutaneous fat of humans to be about 72 hours. Subsequent studies by this author confirmed this estimate and reported the half-life of styrene in adipose tissue to be 24–96 hours (Engstrom et al. 1978a).

p58, line 15 t/2 given as 2-4 days

Give in ppm based on std rm temp at m pressure

Fiberglass factory workers exposed to >215 mg/m^3 of styrene for 8-hour work shifts had blood styrene levels which ranged from 120 to 684 µg/L at the end of the shift (Apostoli et al. 1983). The concentrations of urinary MA and phenylglyoxilic acid (PGA) were 133–2,100 and 107–685 mg/L, respectively. These levels were also determined at the end of the work-shift. Distribution of styrene was also studied in adult men exposed to about 300 mg/m^3 of styrene for 2 hours during light physical exercise (Wigaeus et al. 1983). Blood styrene reached a level of approximately 20 µmol/L after 75 minutes. The concentration of styrene in adipose tissue was about 50 µmol/kg after 30–90 minutes of exposure.

also give in ppm

It would be useful to give comparable units to help with comparisons. Previously blood listed as µg/L

Rats were exposed for 5 hours to styrene at concentrations ranging from 50 to 2,000 ppm (Withey and Collins 1979). Tissue concentrations of styrene in the heart, liver, lung, kidney, spleen, brain, and perirenal fat demonstrated different patterns of distribution as the dose increased. The styrene concentration in perirenal fat was 10 times greater than in other organs. The largest amounts of styrene were found in the subcutaneous fat of male rats exposed to about 45 ppm of radioactively labeled styrene in the inspired air for 1–8 hours (Carlsson 1981). The concentration increased steadily during the first 4 hours of exposure. Styrene concentrations in brain tissue and muscles were about 70% of the arterial blood value. Other investigators (Ramsey and Andersen 1984; Ramsey and Young 1978; Savolainen and Pfaffli 1978; Withey 1976) demonstrated that higher levels of styrene in adipose tissue increase with higher exposures to styrene. Styrene was found to distribute to the fetuses of pregnant rats after inhalation exposure, but at concentrations much lower than those measured in maternal organs and tissues (Withey and Karpinski 1985).

3.4.2.2 Oral Exposure

No studies were located regarding distribution in humans after oral exposure to styrene.

An oral dose of 20 mg/kg of ^{14}C styrene was administered to male and female rats (Plotnick and Weigel 1979). Tissue levels peaked at 4 hours or earlier after dosing. Less than 10% of the administered dose was found in the stomach, small intestine, and large intestine 8 hours after dosing. The kidney had the highest concentration of radioactivity at all time intervals, with decreasing amounts in the liver and pancreas. Fat tissue showed increased levels after 2 hours. All tissue levels were below 1 μg/g at 24 hours and at 48 and 72 hours were below the limit of detection. Excretion data from the Plotnick and Weigel (1979) study are presented in Section 3.4.4.2.

3.4.2.3 Dermal Exposure

No studies were located regarding distribution in humans after dermal exposure to styrene.

Immersion of rats' tails in pure liquid styrene for 1 hour resulted in styrene levels in the liver and brain that were estimated to be between 50 and 70% of the concentrations found in the same organs after 4-hour inhalation exposure to a vapor concentration of 11.8 g/m^3 (Shugaev 1969). A skin:air partition coefficient of 91.9 was calculated ~~using~~ for rat skin (Mattie et al. 1994).

3.4.3 Metabolism

There have been numerous studies, conducted primarily via inhalation, that address the metabolism of styrene in humans and animals (Drummond et al. 1989; Engstrom et al. 1976; Korn et al. 1984, 1987; Leibman 1975; Lof et al. 1983; Withey and Collins 1979; Young et al. 1979). The proposed pathways of styrene metabolism are shown in Figure 3-3. Styrene is metabolized by the microsomal NADPH-cytochrome P-450 dependent mono-oxygenase to styrene oxide. The styrene oxide is then hydrated to phenylethylene glycol (styrene glycol). This transformation is catalyzed by microsomal epoxide hydratase. The styrene glycol is then metabolized directly to MA or to benzoic acid and then hippuric acid. Mandelic acid is also metabolized to PGA. The MA, hippuric acid, and PGA are excreted in the urine. In another pathway, styrene oxide is metabolized by cystolic glutathione-S-transferase to mercapturic acids appearing in the urine as hydroxyphenylethyl mercapturic acid. A minor metabolic pathway of styrene in rats involves the formation of 1- and 2-phenylethanol and ring hydroxlation to form vinyl phenol as urinary metabolites. The presence of 4-vinylphenol has been reported in the urine of workers exposed to styrene, but this may have been due to the contamination of the styrene to which the subjects were exposed (Pfaffli et al. 1981). The urinary metabolites that predominate in humans are MA and PGA. In rats, the predominant urinary metabolites are MA, PGA, hippuric acid, and glucuronide. Metabolic conversion to styrene-7,8-epoxide (styrene oxide) by the microsomal mixed function oxidase

and epoxide hydratase from the liver and spleen of several rodent species has been demonstrated (Belvedere and Tursi 1981; Cantoni et al. 1978; Leibman 1975; Lof et al. 1984; Vainio et al. 1979). However, styrene oxide has only been found at low concentration, close to detection levels (0.02 μmol/L), in the blood of workers exposed to styrene (Lof et al. 1986a; Mendrala et al. 1991) investigated the species differences in the *in vitro* hepatic metabolism of styrene. The results indicated that mice had the greatest capacity to produce styrene oxide (highest styrene epoxidase activity), followed by rats and then humans. In addition, humans may have the highest capacity to metabolize styrene oxide to styrene glycol, since the human form of styrene oxide hydratase had the highest affinity (lowest Km) for styrene oxide. Assuming that styrene oxide is the metabolite responsible for styrene-induced toxicity (see below) the results of this study indicate that care must be taken in extrapolation of data from animal studies to humans for risk assessment.

Needs references

3.4.4 Elimination and Excretion

3.4.4.1 Inhalation Exposure

Several studies have demonstrated that styrene is almost totally excreted as urinary metabolites in humans, and at higher doses, the elimination profile indicates saturation of metabolic excretion or processes (Ramsey and Young 1978; Ramsey et al. 1980). Most of the inhaled styrene is excreted in urine as MA and PGA. In a study of the excretion of styrene and its metabolites resulting from a 100-ppm/8-hour inhalation exposure, 2.6% of the total uptake was excreted as unchanged styrene in exhaled air (Guillemin and Berode 1988). The metabolites MA, PGA, and hippuric acid were excreted in the urine at 56.9, 33, and 7.5% of the absorbed dose, respectively. In exercising volunteers exposed to 50 ppm styrene for 2 hours, 0.7–2.2% of the retained dose was exhaled as unchanged styrene (Johanson et al. 2000). Peak levels of styrene in the urine were measured immediately after exposure termination, whereas urinary excretion of MA and PGA peaked at 2 hours after exposure termination. MA excretion accounted for 6–29% of the estimated retained dose and PGA excretion accounted for 4–6%; the halftime excretion rates of MA and PGA were 2.2–4.2 and 3.5–13.9 hours, respectively. Phenylaceturic acid and hippuric acid was also detected in the urine samples collected 2 hours after exposure termination. At this time point, MA account for 73% of the total excreted metabolites, PGA 18%, phenylaceturic acid 4.5%, and hippuric acid 5.7%. In styrene workers exposed to 29–42 ppm styrene, both R-mandelic acid and S-mandelic acid were detected in the urine (Hallier et al. 1995). The ratio of R- to S-mandelic acid ranged from 0.7 to 1.2 in 19 of the 20 workers; in the last worker, the ratio was 2.2.

An alternative pathway for the metabolism of styrene 7,8-oxide is conjugation with glutathione, resulting in the excretion of mercapturic acids. Low levels of mercapturic acids have been detected in workers exposed to an unspecified amount of styrene (Maestri et al. 1997a). The mean concentrations of styrene metabolites were 580 mg/g creatinine mandelic acid, 174 mg/g creatinine phenylglyocylic acid, 1.517 mg/g N-acetyl-S-(1-phenyl-2-hydroxyethyl)-cysteine S- enantiomer, 0.0637 mg/g N-acetyl-S-(1-phenyl-2-hydroxyethyl)-cysteine R- enantiomer, and 1.519 mg/g N-acetyl-S-(2-phenyl-2-hydroxyethyl)-cysteine. Another study of styrene workers (exposure level of 29–41 ppm) only detected styrene-specific mercapturic acid in 1 of 20 workers (Hallier et al. 1995). Similarly, in volunteers exposed to 50 ppm for 2 hours during exercise, N-acetyl-S-(2-phenyl-2-hydroxyethyl)-cysteine was not detected in urine samples collected up to 5 hours after exposure termination (Norström et al. 1992).

In volunteers exposed to 80 ppm styrene, styrene is cleared from the blood in a biphasic manner, indicating a two-compartment pharmacokinetic model. The half-lives for the rapid and slow clearance phases are 0.58 and 13.0 hours, respectively. The half-life of styrene in subcutaneous adipose tissue of humans is 2–4 days (Engstrom et al. 1978a). The quantities of the major metabolites of styrene in urine compared with the quantity of styrene eliminated unchanged in expired air indicated that approximately 97% is cleared by the metabolic route (Ramsey et al. 1980).

→ p 55 line 5, the values 24 - 96h.

Another human inhalation study determined that between 59 and 66% of inhaled styrene (50–200 ppm) was retained after a 4–8-hour exposure (Guillemin and Bauer 1979). Urinary elimination of MA was biphasic with a half-life for the first phase of 4 hours and for the second phase, 25 hours. These findings were comparable to those reported by Engstrom et al. (1976). The half-life of urinary elimination of PGA was determined to be 11 hours. This was regarded by the authors as being the first phase of elimination since MA is a precursor of PGA.

A lactational transfer pharmacokinetic model developed by Fisher et al. (1997) predicted that exposure to 50 ppm styrene would result in 0.650 mg styrene being ingested by a nursing infant over a 24-hour period.

? Not necessarily elimination. Could also be given as absorption, particularly with current wording.

Styrene is almost totally excreted as urinary metabolites in animals. The blood elimination curve for rats is biphasic exponential at 80 and 200 ppm styrene over 6 hours. For exposures >600 ppm exposure levels for 6 hours duration), a nonlinear blood elimination curve following Michaelis-Menten kinetics was observed. In going from 80 to 1,200 ppm (a 15-fold increase) the area under the blood concentration curves increases by 112-fold (Ramsey and Young 1978; Young et al. 1979). Rats exposed to 50–

2,000 ppm styrene by inhalation for 5 hours exhibited a dose dependent biphasic pattern of elimination (Withey and Collins 1979). Bergman 1979 ^{14}C-styrene inhalation in mice majority radioactivity localized in kidney presumably due to urine metabolites

3.4.4.2 Oral Exposure

No studies were located regarding excretion in humans after oral exposure to styrene.

Excretion of styrene was studied in the same rats for which there was good distribution data (Plotnick and Weigel 1979). Styrene was rapidly excreted in the urine with 90% of the dose detected in the urine within 24 hours of administration. Less than 2% of the dose was found in the feces. Detectable tissue levels were not found 48 and 72 hours after administration. Sbrana et al. 1983 · mice ^{14}C-styrene 80% dose in urine

3.4.4.3 Dermal Exposure

In a study of the absorption of liquid styrene applied to the forearms of male volunteers, about 13% of the absorbed dose was excreted as MA (Dutkiewicz and Tyras 1968).

No studies were located regarding excretion in animals after dermal exposure to styrene.

3.4.5 Physiologically Based Pharmacokinetic (PBPK)/Pharmacodynamic PD) Models

delete here

Insert parenthesis (PB insert (PBPK/

Physiologically based pharmacokinetic (PBPK) models use mathematical descriptions of the uptake and disposition of chemical substances to quantitatively describe the relationships among critical biological processes (Krishnan et al. 1994). PBPK models are also called biologically based tissue dosimetry models. PBPK models are increasingly used in risk assessments, primarily to predict the concentration of potentially toxic moieties of a chemical that will be delivered to any given target tissue following various combinations of route, dose level, and test species (Clewell and Andersen 1985). Physiologically based pharmacodynamic (PBPD) models use mathematical descriptions of the dose-response function to quantitatively describe the relationship between target tissue dose and toxic end points. insert parenthesis

PBPK/PD models refine our understanding of complex quantitative dose behaviors by helping to delineate and characterize the relationships between: 1) the external/exposure concentration and target tissue dose of the toxic moiety, and 2) the target tissue dose and observed responses (Andersen and Krishnan 1994; Andersen et al. 1987a). These models are biologically and mechanistically

based and can be used to extrapolate the pharmacokinetic behavior of chemical substances from high to low dose, from route to route, ~~between~~ among species, and ~~between~~ among subpopulations within a species. The biological basis of PBPK models results in more meaningful extrapolations than those generated with the more conventional use of uncertainty factors.

since more than 2 animal species + subpops of humans

The PBPK model for a chemical substance is developed in four interconnected steps: 1) model representation, 2) model parameterization, 3) model simulation, and 4) model validation (Krishnan and Andersen 1994). In the early 1990s, validated PBPK models were developed for a number of toxicologically important chemical substances, both volatile and nonvolatile (Krishnan and Andersen 1994; Leung 1993). PBPK models for a particular substance require estimates of the chemical ~~substance~~-specific physicochemical parameters, and species-specific physiological and biological parameters. The numerical estimates of these model parameters are incorporated within a set of differential and algebraic equations that describe the pharmacokinetic processes. Solving these differential and algebraic equations provides the predictions of tissue dose. Computers then provide process simulations based on these solutions.

Computers solve the ODEs in addition to providing the simulations

The structure and mathematical expressions used in PBPK models significantly simplify the true complexities of biological systems. If the uptake and disposition of the chemical substances are adequately described, however, this simplification is desirable because data are often unavailable for many biological processes. A simplified scheme reduces the magnitude of cumulative uncertainty. The adequacy of the model is, therefore, of great importance, and model validation is essential to the use of PBPK models in risk assessment.

What is meant by this? A simplified model can still have uncertainty associated with it.

PBPK models improve the pharmacokinetic extrapolations used in risk assessments that identify the maximal (i.e., the safe) levels for human exposure to chemical substances (Andersen and Krishnan 1994). PBPK models provide a scientifically sound means to predict the target tissue dose of chemicals in humans who are exposed to environmental levels (for example, levels that might occur at hazardous waste sites) based on the results of studies where doses were higher or were administered in different species. Figure 3-4 shows a conceptualized representation of a PBPK model.

If PBPK models for styrene exist, the overall results and individual models are discussed in this section in terms of their use in risk assessment, tissue dosimetry, and dose, route, and species extrapolations.

Several investigators have developed toxicokinetic models for styrene (Csanády et al. 1994, 2003; Jonsson and Johanson 2002; Leavens and Bond 1996). The Csanády et al. (1994, 2003) model is useful for evaluating the carcinogenic risk associated with inhalation exposure to styrene. As discussed in Section 3.2.1.7, species differences exist in the metabolism of styrene in the lungs of rats, mice, and humans; these differences result in increased sensitivity of mice. Jonsson and Johanson (2002) developed a population-based PBPK model for styrene, which decreased the intraindividual variability for estimating the metabolic capacity for styrene in humans. Leavens and Bond (1996) described initial work on developing a model for co-exposure to 1,3-butadiene and styrene in mice. Some of these models provide strong support for the observed differences in styrene toxicity between rats, mice, and humans. As discussed further in Section 3.5.3, some have primarily focused on the species differences in the metabolism of styrene and metabolic differences between rats, mice, and humans

- In wrong section, should be given in 3.4.3

3.5 MECHANISMS OF ACTION

3.5.1 Pharmacokinetic Mechanisms

- None of this is relevant for styrene toxicity. Need oxide formation vs elimination, target tissue (lung, liver, brain) PK, species differences in PK

Styrene is rapidly absorbed through the respiratory tract (Ramsey and Andersen 1984; Ramsey and Young 1978; Ramsey et al. 1980; Withey and Collins 1979; Withey and Karpinski 1985) with a mean uptake of approximately 60–70% in humans (Johanson et al. 2000; Norström et al. 1992). A concentration-dependent uptake efficiency was found in the upper respiratory tract of rats and mice (Morris 2000). In rats, the uptake efficiency was 23.7% at 5 ppm and 10.1% at 200 ppm; in mice, uptake efficiency decreased from 41.7% at 5 ppm to 9.6% at 200 ppm. Based on the decreased uptake efficiency observed in rats and mice following exposure to the cytochrome P450 inhibitor, metyrapone, Morris (2000) suggested that styrene was metabolized *in situ* and this metabolism enhanced styrene uptake. In humans, blood styrene levels reached steady state after 75 minutes of exposure to 70 ppm (Wigaeus et al. 1983). The elimination of styrene from blood was biphasic, with a half-time of 1 minute for the rapid distribution phase and 40.8 minutes for the elimination phase. Styrene is rapidly distributed throughout the body with the highest concentrations found in adipose tissue. In rats, the styrene concentration in the adipose tissue was approximately 50-fold higher than in muscle; the biological half-time was 6.3 hours in adipose tissue and 2.4–2.0 hours in the blood, liver, kidney, spleen, muscle, and brain (Teramoto and Horiguchi 1979). Wigaeus et al. (1983) estimated a human adipose tissue:blood partition coefficient of 74. In humans, styrene is primarily excreted in the urine as mandelic acid and phenyoxyglylic acid. The half-times of mandelic acid and phenylglyoxylic acid in the urine were 3.6 and 8.8 hours, respectively, in humans exposed to 70 ppm for 2 hours (Wigaeus et al. 1983); another study reported

elimination half-times of 2.2–4.2 hours for mandelic acid and 3.5–13.9 hours for phenylglyoxylic acid following a 2-hour exposure to 50 ppm styrene (Johanson et al. 2000).

3.5.2 Mechanisms of Toxicity

– ? For carcinogenic effects only. Text spent a significant portion on neurotoxic effects, presumably due to styrene, but those aren't mentioned here

A large number of studies have investigated the mechanism of styrene carcinogenic activity, particularly the increased susceptibility of mice. Increases in lung tumors have been observed in mice exposed to 160 ppm 6 hours/day, 5 days/week for approximately 2 years (Cruzan et al. 2001) and following gavage exposure to 300 mg/kg/day administered 5 days/week (NCI 1979b); however, neoplastic tumors have not been observed in rats exposed to concentrations as high as 1,000 ppm 6 hours/day, 5 days/week for 2 years (Cruzan et al. 1998) or 2,000 mg/kg/day 5 days/week for 2 years (NCI 1979b). As reviewed by IARC (2002), Cohen et al. (2002), and Cruzan et al. (2002), genotoxic and nongenotoxic modes of action have been proposed. Although styrene itself does not appear to be DNA reactive, styrene 7,8-oxide is DNA reactive and has been shown to form stable N_2 and O_6 adducts of deoxyguanosine. Styrene oxide, DNA adducts, and genotoxic effects have been detected in humans, rats, and mice. Styrene (styrene 7,8-oxide is the likely causative agent) has been shown to be mutagenic in bacteria, and exposure can result in increased frequency of sister chromatid exchange, chromosomal aberrations, micronucleated cells, and DNA strand breaks. However, elevated levels of blood styrene oxide do not explain the species differences in tumor formation. In humans, styrene 7,8-oxide is rapidly hydrolyzed by epoxide hydrolase as evidenced by the high levels of mandelic acid, phenylglyoxylic acid, and hippuric acid detected in the urine. Styrene 7,8-oxide is relatively stable in rats and mice, and elevated levels have been detected in blood. The blood levels of styrene oxide in rats exposed to 1,000 ppm is 100-fold higher than the levels in mouse exposed to 20–40 ppm; however, tumors have not been detected in rats.

This is PK information that needed to be in 3.5.1

Although the nongenotoxic potential mode of action also implicates styrene 7,8-oxide as the causative agent, it involves cytotoxic damage at the target tissue, the lung. In the lung, the cytotoxic effects of repeated exposure to styrene 7,8-oxide results in bronchiolar epithelial hyperplasia, which eventually results in the formation of neoplastic tumors. In the mouse lung, styrene is primarily metabolized by cytochrome P450, particularly the CYP2F2 isoform, in the Clara cell. Humans appear to have a lower capacity to metabolize styrene in the lung compared to rats and a much lower capacity compared to mice. Mouse Clara cells metabolize higher levels of styrene than rat Clara cells and produce a higher ratio of R-enantiomer styrene oxide-to S entantiomer styrene oxide, as compared to rats. It has been estimated that mice produce 15 times more R-enantiomers styrene oxide than rats. This is particularly important since R-styrene oxide is a more potent pneumotoxicant than S-styrene oxide. In mice and rats, a portion

These lines to line 3, p 63 are PK info that belongs in 3.5.1. In addition the lung metabolism, gsh conjugation + stereochemistry should have been discussed more thoroughly in 3.4.3

of the styrene oxide generated is metabolized via glutathione conjugation. Mice appear to be more susceptible to glutathione depletion than rats, and glutathione depletion has been observed in mouse lung tissue at exposure concentrations of 80–300 ppm.

IARC (2002) concluded that the proposed mechanism involving the metabolism of styrene to styrene 7,8-oxide is the mouse Clara cell is the likely mode of action resulting in lung tumors in mice. This mode of action is not likely to be relevant to humans to a biologically significant extent. However, this mechanism and the genotoxic mode of action has not been excluded for humans, and styrene is considered a possible human carcinogen.

3.5.3 Animal-to-Human Extrapolations

Species differences in exist in the metabolism of styrene in humans, rats, and mice. In all three species, styrene in predominantly metabolized styrene 7,8-oxide. Species differences in the subsequent metabolism of styrene 7,8-oxide exist. In humans, styrene 7,8-oxide is primarily hydrolyzed to mandelic acid via epoxide hydrolase. Conjugation with glutathione also appears to be an important pathway in rats and mice. In rats, 68–72% of the styrene metabolites in urine are generated from the epoxide hydrolase pathway and 26–35% are from the glutathione transferase pathway; in mice, 48–59 and 20–35% arise from the epoxide hydrolase and glutathione transferase pathways, respectively (Cruzan et al. 2002). In contrast, 95–100% of the styrene 7,8-oxide is metabolized via the epoxide hydrolase pathway; only trace amounts of mercapturic acids (from the glutathione transferase pathway) have been detected in human urine. The difference in metabolism could result in significant increases in styrene 7,8-oxide levels in the body following exposure to high levels of styrene which may result in depletion of gluthatione. Additionally, a small percentage of styrene can under ring oxidation resulting in the formation of 4-vinylphenol. Ring-opened compounds account for 4–8% of the urinary metabolites in mice, less than 1% in rats, and were not detected in humans. The production of 4-vinylphenol is potentially significant mode of action because it is considered to be more toxic to the liver and lung than styrene or styrene oxide (Cruzan et al. 2005b).

As discussed in above, differences in the metabolism of styrene between humans, rats, and mice have resulted in toxicity differences. Species differences in toxicity have been detected in the nasal epithelium, lungs, and liver. In the respiratory tract, the species differences between rats and mice are due to local metabolism of styrene to styrene oxide. The higher rate of metabolism in mice and higher production of the more reactive enantiomer likely results in increased susceptibility. The fact that humans have a more

limited ability to metabolize styrene in the respiratory tract and possibly a higher potential to detoxify styrene oxide suggests that mice are not a good model for end points in which styrene oxide is the causative agent.

3.6 TOXICITIES MEDIATED THROUGH THE NEUROENDOCRINE AXIS

Recently, attention has focused on the potential hazardous effects of certain chemicals on the endocrine system because of the ability of these chemicals to mimic or block endogenous hormones. Chemicals with this type of activity are most commonly referred to as *endocrine disruptors*. However, appropriate terminology to describe such effects remains controversial. The terminology *endocrine disruptors*, initially used by Thomas and Colborn (1992), was also used in 1996 when Congress mandated the EPA to develop a screening program for "...certain substances [which] may have an effect produced by a naturally occurring estrogen, or other such endocrine effect[s]...". To meet this mandate, EPA convened a panel called the Endocrine Disruptors Screening and Testing Advisory Committee EDSTAC), and in 1998, the EDSTAC completed its deliberations and made recommendations to EPA concerning *endocrine disruptors*. In 1999, the National Academy of Sciences released a report that referred to these same types of chemicals as *hormonally active agents*. The terminology *endocrine modulators* has also been used to convey the fact that effects caused by such chemicals may not necessarily be adverse. Many scientists agree that chemicals with the ability to disrupt or modulate the endocrine system are a potential threat to the health of humans, aquatic animals, and wildlife. However, others think that endocrine-active chemicals do not pose a significant health risk, particularly in view of the fact that hormone mimics exist in the natural environment. Examples of natural hormone mimics are the isoflavinoid phytoestrogens (Adlercreutz 1995; Livingston 1978; Mayr et al. 1992). These chemicals are derived from plants and are similar in structure and action to endogenous estrogen. Although the public health significance and descriptive terminology of substances capable of affecting the endocrine system remains controversial, scientists agree that these chemicals may affect the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body responsible for maintaining homeostasis, reproduction, development, and/or behavior (EPA 1997). Stated differently, such compounds may cause toxicities that are mediated through the neuroendocrine axis. As a result, these chemicals may play a role in altering, for example, metabolic, sexual, immune, and neurobehavioral function. Such chemicals are also thought to be involved in inducing breast, testicular, and prostate cancers, as well as endometriosis (Berger 1994; Giwercman et al. 1993; Hoel et al. 1992).

developmental age (Guzelian et al. 1992; NRC 1993). Vulnerability often depends on developmental stage. There are critical periods of structural and functional development during both prenatal and postnatal life, and a particular structure or function will be most sensitive to disruption during its critical periods). Damage may not be evident until a later stage of development. There are often differences in pharmacokinetics and metabolism between children and adults. For example, absorption may be different in neonates because of the immaturity of their gastrointestinal tract and their larger skin surface area in proportion to body weight (Morselli et al. 1980; NRC 1993); the gastrointestinal absorption of lead is greatest in infants and young children (Ziegler et al. 1978). Distribution of xenobiotics may be different; for example, infants have a larger proportion of their bodies as extracellular water, and their brains and livers are proportionately larger (Altman and Dittmer 1974; Fomon 1966; Fomon et al. 1982; Owen and Brozek 1966; Widdowson and Dickerson 1964). The infant also has an immature blood-brain barrier (Adinolfi 1985; Johanson 1980) and probably an immature blood-testis barrier (Setchell and Waites 1975). Many xenobiotic metabolizing enzymes have distinctive developmental patterns. At various stages of growth and development, levels of particular enzymes may be higher or lower than those of adults, and sometimes unique enzymes may exist at particular developmental stages (Komori et al. 1990; Leeder and Kearns 1997; NRC 1993; Vieira et al. 1996). Whether differences in xenobiotic metabolism make the child more or less susceptible also depends on whether the relevant enzymes are involved in activation of the parent compound to its toxic form or in detoxification. There may also be differences in excretion, particularly in newborns who all have a low glomerular filtration rate and have not developed efficient tubular secretion and resorption capacities (Altman and Dittmer 1974; NRC 1993; West et al. 1948). Children and adults may differ in their capacity to repair damage from chemical insults. Children also have a longer remaining lifetime in which to express damage from chemicals; this potential is particularly relevant to cancer.

Certain characteristics of the developing human may increase exposure or susceptibility, whereas others may decrease susceptibility to the same chemical. For example, although infants breathe more air per kilogram of body weight than adults breathe, this difference might be somewhat counterbalanced by their alveoli being less developed, which results in a disproportionately smaller surface area for alveolar absorption (NRC 1993).

No studies were identified that examined the toxicity of styrene in children ~~or young laboratory animals~~. The discussion in this paragraph is humans, the next paragraph has lab animals
Several occupational exposure studies have examined the developmental toxicity of styrene; these studies did not find statistically significant alterations in the occurrence of stillbirths, infant deaths,

3.8 BIOMARKERS OF EXPOSURE AND EFFECT

Biomarkers are broadly defined as indicators signaling events in biologic systems or samples. They have been classified as markers of exposure, markers of effect, and markers of susceptibility (NAS/NRC 1989).

Due to a nascent understanding of the use and interpretation of biomarkers, implementation of biomarkers as tools of exposure in the general population is very limited. A biomarker of exposure is a xenobiotic substance or its metabolites) or the product of an interaction between a xenobiotic agent and some target molecules) or cells) that is measured within a compartment of an organism (NAS/NRC 1989). The preferred biomarkers of exposure are generally the substance itself, or substance-specific metabolites in readily obtainable body fluids), or excreta. However, several factors can confound the use and interpretation of biomarkers of exposure. The body burden of a substance may be the result of exposures from more than one source. The substance being measured may be a metabolite of another xenobiotic substance (e.g., high urinary levels of phenol can result from exposure to several different aromatic compounds). Depending on the properties of the substance (e.g., biologic half-life) and environmental conditions (e.g., duration and route of exposure), the substance and all of its metabolites may have left the body by the time samples can be taken. It may be difficult to identify individuals exposed to hazardous substances that are commonly found in body tissues and fluids (e.g., essential mineral nutrients such as copper, zinc, and selenium). Biomarkers of exposure to styrene are discussed in Section 3.8.1.

Biomarkers of effect are defined as any measurable biochemical, physiologic, or other alteration within an organism that, depending on magnitude, can be recognized as an established or potential health impairment or disease (NAS/NRC 1989). This definition encompasses biochemical or cellular signals of tissue dysfunction (e.g., increased liver enzyme activity or pathologic changes in female genital epithelial cells), as well as physiologic signs of dysfunction such as increased blood pressure or decreased lung capacity. Note that these markers are not often substance specific. They also may not be directly adverse, but can indicate potential health impairment (e.g., DNA adducts). Biomarkers of effects caused by styrene are discussed in Section 3.8.2.

A biomarker of susceptibility is an indicator of an inherent or acquired limitation of an organism's ability to respond to the challenge of exposure to a specific xenobiotic substance. It can be an intrinsic genetic or other characteristic or a preexisting disease that results in an increase in

absorbed dose, a decrease in the biologically effective dose, or a target tissue response. If biomarkers of susceptibility exist, they are discussed in Section 3.10, Populations That Are Unusually Susceptible.

3.8.1 Biomarkers Used to Identify or Quantify Exposure to Styrene

The elimination of styrene via expired air may be used to identify exposure to styrene (Guillemin and Berode 1988; Stewart et al. 1968). Only a small percentage of unchanged styrene is expired after cessation of exposure. There are no adequate studies correlating post-exposure exhaled styrene with previous exposure levels. Assessment of occupational exposure involving measurement of unchanged styrene in urine has been reported (Dolara et al. 1984). In this study of workers, the styrene air concentrations were 16–61 mg/m^3 and the urinary concentrations of styrene were 0.7–4.1 μg/L. Urinary mutagenic activity was also evaluated in this study and was not a good indication of exposure to styrene. Only a small fraction of unchanged styrene is recovered in the urine. However, measurement of styrene in urine is a reliable indicator of styrene exposure if the exposure is recent (Dolara et al. 1984; Gobba et al. 1993; Guillemin and Berode 1988; Pezzagno et al. 1985).

Analysis of unchanged styrene in blood may be used as a qualitative indicator of styrene exposure (Antoine et al. 1986). In one study, styrene was detected in the blood of humans exposed to 80 ppm (Ramsey et al. 1980). The maximum blood concentration at the end of exposure was 0.92±0.26 μg/mL. The half-life values for rapid and slow clearance curves were 0.58 and 13 hours, respectively. In another study, the concentration of styrene in blood (0.2–3.7 mg/L) increased with the level and duration of styrene exposure (Baselt 1988a).

The presence of styrene in adipose tissue is also an indicator of exposure. The concentration of styrene in the adipose tissue of two workers exposed to 32–85 mg/m^3 of styrene during a work week suggested a half-life of 5.2 days for one worker and 2.8 days for the other worker. The elimination time was estimated to be 5 weeks (Engstrom et al. 1978b).

Levels of occupational exposure to styrene may also be estimated by measurement of styrene metabolites such as MA and PGA in urine (Bartolucci et al. 1986; Elia et al. 1980; Engstrom et al. 1976; Sedivec et al. 1984; Sollenberg et al. 1988). ~~It should be noted that~~ However, large intra-individual differences in MA and PGA urinary concentrations have been reported. A study of the inter- and intra-individual differences found that PGA levels were less variable than MA levels (Symanski et al. 2001) and variability was

higher in post-shift urine samples compared to pre-shift urine samples. Expressing MA and PGA levels in units of mg per gram creatinine decreased the source intra-individual variability. Some studies found a good correlation between the time-weighted styrene exposure and urinary MA concentrations (Chua et al. 1993; Engstrom et al. 1976; Harkonen et al. 1979), while other studies found a better correlation with the sum of urinary MA and PGA at the end of the work period (Elia et al. 1980; Ong et al. 1994; Sollenberg et al. 1988). A good correlation between environmental styrene levels and urinary PGA levels has also been found (Chua et al. 1992). Total MA and PGA measured the morning after exposure may be a more reliable biological indicator of styrene exposure in factories where there is high variability in the environmental styrene concentration (Bartolucci et al. 1986). So how was the MA in Benignus (2005) converted to styrene for the metaanalysis

Reference levels of styrene likely to be observed in workers exposed to the time-weighted average concentrations by inhalation have been reported. The American Conference of Governmental Industrial Hygienists (ACGIH 2006) recommends a biological exposure index of 400 mg/g creatinine for the sum of MA and PGA in urine.

? should this be MA + PGA or styrene urinary metabolites

3.8.2 Biomarkers Used to Characterize Effects Caused by Styrene

The most common symptom of styrene exposure is depression of the central nervous system. Other organic solvent vapors cause similar effects. However, neurological symptoms can be used with caution to estimate styrene exposure and adverse effects. Central nervous system depression induced by styrene has been correlated with a urinary MA concentration in excess of 800 mg/L. A measured decrement in psychomotor performance has been associated with urinary MA concentrations of >1,200 mg/L (Harkonen et al. 1978).

Logic, memory, and visuo-constructive abilities were significantly affected in 50 workers with MA and PGA levels corresponding to >50 ppm of styrene in air (Mutti et al. 1984a). Reaction time to a sequence of light stimuli in two female workers resulted in marked impairment in workers with the highest MA excretion. The correlation coefficient for reaction time versus urinary MA measured as mmol/mmol creatinine) was 0.86 (Mackay and Kelman 1986).

Need to add text here to indicate these would be biomarkers for possible carcinogenic effects to transition from the neurotoxic effects

Cytogenetic monitoring of peripheral lymphocytes as a biomarker of effect has been proposed (DeJong et al. 1988; Pero et al. 1982). Future biomarkers may include hemoglobin adducts. Using unscheduled DNA synthesis (UDS) as an indicator of DNA damage, the lymphocytes of 38 individuals occupationally exposed to styrene were evaluated. The induced UDS was significantly increased for the group exposed

to 1–40 ppm styrene (Pero et al. 1982). Measurement of chromosome aberration in peripheral blood lymphocyte has been used for many years to monitor the biologic effects of genotoxic chemicals. However, due to high background levels of chromosomal aberration and exposures to other genotoxic workplace chemicals, the sensitivity of this biomarker for the effects of styrene is probably not adequate (DeJong et al. 1988). The role of hepatic glutathione in the toxicity of styrene has been proposed as inhibiting the covalent binding of styrene. This has been confirmed in animal studies by decreased glutathione in styrene-exposed animals (Parkki 1978). However, its use as a biomarker of effect in humans remains to be demonstrated since data on the adverse effects of styrene on the human liver are insufficient.

Levels of styrene oxide may also be a useful biomarker of effect, since this metabolic intermediate may be responsible for many of styrene's toxic effects. However, no data were located regarding a correlation between styrene oxide and any adverse health effect.

Disregard annotation

3.9 INTERACTIONS WITH OTHER CHEMICALS

? Butadiene is missing
Acrylonitrile is missing

Styrene metabolism is known to be inhibited by the presence of other chemicals such as toluene, trichloromethylene, and ethyl benzene. The biotransformation of styrene in rats to PGA, MA, and hippuric acid was suppressed by co-administration of toluene (Ikeda et al. 1972). This may be due to competitive inhibition of oxidative mechanisms. Similar results were reported by Ikeda and Hirayama (1978) in rats when styrene metabolism was inhibited by the administration of trichloroethylene. Urinary metabolites of styrene may be markedly reduced when humans or animals are concurrently exposed to organic solvents that inhibit styrene metabolism.

3.10 POPULATIONS THAT ARE UNUSUALLY SUSCEPTIBLE

A susceptible population will exhibit a different or enhanced response to styrene than will most persons exposed to the same level of styrene in the environment. Reasons may include genetic makeup, age, health and nutritional status, and exposure to other toxic substances (e.g., cigarette smoke). These parameters result in reduced detoxification or excretion of styrene, or compromised function of organs affected by styrene. Populations who are at greater risk due to their unusually high exposure to styrene are discussed in Section 6.7, Populations with Potentially High Exposures.

Styrene is a hazardous substance found in the workplace with much lower levels found in the environment. Therefore, the populations at risk are workers in industries making polystyrene plastics,

emetics, but recommend administration of water for dilution of gastric lavage (Bronstein and Currance 1988; Haddad and Winchester 1990). Others suggest administration of syrup of ipecac to induce vomiting, but consider the usefulness of activated charcoal to bind the styrene and cathartics to speed fecal excretion as questionable (Ellenhorn and Barceloux 1988). Following acute inhalation exposure, administration of oxygen and use of mechanical ventilation to support respiration have been suggested (Bronstein and Currance 1988; Ellenhorn and Barceloux 1988; Haddad and Winchester 1990). Administration of aminophylline and inhaled bronchodilators may be required to treat bronchospasm (Ellenhorn and Barceloux 1988). Furthermore, cardiac monitoring has been suggested. Supportive treatment may be needed for neurological effects of styrene exposure (Haddad and Winchester 1990).

* The position on use of ipecac has been debated + needs to be qualified here. Manoguerra et al. (2005)

3.11.2 Reducing Body Burden

Styrene is metabolized by the body, and most styrene that is absorbed is excreted in the urine as metabolites of the parent compound. Styrene is cleared rapidly from the human body. Its half-life is several hours in the blood and about 2–4 days in subcutaneous adipose tissue (see Section 3.4). No method is commonly used to enhance the elimination of the absorbed dose of styrene.

3.11.3 Interfering with the Mechanism of Action for Toxic Effects

In humans, central nervous system depression and upper respiratory tract irritation were reported following acute exposure to higher styrene concentrations (see Section 3.2). Studies in animals indicate that chronic styrene exposure causes liver and kidney effects and may induce cancer. Styrene oxide was found to be the active mutagenic metabolite of styrene in several studies (de Raat 1978; Donner et al. 1979; Norppa et al. 1979, 1980a, 1980b, 1981, 1984, 1988; Pohlova et al. 1985; Vainio et al. 1976). Based on these studies, it can be concluded that styrene is a typical indirect mutagen that needs metabolic activation to be able to bind covalently to macromolecules (e.g., nucleic acids). In one of the possible metabolic pathways, styrene oxide is further metabolized to hydroxyphenylethyl mercapturic acid. The reaction utilizes glutathione (Bond 1989). ~~It has been demonstrated that~~ The mutagenic activity of styrene oxide was decreased in the presence of glutathione in *S. typhimurium* TA100 (Yoshikawa et al. 1980). This experiment, therefore, suggests that glutathione may reduce the mutagenic effects of styrene oxide.

The formation of styrene oxide may also contribute to other effects following styrene exposure. ~~It is well established that~~ glutathione decreases the cytotoxicity of many reactive chemicals by acting as a scavenger of toxic metabolites. ~~It was found that~~ Liver exposure of rodents to high levels of styrene caused depletion of glutathione content in the liver cells of these animals (Das et al. 1983; Vainio et al. 1979). ~~It~~

~~was suggested that~~ has been suggested glutathione decrease~~s~~ to the hepatoxicity by preventing styrene oxide reaction with other endogenous macromolecules. Similarly, depletion of glutathione was found in all regions of rat brain following exposure to styrene oxide (Dixit et al. 1982; Trenga et al. 1991). The authors speculated that the depletion of brain glutathione may lead to an increased concentration of free styrene oxide with increased binding to cellular nucleophiles. This process would contribute to oxidative injury to neuronal and glial cells and may be a part of styrene-induced neurotoxicity. ~~It should be noted~~, however, ~~that~~ styrene itself, being a lipophilic compound, may disrupt the nerve membrane function in a manner similar to anesthetic agents.

Although results from *in vitro* studies in bacteria and *in vivo* animal studies demonstrate that exogenous glutathione precursors may decrease the effects of styrene toxicity, ~~it is not known whether~~ the benefit of this treatment ~~would be beneficial in~~ in humans is not known. For low-level exposure cases, ~~it is unlikely that~~ the endogenous glutathione levels ~~would~~ are unlikely to be decreased to a significant extent. Therefore, ~~it is unlikely that~~ exogenous glutathione precursors such as N-acetylcysteine ~~may~~ are not likely to be effective in mitigating the toxic effects of styrene. Exogenous doses of reducing agents may be useful following acute high dose exposure to styrene. In this case, a significant depletion of glutathione may occur as a result of the presence of high levels of styrene oxide. However, there are no clinical data available to date that support the use of this treatment.

3.12 ADEQUACY OF THE DATABASE

Section 104(i)(5) of CERCLA, as amended, directs the Administrator of ATSDR in consultation with the Administrator of EPA and agencies and programs of the Public Health Service, to assess whether adequate information on the health effects of styrene is available. Where adequate information is not available, ATSDR, in conjunction with the National Toxicology Program (NTP), is required to assure the initiation of a program of research designed to determine the health effects and techniques for developing methods to determine such health effects, of styrene.

The following categories of possible data needs have been identified by a joint team of scientists from ATSDR, NTP, and EPA. They are defined as substance-specific informational needs that if met would reduce the uncertainties of human health assessment. This definition should not be interpreted to mean that all data needs discussed in this section must be filled. In the future, the identified data needs will be evaluated and prioritized, and a substance-specific research agenda will be proposed.

3.12.1 Existing Information on Health Effects of Styrene

The existing data on health effects of inhalation, oral, and dermal exposure of humans and animals to styrene are summarized in Figure 3-5. The purpose of this figure is to illustrate the existing information concerning the health effects of styrene. Each dot in the figure indicates that one or more studies provide information associated with that particular effect. The dot does not necessarily imply anything about the quality of the study or studies, nor should missing information in this figure be interpreted as a "data need". A data need, as defined in ATSDR's *Decision Guide for Identifying Substance-Specific Data Needs Related to Toxicological Profiles* Agency for Toxic Substances and Disease Registry 1989), is substance-specific information necessary to conduct comprehensive public health assessments. Generally, ATSDR defines a data gap more broadly as any substance-specific information missing from the scientific literature.

There is information on most categories of human toxicity via the inhalation route from occupational studies. However, there are limited data on humans exposed to styrene by the oral or dermal routes. Data from animal studies are more extensive, with studies available for most areas of toxicity resulting from exposure via the oral and inhalation routes. Little is known about the effects of dermal exposure to styrene in animals.

3.12.2 Identification of Data Needs

Acute-Duration Exposure. The possibility for brief human exposure to high concentrations of styrene exists in occupational settings, and might also exist near major spills. Exposure of the general public to episodic high concentrations of styrene at hazardous waste sites, in the home, or in the general environment is unlikely. The respiratory tract and central nervous system are the likely target organ systems for inhaled styrene (Alarie 1973; Carpenter et al. 1944; DeCeaurriz et al. 1983; Kankaanpaa et al. 1980; Murray et al. 1978; Sceber et al. 2004; Spencer et al. 1942; Stewart et al. 1968). Animal studies have reported hepatic (Cruzan et al. 1997, 2001; Morgan et al. 1993a, 1993b, 1993c; Vaino et al. 1979) and nasal (Cruzan et al. 2001) effects and hearing impairments (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003). Available toxicokinetic data suggest that the mouse may be more sensitive to the hepatic and nasal toxicity of styrene than humans; thus, these data are not suitable for derivation of an acute-duration inhalation MRL. Studies have also examined potential reproductive (Salomaa et al. 1985) and developmental (Kankaapää et al. 1980; Murray et al. 1978) effects; the highest doses tested in these studies were NOAELs. The data are not considered sufficient to establish an inhalation acute-duration MRL. Episodic high-level exposures to styrene from contaminated food or water are unlikely. Few

– but an acute-duration MRL for inhalation was established on pg 13

studies have examined the toxicity of styrene following exposure to an acute oral dose. Abdominal discomfort was observed in residents exposed to high levels of styrene in drinking water (Arnedo-Pena et al. 2003); concomitant inhalation exposure to styrene limits the utilization of this study for MRL derivation. A study in rats identified a LOAEL for neurotoxicity (Husain et al. 1985) and another rat study examined potential developmental effects, but found no adverse effects (Daston et al. 1991). The animal data are not considered sufficient to derive an oral acute-duration MRL. Thus, additional single-dose oral and inhalation studies are needed to better define toxicity thresholds. However, the potential carcinogenicity of styrene prevents the design of controlled laboratory exposures in humans. Dermal exposure to styrene at significant levels is unlikely except in the case of workplace spills and dermal absorption is probably low based on limited human studies. However, the almost complete lack of dermal toxicity data in animals and humans creates a degree of uncertainty on this issue. Therefore, single-dose dermal studies would be useful in determining target organs and thresholds for dermal exposure. In designing these types of studies, precautions should be taken to avoid concomitant inhalation exposure.

Yet one was given on p17. Please clarify

Intermediate-Duration Exposure. Intermediate-duration inhalation exposure studies in humans is limited to a study examining potential reproductive effects in workers (Lindbolm et al. 1985); exposure information was not provided. Inhalation studies in animals have reported damage to the nasal olfactory epithelium in rats (Cruzan et al. 1997, 2005a, 2005b; Ohashi et al. 1986) and mice (Cruzan et al. 1997, 2001), liver damage in mice (Cruzan et al. 1997), eye irritation in rats (Cruzan et al. 1997) and guinea pigs (Spencer et al. 1942), ototoxicity in rats (Campo et al. 2001; Lataye et al. 2000; Loquet et al. 1999, 2000; Makitie et al. 2002; Pouyatos et al. 2002; Pryor et al. 1987; Yano et al. 1992), and impaired nerve conduction velocity (Yamamoto et al. 1997). A two-generation study in rats did not find reproductive, developmental, or neurodevelopmental effects (Cruzan et al. 2005a, 2005b), but another study did find neurodevelopmental effects (Katakura et al. 1999, 2001). However, additional studies are needed, as the data are not considered sufficient to derive an intermediate-duration inhalation MRL. Oral exposure studies of intermediate-duration are limited. Animal studies indicate that respiratory, hepatic, neurological, and neurodevelopmental end points need further evaluation. There are limited animal data and no human data on the toxicity of styrene following intermediate-duration oral exposure. Studies by Srivastava et al. (1982), Ponmarkov and Tomatis (1978), and Wolf et al. (1956) have examined a limited number of systemic end points. Other studies have identified adverse effect levels for neurological effects (Agrawal et al. 1982; Bushnell 1994; Husain et al. 1980), impaired development of the reproductive system (Srivastava et al. 1992a, 1992b), or neurodevelopmental effects (Zaidi et al. 1985); no reproductive effects were observed in a three-generation reproduction study (Beliles et al. 1985). The

for intermediate-duration

current information is not sufficient to develop an oral MRL. One study examined the dermal toxicity of styrene in rabbits (Spencer et al. 1942); basic information on the adverse effects of intermediate-duration dermal exposure to styrene in animals is also needed due to the sparsity of available data.

Chronic-Duration Exposure and Cancer. A large number of occupational exposure studies have examined the chronic toxicity of styrene. Systemic toxicity studies have examined endocrine (Bergamaschi et al. 1997; Mutti et al. 1984b), hematological (Checkoway and Williams 1982; Thiess and Friedheim 1978), hepatic (Hotz et al. 1980; Lorimer et al. 1978), or renal (Verplanke et al. 1998; Viau et al. 1987; Vyskocil et al. 1989) end points; most studies relied on biomarkers of toxicity. The most widely examined end point is neurotoxicity, and the available data suggest that this is the most sensitive end point. Examined neurological end points included color vision (Campagna et al. 1995, 1996; Chia et al. 1994; Eguchi et al. 1995; Fallas et al. 1992; Gobba et al. 1991; Gong et al. 2002; Kishi et al. 2001; Mutti et al. 1984a), vestibular effects (Calabrese et al. 1996; Moller et al. 1990), hearing impairment (Morata et al. 2002; Morioka et al. 1999; Muijser et al. 1988; Sliwinska-Kowalska et al. 2003), symptoms of neurotoxicity (Checkoway et al. 1992; Cherry et al. 1980; Edling et al. 1993; Viaene et al. 1998, 2001), performance on neurobehavioral tests (Cherry et al. 1980; Edling et al. 1993; Gamberale et al. 1976; Jegaden et al. 1993; Lindstrom et al. 1976; Mutti et al. 1984a; Tsai and Chen 1996; Viaene et al. 1998, 2001), nerve conduction velocity (Behari et al. 1986; Murata et al. 1991; Rosen et al. 1978), olfactory alterations (Dalton et al. 2003), and EEG alterations (Harkonen et al. 1984; Seppalainen and Harkonen 1976). Other human studies have examined reproductive (Harkonen and Holmberg 1982; Hemminki et al. 1980) and developmental (Ahlborg et al. 1987; Lemasters et al. 1989) end points. The chronic toxicity of styrene has also been examined in rat (Cruzan et al. 1998; Jersey et al. 1978) and mouse (Cruzan et al. 2001) studies. The occupational exposure studies were considered adequate for derivation of a chronic-duration inhalation MRL for styrene. Further research to define the dose-response curve more fully and to identify a chronic inhalation NOAEL for neurological effects would be valuable and would help to reduce uncertainty in the MRL. Data on chronic oral exposure to styrene is only available through animal studies (Beliles et al. 1985; Conti et al. 1988; NCI 1979b; Quast et al. 1979). In these studies, the most sensitive indicator of toxicity appears to be Heinz body formation in red blood cells (Quast et al. 1979), and the EPA has calculated a chronic oral RfD based on this study (IRIS 2007). However, there is some doubt regarding the chronic oral NOAEL, and whether hematological effects are really more sensitive than neurological effects. Moreover, decreased survival has been noted in rats at exposure levels only slightly higher than the no-effect level for hematological effects (Conti et al. 1988). Therefore, no chronic oral MRL has been derived. Further studies on the effects of oral exposure, with special emphasis on neurological or neurobehavioral effects, would be valuable. Although chronic dermal

have been reported for spontaneous abortions with some studies reporting significant increases (Harkonen and Holmberg 1982; Hemminki et al. 1980; McDonald et al. 1988) and others reporting no effect (Harkonen and Holmberg 1982; Hemminki et al. 1980, 1984; Lindbohm et al. 1985). Oligomenorhhea was observed in one study of workers (Cho et al. 2001), but not in another study (Lemasters et al. 1985). Studies in male workers have found alterations in sperm parameters (Kolstad et al. 1999a), but no alterations in time-to-pregnancy (Kolstad et al. 2000; Sallmén et al. 1998) or fertility rates (Kolstad et al. 1999c). A two-generation inhalation study (Cruzan et al. 2005b) and three-generation oral study (Beliles et al. 1985) in rats showed no styrene-related reproductive effects. However, testicular effects have been observed in an oral exposure study (Srivastava et al. 1989), but not in an inhalation study (Salomaa et al. 1985). Additional reproductive data on occupationally-exposed males would be useful in evaluating the existing animal data that indicates altered testicular function and studies in females would be useful in evaluating the inconsistent findings in the existing studies.

Developmental Toxicity. Data on the developmental effects of inhalation exposure to styrene are available in humans and animals. Occupational exposure studies (Ahlborg et al. 1987; Härkönen et al. 1984; Lemasters et al. 1989) have not found increases in the occurrence of birth defects or birth weight. However, interpretation of the results are complicated by exposure to other chemicals and lack of information on exposure levels. Additional occupational studies are needed to adequately assess this end point. Developmental studies in animals via inhalation (Cruzan et al. 2005b; Kankaapää et al. 1980; Murray et al. 1978) or oral (Beliles et al. 1985) exposure have not found effects on fetal outcome, birth weight, or incidence of abnormalities. However, several studies have reported neurodevelopmental (Katakura et al. 1999, 2001; Zaidi et al. 1985) or reproductive (Srivastava et al. 1992a, 1992b) effects. Additional studies are needed to examine the potential effects on the nervous and reproductive systems of developing organisms. No studies examined the developmental toxicity of styrene following dermal exposure.

Immunotoxicity. Occupational exposure studies have found alterations in lymphocyte subsets (Bergamaschi et al. 1995b; Biró et al. 2002), which may be indicative of reduced cell-mediated immunity and an impaired immune response to concanavalin (Tulinska et al. 2000); another study found no alterations in immunoglobulin levels (Chmielewski et al. 1977). Limited data in animals indicate that inhalation (Ban et al. 2006) and oral (Sinitskij 1969) exposure can also result in impaired immune response. No dermal exposure studies examining immunotoxicity were identified. Human and animal studies provide suggestive evidence that the immune system is a target; additional studies would be useful to further investigate the effect of styrene on immune function.

characterization of exposure levels and possible exposure to other chemicals. Some studies provided no data on styrene exposure levels and other studies provide current exposure levels with limited or no data on past exposure levels. Occupational exposure and experimental studies also provide suggestive evidence of acute upper respiratory tract irritation and eye irritation (Carpenter et al. 1944; NIOSH 1983) Stewart et al. 1968) and possible endocrine effects (elevated levels of serum prolactin) (Arfini et al. 1987; Bergamaschi et al. 1996, 1997; Luderer et al. 2004; Mutti et al. 1984b); additional studies are needed to confirm the results of these studies and to establish dose-response relationships. Additionally, there are suggestive findings that styrene has the potential to induce reproductive effects (Cho et al. 2001; Harkonen and Holmberg 1982; Hemminki et al. 1980; Kolstad et al. 1999c; McDonald et al. 1988); however, poor characterization of styrene exposure and the low statistical power of the studies limit the usefulness of the studies; studies of males and female styrene workers that examined a variety of reproductive end points and adequately characterized exposure would be useful.

Biomarkers of Exposure and Effect.

Should this be qualified because of variability

Exposure. Available studies indicate that there are good quantitative relationships between styrene metabolites (MA and PGA) in the urine and styrene exposure levels in humans (Bartolucci et al. 1986; Chua et al. 1992; Elia et al. 1980; Engstrom et al. 1976; Harkonen et al. 1978; Ong et al. 1994; Sedivec et al. 1984; Sollenberg et al. 1988; Symanski et al. 2001). Levels of styrene in blood have also been used as a biomarker of exposure (Antoine et al. 1986; Baselt et al. 1988a; Ramsey et al. 1980).

Effect. There are currently no biomarkers specific for the effects of styrene that are not also typical of other central nervous system depressants. Further research is needed to evaluate potential biomarkers of effect in the areas of chromosome aberrations, psychomotor decrement, hepatic glutathione depletion, and adipose tissue retention of styrene. These potential biomarkers should be evaluated in terms of long-term or chronic exposure periods, and their specificity for exposure to styrene.

Absorption, Distribution, Metabolism, and Excretion. Styrene oxide (styrene epoxide) has been identified as an intermediate metabolite of styrene (Drummond et al. 1989; Engstrom et al. 1976; Korn et al. 1984, 1987; Leibman 1975; Lof et al. 1983; Withey and Collins 1979; Young et al. 1979). However, styrene oxide has only been found in minute amounts in human studies (Lof et al. 1986a). The presence of styrene oxide, a mutagen and carcinogen, may account for some conflicting results and/or interspecies variation in mutagenicity tests and cancer bioassays. The role, if any, of styrene oxide in the overall toxicity of styrene needs to be evaluated by additional metabolism studies to confirm its presence, level,

and duration in human tissues. The toxicokinetics of styrene exposure via inhalation are reasonably well defined. However, oral and dermal exposure data are needed to better characterize absorption rates and the elimination ratios of the metabolites (MA and PGA).

Comparative Toxicokinetics. Interspecies variations in styrene metabolism have been established by noting, for example, different ratios of MA and PGA in different species (Mendrala et al. 1991; Ramsey et al. 1980; Ramsey and Young 1978; Young et al. 1979) reported species differences in the *in vitro* metabolism of styrene and styrene oxide, which indicated that mice and rats had a higher capacity to produce styrene oxide from than humans. Efforts should continue to identify which animal model best approximates human metabolism of styrene. Although urinary metabolites of styrene in humans are known, the occurrence and significance of styrene oxide needs to be evaluated.

Edit sentence

Methods for Reducing Toxic Effects. Recommended methods for the mitigation of acute effects of styrene intoxication include mechanical ventilatory support, administration of oxygen, and drug therapy for bronchospasm, if exposure is by inhalation (Bronstein and Currance 1988; Ellenhorn and Barceloux 1988). Thorough washing or flushing with water is recommended for dermal/ocular exposure. There is disagreement concerning the use of emetics to prevent absorption of styrene following ingestion due to potential of aspiration into the lung (Bronstein and Currance 1988; Ellenhorn and Barceloux 1988; Haddad and Winchester 1990). Supportive treatment is indicated for neurological effects of styrene exposure (Haddad and Winchester 1990). No information was located concerning mitigation of effects of lower-level or longer-term exposure to styrene. Further information on techniques to mitigate such effects would be useful in determining the safety and effectiveness of possible methods for treating styrene-exposed populations in the vicinity of hazardous waste sites. This includes further studies on the mechanism(s) of styrene toxicity, so that methods may be developed to interfere with or block styrene's toxic actions in the body.

This isn't give previously - should be

Children's Susceptibility. **Data needs relating to both prenatal and childhood exposures, and developmental effects expressed either prenatally or during childhood, are discussed in detail in the Developmental Toxicity subsection above.**

No studies were identified that examined the toxicity of styrene in children or young laboratory animals. No consistently observed developmental effects have been reported in occupational exposure studies (Ahlborg et al. 1987; Härkönen et al. 1984; Lemasters et al. 1989) or in animal studies (Cruzan et al. 2005b; Daston et al. 1991; Kankaanpää et al. 1980; Murray et al. 1978). The nervous system is the most

5. PRODUCTION, IMPORT/EXPORT, USE, AND DISPOSAL

5.1 PRODUCTION

In the United States, styrene is produced principally by the catalytic dehydrogenation of ethylbenzene. Hence, ethylbenzene is a common contaminant. Styrene is also produced by oxidation of ethylbenzene to ethylbenzene hydroperoxide, which is then reacted with propylene to produce propylene oxide and α-methylphenyl carbinol. The carbinol is then further dehydrated to produce styrene (Dickson et al. 1983; HSDB 2007; IARC 1979). The first route of manufacture (dehydrogenation of ethylbenzene) represents 90% of styrene production. The other described method is the second most commonly used route of styrene synthesis. Other methods of styrene production are rarely used.

Styrene has been manufactured in the United States since 1938, with production increasing dramatically over the last 30 years. Production increased 16% in the decade between 1977 and 1987, but production increased >32% between 1987 and 1999, and rose again by another 28% between the years 1999 and 2006 (HSDB 2007; SRI 2006). Specifically, U.S. production of styrene in 1978 was 6.8 billion pounds, and then in 1987, production was approximately 8 billion pounds. In 1999, U.S. styrene production was over 10 billion pounds, and in 2006, the United States produced >13 billion pounds (Dickson et al. 1983; Heylin 1989; HSDB 2007; SRI 1988a, 1989, 2006; USITC 1987, 1988). ~~Therefore,~~ since 1977, U.S. total styrene production has more than doubled.

Mix up here r combine.

Information regarding the locations of the numerous styrene production facilities and the amounts of styrene that may be present on-site is presented in Table 5-1. Current domestic producers of styrene include Chevron Phillips Chemical Company LP (Aromatics and Styrenics Business Unit), St. James, Louisiana; Cos-Mar Company, Carville, Louisiana; the Dow Chemical Company, Freeport, Texas; INEOS Americas, LLC, Texas City, Texas; Lyondell Chemical Company, Channelview, Texas; NOVA Chemicals Corp, Bayport, Texas; Sterling Chemicals, Inc., Texas City, Texas; and Westlake Styrene Corporation, Sulphur, Louisiana (SRI 2006). The production of styrene at these facilities is directed primarily for captive processes (on-site conversion to other materials) or for merchant sales to other entities (these include export). The information presented in Table 5-1 reflects the locations of these production plants, where it can be noted that the greatest production capacity occurs primarily in Texas and Louisiana.

5.2 IMPORT/EXPORT

Imports of styrene have generally been <1% of U.S. domestic production volumes, with imported styrene amounts decreasing over the last decades, and exported amounts increasing during the same time period. Styrene imports were reported to be 26.4 million pounds for 1976, 320 million pounds in 1986, (Dickson et al. 1983; IARC 1979), but only 1 million pounds in 1999 (HSDB 2007). These trends indicate a higher capacity for domestic producers to meet industry needs. Styrene exports were <1 billion pounds in 1978, but had exceeded 1 billion pounds by 1983. Exports have slowly increased such that recent export data (2000) indicate that the U.S. exports >2 billion pounds of styrene annually (HSDB 2007), also indicating that domestic production is more than capable to serve domestic needs.

5.3 USE

Styrene is used predominantly (65% of total product) in the production of polystyrene plastics and resins (James and Castor 2005). Some of these resins are used for construction purposes such as in insulation or in the fabrication of fiberglass boats. Styrene is also used as an intermediate in the synthesis of materials used for ion exchange resins and to produce copolymers such as styrene-acrylonitrile (SAN) and acrylonitrile-butadiene-styrene (ABS), both representing approximately 9% of styrene use, and styrene-butadiene rubber (SBR), representing approximately 6% of styrene use. SBR is used for such products as car tires, hoses used for industrial applications, and shoes. A related polymer, styrene-butadiene latex (approximately 7%), is used in making carpet, coatings for paper, and as part of latex paints. ~~Approximately 9% goes into SAN copolymer and polymers of AB~~ already listed S. SAN and ABS are used for materials such as piping, automotive components, refrigerator liners, plastic drinking glasses, and car battery enclosures. An additional 7% of styrene is formulated with unsaturated polyester resins in such things as boat hulls (fiberglass reinforcement materials). The remaining amounts of styrene produced are used for several types of applications, including less common thermoplastics and even for laboratory and water purification uses (ion-exchange resins) and glues and adhesives (James and Castor 2005). Styrene copolymers are also frequently used in liquid toner for photocopiers and printers (HSDB 2007).

The Food and Drug Administration (FDA) permits styrene to be used as a direct additive for synthetic flavoring and an indirect additive in polyester resins, ion-exchange membranes, and in rubber articles (5% by weight maximum) intended for use with foods (HSDB 2007; IARC 1979; NIOSH 1983).

5.4 DISPOSAL

Typical means of styrene disposal include absorption on vermiculite or similar material, followed by disposal in an EPA-permitted landfill. Incineration is also a useful disposal method, but this must be carefully controlled since pure styrene is highly flammable (HSDB 2007). No data were located regarding the quantities of styrene disposed by these means on a national level, but the state of Massachusetts reported that most styrene disposal occurred via incineration (95.5%), followed by smaller amounts being disposed of in landfills (0.5%), a slightly greater amount being subjected to solvent recovery (0.7%), and slightly more being transferred to waste/energy brokers (3.3%) (Keenan and Harriman 1993). The total amounts represented were ~250,000 pounds. ~~It is unknown if~~ Whether the data reported for Massachusetts are representative of the proportions disposed of by these means in other states is not known.

6. POTENTIAL FOR HUMAN EXPOSURE

6.1 OVERVIEW

Styrene has been identified in at least 31 of the 1,689 hazardous waste sites that have been proposed for inclusion on the EPA National Priorities List (NPL) (HazDat 2007). However, the number of sites evaluated for styrene is not known. The frequency of these sites can be seen in Figure 6-1. Of these sites, 30 are located within the United States and one is located in the Commonwealth of Puerto Rico (not shown).

p 59 lists 51

Styrene is a widely used industrial chemical with reported atmospheric emissions of >54 million pounds annually in the United States (TRI05 2007). Styrene photodegrades in the atmosphere, with a half-life ranging between 7 and 16 hours (which are the degradation half-lives catalyzed by reactions with hydroxyl radical and ozone, respectively). Styrene is moderately mobile in soil and volatilizes from water to the atmosphere. Styrene will undergo biodegradation in most top soils and aquatic environments, but degradation will be much slower in environments that are anaerobic. Bioconcentration does not appear to be significant.

The principal route of styrene exposure for the general population is probably by inhalation of contaminated indoor air. Mean indoor air levels of styrene have been reported in the range of 0.1–50 $\mu g/m^3$, and can be attributed to emissions from building materials, consumer products, and tobacco smoke. It should be pointed out that the workplace or home office may have substantially higher levels of airborne styrene, due to emissions from laser printers and photocopiers. General workplace styrene concentrations ranged from 89 to 1.5×10^6 $\mu g/m^3$. The most significant exposure route in these settings is also likely by inhalation. The industries with the highest potential exposure are probably the reinforced plastics factories, boatbuilding facilities, and polystyrene factories. Exposure may also be high in areas near major spills. Exposure to styrene from hazardous waste sites is potentially important, but the magnitude of the problem is unknown. The potential for outdoor exposure to styrene is lower than indoor exposure, with reported mean air levels ranging from 0.28 to 20 $\mu g/m^3$.

also give range in ppm

give in ppm.

6.2 RELEASES TO THE ENVIRONMENT

The Toxics Release Inventory (TRI) data should be used with caution because only certain types of facilities are required to report (EPA 2005). This is not an exhaustive list. Manufacturing and processing facilities are required to report information to the TRI only if they employ 10 or more

full-time employees; if their facility is included in Standard Industrial Classification (SIC) Codes 10 (except 1011, 1081, and 1094), 12 (except 1241), 20–39, 4911 (limited to facilities that combust coal and/or oil for the purpose of generating electricity for distribution in commerce), 4931 (limited to facilities that combust coal and/or oil for the purpose of generating electricity for distribution in commerce), 4939 (limited to facilities that combust coal and/or oil for the purpose of generating electricity for distribution in commerce), 4953 (limited to facilities regulated under RCRA Subtitle C, 42 U.S.C. section 6921 et seq.), 5169, 5171, and 7389 (limited S.C. section 6921 et seq.), 5169, 5171, and 7389 (limited to facilities primarily engaged in solvents recovery services on a contract or fee basis); and if their facility produces, imports, or processes ≥25,000 pounds of any TRI chemical or otherwise uses >10,000 pounds of a TRI chemical in a calendar year (EPA 2005).

6.2.1 Air

p 88 Well had >54 million pounds

Estimated releases of more than 51 million pounds (>23 thousand metric tons) of styrene to the atmosphere from 1,563 domestic manufacturing and processing facilities in 2005, accounted for >93% of the estimated total environmental releases from facilities required to report to the TRI (TRI05 2007). These releases are summarized in Table 6-1.

Styrene may be emitted to the atmosphere from industrial production and usage processes, motor vehicle operation, combustion processes, building materials, and consumer products. Estimated atmospheric industrial styrene emissions reported to EPA for the 2005 TRI totaled >51 million pounds, with >41 million pounds released from point sources and >10 million pounds released as fugitive emissions (EPA 2007). Styrene ranked 16th among air emissions for reported chemicals and chemical group compounds in the United States in 2005. Since EPA regulations that require reporting of toxic chemical emissions apply only to selected facilities producing and/or using substantial quantities of the chemical (EPA 1988a), the total air emissions of styrene are probably greater than those reported. Typical sources of industrial styrene emissions are those facilities producing styrene, polystyrene, other plastics, synthetic rubber, and resins (EPA 1975, 1987d; Graedel 1978; IARC 1979; NIOSH 1983). The number of facilities reporting styrene emissions to the TRI are listed in Table 6-1, along with number of reporting facilities in each state and Puerto Rico, and the primary routes of styrene release from those facilities.

Styrene has been identified as a component of motor vehicle emissions from both gasoline- and diesel-powered engines (Hampton et al. 1982, 1983). Styrene emission rates ranging from 6.2 to 7.0 mg/km distance for gasoline-powered vehicles and 1.4–2.1 mg/km for diesel trucks have been reported (Hampton

et al. 1983). Styrene may also be emitted into the air by other combustion processes. Styrene has been identified in the stack emissions from waste incineration (Junk and Ford 1980), and Kleindienst et al. (1986) reported the presence of styrene in wood smoke emissions, but no quantitative data were reported.

Emissions of styrene from building materials (carpets, floor tiles, insulation), office copiers, and consumer products (disinfectants, plastics, paint, cigarettes) may contribute significantly to indoor air pollution (Crump 1995). A styrene emission rate from glued carpet of 98 ng/minute/m^2 was calculated by Wallace et al. (1987b), and Girman et al. (1986) identified styrene as a major emittant from adhesives used in the constructing and finishing of buildings. Hodgson et al. (1993) determined an average styrene emission rate from new carpets of 410 ng/minute/m^2 over a 24-hour time period, but this was reduced to 30 ng/minute/m^2 when emissions were measured over 168 hours. Carpet cushioning material showed higher styrene emission rates of 2,300 ng/minute/m^2 when measured over 6 hours, but this material also showed significantly lower emission rates of 83 ng/minute/m^2 when measured over a longer span of 96 hours (Schaeffer et al. 1996). Polystyrene products such as packaging materials, toys, housewares, and appliances that may contain small amounts of the monomer also contribute to air levels. The workplace or home office may have substantial levels of airborne styrene due to emissions from laser printers and photocopiers. In the case of laser printers, styrene concentrations measured in test chambers during printer operation were reported to be as high as 380 μg/m^3 (Kagi et al. 2007). For photocopiers, emission rates from four different copiers averaged 3,300 μg/hour, but one copier had an emission rate of 12,000 μg/hour (Leovic et al. 1996). General workplace styrene concentrations ranged from 89 to 1.5×10^6 μg/m^3. Styrene has also been detected in sidestream smoke emitted from cigarettes but concentrations were not reported (IARC 1979).

also list ppm

6.2.2 Water

Estimated releases of 4,858 pounds (~2.2 metric tons) of styrene to surface water from 1,563 domestic manufacturing and processing facilities in 2005, accounted for about 0.01% of the estimated total environmental releases from facilities required to report to the TRI (TRI05 2007). No styrene was reported to have been released to publicly owned treatment works (POTWs) (TRI05 2007). These releases are summarized in Table 6-1.

The principal sources of styrene releases to water are industrial effluents. Styrene has been detected in effluents from chemical, textile, latex, and coal gasification plants (EPA 1976; Pellizzari et al. 1979). Styrene was also identified in one of 63 industrial effluents at a concentration of <10 μg/L (EPA 1979b).

Styrene occurred at concentrations up to 83 μg/L in coal gasification effluents (Pellizzari et al. 1979), and King and Sherbin (1986) reported styrene concentrations up to 970 μg/L in chemical plant effluents. The daily styrene loading from a single chemical plant into the St. Clair River (just south of Lake Huron on the Michigan/Ontario border) was estimated at 133 kg (King and Sherbin 1986). Styrene was detected (but not quantified) in the leachate from an industrial landfill in a study of 58 municipal and industrial landfill leachates (Brown and Donnelly 1988). Styrene has also been detected at trace concentrations in the River Elbe at two different sampling locations, with concentrations ranging from 6.1 to 46 ng/L (Gotz et al. 1998).

6.2.3 Soil

Estimated releases of more than two million pounds (~1,000 metric tons) of styrene to soils from 1,563 domestic manufacturing and processing facilities in 2005, accounted for about 4.1% of the estimated total environmental releases from facilities required to report to the TRI (TRI05 2007). An additional 0.980 million pounds (~444 metric tons), constituting about 1.8% of the total environmental emissions, were released via underground injection (TRI05 2007). These releases are summarized in Table 6-1.

Soil and sediments may become contaminated with styrene by chemical spills, landfill disposal of styrene-containing wastes, or discharge of styrene-contaminated water. A small amount of styrene is produced naturally through the activities of microorganisms, and some plants also produce styrene that may be released to soil. The amounts released to soil through these processes, however, are not expected to be significant in comparison to human activities that generate and release styrene to soil.

6.3 ENVIRONMENTAL FATE

6.3.1 Transport and Partitioning

Should styrene be released to the environment, its high vapor pressure would lead to its rapid partitioning to the atmosphere. In the atmosphere, styrene exists as a vapor. Styrene is an oily liquid that is slightly volatile; its vapor pressure has been determined to be approximately 5 mmHg at 20 °C (Verschueren 2001). A small fraction of the styrene released to the atmosphere may dissolve into condensed water vapor such as clouds and raindrops. A Henry's law constant (H) is a measure of the tendency of a chemical to partition between its gas phase and water. A value for H has not been experimentally measured for styrene, but it may be estimated by dividing the vapor pressure of styrene by its solubility in

This sentences seem contradictory. If it has a high vapor pressure it is very volatile. However 5mmHg isn't a high vapor pressure (< water)

6.4 LEVELS MONITORED OR ESTIMATED IN THE ENVIRONMENT

Reliable evaluation of the potential for human exposure to styrene depends in part on the reliability of supporting analytical data from environmental samples and biological specimens. Concentrations of styrene in unpolluted atmospheres and in pristine surface waters are often so low as to be near the limits of current analytical methods. In reviewing data on styrene levels monitored or estimated in the environment, it should also be noted that the amount of chemical identified analytically is not necessarily equivalent to the amount that is bioavailable. The analytical methods available for monitoring styrene in a variety of environmental media are detailed in Chapter 7.

6.4.1 Air

Styrene is a common contaminant of ambient urban air. Concentrations of styrene greater than rural air concentrations have been identified in urban and industrial source areas, near hazardous waste sites, in motor vehicle tunnels, in indoor air, and in workplace environments. A summary of monitoring data for these locations is presented in Table 6-2. The data suggest that indoor air concentrations of styrene can be considerably higher than outdoor concentrations. Cigarette smoke has also been implicated as a significant source of styrene in indoor air (EPA 1987e; Wallace et al. 1986a), as has the operation of photocopying machinery (Stefaniak et al. 2000; Leovic et al. 1996; Leovic et al. 1998) and laser printers (Kagi et al. 2007).

Monitoring studies in Minnesota detected styrene in over 1,400 air samples collected from a total of 2,507 samples (there were 1,004 samples where styrene was below the detection limits) over an 8-year period. The average concentration detected was 0.1 $\mu g/m^3$; the median concentration detected was 0.08 $\mu g/m^3$; and the maximum detected amount was 1.49 $\mu g/m^3$ (Pratt et al. 2000). Styrene monitoring in ambient air conducted in Chiba City, Japan over an 8-week period showed slightly higher mean concentrations, ranging from 0.11 to 0.36 $\mu g/m^3$ (Uchiyama and Hasegawa 2000).

ppm comparisons would be useful since most effects data gave ppm exp.

6.4.2 Water

Styrene is not frequently found in U.S. water supplies. Styrene was not detected in any of the >1,000 samples of drinking water analyzed during three federal surveys (EPA 1988b), but had been reported occasionally in drinking water supplies in several states (Coleman et al. 1984; EPA 1975, 1976; Kleopfer and Fairless 1972; Kool et al. 1982; Sanjivamurthy 1978;) well water (EPA 1985; Krill and

Sonzogni 1986), river water (EPA 1976; Sheldon and Hites 1978), and Lake Erie (Konasewich et al. 1978). Quantitative data were not available in these reports. A more recent survey provided by the EPA National Contaminant Occurrence Database (EPA 2006) noted that styrene was detected rarely in groundwater, where it was detected only 295 times out of over 174,000 analyses (<0.2% of the samples); detected concentrations ranged from <10 to 40 μg/L. Styrene concentrations in raw and treated waters ranged from 0.1 to ≥1.0 μg/L in an evaluation of organic compounds in Canadian water supplies at nine municipalities along the Great Lakes (Otson 1987). A survey conducted on 455 NPL and non-NPL hazardous waste sites documented the presence of styrene in 2.2% of the groundwater sites (n=405) and 1.6% of the surface water sites (n=383). The styrene concentrations measured in these samples were approximately an order of magnitude lower than the concentrations measured in the sediment and soils, with a styrene concentration of 5.3 μg/L (geometric mean) in the groundwater and 9.3 μg/L in the surface waters at these sites (CLPSD 1986). Styrene was rarely detected in aquifer materials in a large evaluation of U.S. groundwater and wells conducted by the U.S. Geological Survey (USGS). For >3,400 test evaluations of aquifer materials, there was a very low frequency of detections, with the median concentration being 0.015 μg/L. It was detected less frequently and also at low concentrations in domestic wells (0.014 μg/L), and at slightly higher concentrations in public wells (median=0.13 μg/L) (USGS 2006).

Other bodies of water—those potentially highly contaminated and those that are used for direct ingestion—likewise do not usually contain styrene. Styrene is not commonly detected in groundwater even near superfund sites, and was not found in a drinking water evaluation of public water sources in Torino, Italy (Carter and Sabatini 1994; Zelano et al. 1998). In a study of wells near a superfund site in Florida, styrene was detected at a very low concentration (the maximum detected concentration was 6.3 μg/L) near the site, but it was not detected in any treated water effluents (Canter and Sabatini 1994). Squillance et al. (1999) surveyed a vast number of drinking water wells in the United States over a 10-year period. Styrene was detected in <1% of the 2,900 surveyed urban and suburban wells between 1985 and 1995, and the concentrations detected were >2 orders of magnitude lower than the health advisory level. ~~It is of note that~~ Interestingly, styrene was only detected in rural wells, not in urban ones. Finally, Zelano et al. (1998) did not report the detection of any styrene in an evaluation of 21 public drinking water fountains in Torino, Italy.

6.4.3 Sediment and Soil

Limited data were located regarding estimation of styrene in sediments and soils (see Section 6.2.3). Water and sediment samples from the Lower Tennessee River were evaluated for styrene, and low concentrations (4.2 ppb) were found (Goodley et al. 1976). A soil survey conducted on 455 NPL and non-NPL hazardous waste sites documented the presence of styrene in either sediments or soils at 3.5% of the sites; the geometric mean concentration of styrene in those samples was 530 μg/kg (CLPSD 1986).

6.4.4 Other Environmental Media

Styrene has been detected as a component of many foods, where it occurs as a natural component of these foods at low levels or slightly higher levels, but most styrene associated with food is the result of packaging of the food material in polystyrene containers. Styrene has been found as a natural component of roasted filberts, dried legumes, fried chicken, cooked pork, roasted beef, mussels, clams, eggs, nectarines, and Beaufort cheese (Dumont and Adda 1978; Kinlin et al. 1972; Lovegren et al. 1979; Takeoko et al. 1988; Tang et al. 1983, 2000), but detected concentrations were often very low (Tang et al. 2000), except for turkey sausage, where detected levels were 100 ppb, and in some cheeses, where concentrations detected were up to 5 ppm (Tang et al. 2000). In contrast, styrene is a natural component of cinnamon, with concentrations up to 40 ppm (Tang et al. 2000).

reorder sentence for clarity

Not clear at first that the contrast is being made concerning natural concentrations. Maybe list all as ppm

Styrene may enter packaged foods by migration from polystyrene food containers and packaging materials, with concentrations ranging from <100 to >3,000 ppm, but common levels being much lower (5–30 ppb) (EPA 1988b; Tang et al. 2000). Concentrations of styrene measured in yogurt packaged in polystyrene containers ranged from 5.5 to 150 ~~μg/L~~ ppb (Withey 1976). Mean levels of styrene in foods packaged in plastic in the United Kingdom ranged from <1 to 180 ~~μg/kg~~ ppb (Gilbert and Startin 1983). Similar concentrations of styrene were detected in other dairy products packaged in polystyrene containers (IARC 1979). The rate of styrene migration into food is mainly a function of the diffusion coefficient of the monomer in the polymer and of the lipophilicity of the food (Till et al. 1987). For example, 4–6% of the free monomer in polystyrene packaging migrated into corn oil or sunflower oil within 10 days, while only 0.3–0.6% migrated into milk, beef, or water. Similarly, migration of styrene from foam cups into liquids such as water, tea, or coffee was about 8 ng/cm^2, while migration into 8% ethanol (as might be encountered in wine or other alcoholic drinks) was 36 ng/cm^2 (Varner and Breder 1981). However, Withey and Collins (1978) found no clear relationship between the styrene monomer content of packaging material (which varied widely) and the amount leached into food after comparable

residence times. Styrene was detected, but not quantified, in samples of mother's milk from four urban areas (Pellizzari et al. 1982).

Styrene has been identified as a component of cigarette smoke (EPA 1984b) and has been detected in concentrations of 18 μg/cigarette in the smoke of cigarettes made in the United States (IARC 1979). Indoor air concentrations of styrene may be significantly higher in homes of smokers than nonsmokers (EPA 1987e).

Consider moving to air

6.5 GENERAL POPULATION AND OCCUPATIONAL EXPOSURE

Exposure to styrene may occur by inhalation, ingestion, or dermal absorption. The most likely mode of exposure of the general population to styrene is by inhalation of indoor air (EPA 1988b). Based on the EPA (1989e) estimate that the average person spends 20.4 hours/day indoors (inhaling about 17 m^3 of air during that time based on an air inhalation rate of 20 m^3/day) and the range of mean indoor air concentrations presented in Table 6-2, typical indoor exposure levels to styrene may range from 1.7 to 850 μg/day. Additional exposures may occur from inhalation of outdoor air and ingestion of food that was stored in polystyrene containers. Based on estimated food consumption rates, Tang et al. (2000) reported an estimated annual general population exposure to styrene ranging from 0.8 to 4.5 mg/person from food. Outdoor air concentrations are likely to be lower in rural than urban areas and are likely to be small compared to indoor air concentrations. Exposure from municipal drinking water is probably insignificant. However, groundwater at hazardous waste sites where styrene has been detected may provide significant exposure to styrene if used as a local water supply.

70 kg?

The exposure of the population to styrene varies significantly from the typical to the worst-case scenario. The daily general population exposure to styrene via food has been estimated at 0.2–1.2 μg/person and the exposure via inhalation has been estimated at 18–54 μg/person, with a total estimated exposure ranging from 18.2 to 55.2 μg/day. This is equivalent to 6.7–20.2 mg/year. ~~It can be seen that~~ Therefore, the primary route of exposure for the general population is via inhalation (Tang et al. 2000). Worst-case exposure estimates, on the other hand, are 0–0.5 μg/day from drinking water, 30 μg/day from food, and 65,000 μg/day from air (EPA 1988b). These estimates are based on the highest levels estimated or monitored and, therefore, reflect the highest potential exposure rather than typical exposure for the general population.

Exposure of the general population to styrene is confirmed by human monitoring data. Styrene has been identified in adipose tissue at concentrations of 8–350 ng/g (EPA 1986d), in blood at a mean

relatives smoke. Children may be also be exposed to higher levels of styrene indoors at home during painting of indoor rooms, especially during winter months (such as over winter school vacations) when the child stays indoors more and during which time, windows may not be opened.

From a food-based exposure perspective, infants may be exposed to styrene from nursing. In a study on chemicals in mother's milk, styrene was a common contaminant (found in 8/12 samples; no concentration data provided) in mother's milk samples collected from mothers living in four U.S. urban areas. The findings, while lacking concentration value, indicate that styrene exposure may occur to nursing children. Food-based exposure of older children is not expected to be significant, except in cases of very high consumption of cinnamon-containing foods or sweets. Typical intake rates of cinnamon, however, are not expected to lead to high levels of styrene ingestion (BFR 2006).

What about formula? since nursing is highly beneficial often misleads to say children are being exposed to chemicals from nursing.

Aside from food-related intake, children's exposure to styrene may also differ from exposures to adults, especially during school, home, or play activities that may expose the children to styrene sources. For example, for elementary aged children (grades 2, 3, 4, and 5) attending inner city schools in Minneapolis, it was found that the lowest exposure to styrene occurred either outdoors or in school, and the highest exposure occurred at home. The latter can be substantially influenced (increased) if smoking occurs in the home. Exposures to styrene while outside, in either winter or spring, were very low (winter: 0.0 $\mu g/m^3$; spring: 0.1 $\mu g/m^3$), whereas exposures were much higher at school (winter: 31.3 $\mu g/m^3$; spring: 39.7 $\mu g/m^3$), but were almost three times higher at home (winter: 91.9 $\mu g/m^3$; spring: 91.9 $\mu g/m^3$) (Adgate et al. 2004). These exposures led to blood level concentrations of styrene that were generally twice as high as the general population (Sexton et al. 2005).

6.7 POPULATIONS WITH POTENTIALLY HIGH EXPOSURES

People working in various styrene industries are likely to have the highest exposures to styrene. Lower levels may be encountered near industrial facilities or hazardous waste sites emitting styrene to outdoor air. High indoor styrene concentrations in the home may be due to emissions from building materials, consumer products, tobacco smoke, photocopiers and laser printers. Smokers and those eating a high proportion of foods packaged in polystyrene may also have above average exposure to styrene, with the amounts estimated by smoking (100 μg from 20 cigarettes) more than doubling the normal estimated exposure to styrene (Tang et al. 2000). In addition, workers with long-term employment at photocopy centers may also be exposed to high concentrations of styrene.

6.8 ADEQUACY OF THE DATABASE

Section 104(i)(5) of CERCLA, as amended, directs the Administrator of ATSDR (in consultation with the Administrator of EPA and agencies and programs of the Public Health Service) to assess whether adequate information on the health effects of styrene is available. Where adequate information is not available, ATSDR, in conjunction with NTP, is required to assure the initiation of a program of research designed to determine the health effects (and techniques for developing methods to determine such health effects) of styrene.

The following categories of possible data needs have been identified by a joint team of scientists from ATSDR, NTP, and EPA. They are defined as substance-specific informational needs that if met would reduce the uncertainties of human health assessment. This definition should not be interpreted to mean that all data needs discussed in this section must be filled. In the future, the identified data needs will be evaluated and prioritized, and a substance-specific research agenda will be proposed.

6.8.1 Identification of Data Needs

Physical and Chemical Properties. The solubility of an organic compound in water is indicative of how that chemical will partition between water, soil, and organisms (Banerjee et al. 1980; Hassett et al. 1983; Valvani et al. 1981). Clarification of the exact solubility of styrene in water would be helpful because a range of values is currently reported (Table 4-2). The Henry's law constant and K_{oc} value for styrene need to be verified experimentally to provide more accurate predictions of air-water and soil-water partitioning.

Production, Import/Export, Use, Release, and Disposal. According to the Emergency Planning and Community Right-to-Know Act of 1986, 42 U.S.C. Section 11023, industries are required to submit substance release and off-site transfer information to the EPA. The TRI, which contains this information for 2005, became available in May of 2007. This database is updated yearly and should provide a list of industrial production facilities and emissions.

Substantial quantities of styrene are currently produced and used in the United States (Heylin 1989; HSDB 1989; SRI 1989; USITC 1988). Production and import quantities, producers, and uses are well documented, with 4.7×10^{12} g produced in 2000 (HSDB 2007), representing a slight increase in U.S. production since 1997 (3.12×10^{12} g). The United States has imported less styrene over the last

Not needed here

Not needed here

several decades, with amounts decreasing more than an order of magnitude, from 1.4×10^{10} g imported in 1978 to 5.7×10^{8} g being imported in 2001 (HSDB 2007). Interestingly, styrene exports increased from 3.6×10^{10} g in 1978 (representing 1% of total U.S. production) to $>1.2 \times 10^{12}$ g exported in 2001 (representing >26% of total U.S. production) (HSDB 2007).

Quantities of styrene disposed of by various disposal methods, other than those reported to the TRI, are not known. Styrene releases into water are regulated by EPA, but styrene is not listed as a hazardous waste constituent and, therefore, land disposal restrictions do not apply to this compound. Additional information on disposal methods used for styrene and styrene-containing products and the quantities disposed of by each method would help to better characterize the potential for human exposure to this compound from disposal at waste sites or other locations.

Environmental Fate. Styrene will partition among the environmental media, with a tendency to volatilize from water to air and to adsorb to soils (EPA 1984b; Roberts et al. 1980; Sato and Nakajima 1978). However, data on styrene volatilization from water and confirmation of the estimated K_{oc} value by adsorption/desorption data would be useful to estimate more accurately the tendency of styrene to partition to air and soil. Confirmation of the K_{oc} would also provide a more reliable basis for estimating the mobility of styrene in the various types of soil.

Although the reaction mechanisms of styrene transformations in the atmosphere are fairly well understood (Atkinson et al. 1982; Bignozzi et al. 1981; EPA 1979a; Sloane and Brudzynski 1979), more information regarding the environmental fates of the transformation products would allow a more accurate prediction of the atmospheric fate of this compound. Biodegradation data are available for styrene under both aerobic and anaerobic conditions (Bridie et al. 1979; Grbic-Galic et al. 1990).

Bioavailability from Environmental Media. Styrene is known to be absorbed following inhalation, oral, and dermal contact (Dutkiewicz and Tyras 1968; Engstrom et al. 1978a, 1978b; Ramsey and Andersen 1984; Ramsey and Young 1978; Withey 1976; Withey and Collins 1979). Absorption rates via inhalation are known (Withey and Collins 1978). Additional data are needed to evaluate absorption rates following oral and dermal exposure. ~~It is believed that~~ absorption of styrene from the gut is believed to be generally rapid and therefore, contact with styrene contaminated food, soil, or water will probably also result in significant absorption. However, this may depend on the medium in which it is contained.

2003), and NIOSH (1984). As shown by the data in Table 7-2, relatively low detection limits can be achieved for the determination of styrene in environmental samples and the accuracy appears to be acceptable for those limited cases in which accuracy data are available. For example, the most sensitive for styrene detection limits were 0.002 μg/L in water, 4 μg/kg in soil, and 500 μg/kg in solid waste. No significant reports were found pertaining to styrene degradation products in environmental samples. Additional information for methods for the determination of styrene in environmental samples are summarized in Table 7-2.

7.3 ADEQUACY OF THE DATABASE

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The following categories of possible data needs have been identified by a joint team of scientists from ATSDR, NTP, and EPA. They are defined as substance-specific informational needs that if met would reduce the uncertainties of human health assessment. This definition should not be interpreted to mean that all data needs discussed in this section must be filled. In the future, the identified data needs will be evaluated and prioritized, and a substance-specific research agenda will be proposed.

7.3.1 Identification of Data Needs

Methods for Determining Biomarkers of Exposure and Effect. Styrene and its primary metabolites, MA and PGA, can be detected in several human tissues (blood, urine, adipose tissue, and in several organs [Table 7-1]), as well as in exhaled breath. The detection limits range in the parts per billion to parts per trillion range (Ashley et al. 1992). Approaches have been developed to provide more efficient and rapid assessment of exposure, such as where the subject breathes for short periods of time into different types of sample collectors (tedlar bags, evacuated canisters, even directly into a mass spectrometer interface [Wallace et al. 1996]), allowing samples to be collected efficiently for subsequent or immediate analysis. Personal monitoring badges containing charcoal have also been developed for

Not a data need + seems out of place

However, the design of studies involving controlled inhalation exposures in humans is precluded by the potential carcinogenicity of styrene.

Methods for Determining Parent Compounds and Degradation Products in Environmental Media. In an occupational setting, the medium that is of most concern for human exposure to styrene is air, although at Superfund sites, contaminated groundwater may pose a danger. Methods are well developed for the determination of styrene in water and air with excellent selectivity and sensitivity (ASTM 1989a; EPA 1989f, 1989g, 1989i, 1995, 1996; NIOSH 1984; USGS 1998). Methods for the determination of styrene in soil and waste samples are not as well developed and may require additional testing and validation (EPA 1986b, 1986c, 2001).

The detection limits for styrene in environmental media cited in Table 7-2 (0.01 mg/sample, typically 10 L, NIOSH 1984; 0.002 μg/L in water, Miermans et al. 2000; and 4 μg/kg in soil, EPA 1986c) are low enough to enable the determination of styrene in any environmental medium likely to pose a hazard to health based upon information currently available in the literature. These detection limits are probably below most ambient background levels of styrene.

Sampling methodologies for compounds such as styrene pose typical collection problems that include the collection of samples that are nonrepresentative, may be of insufficient sample volume, may contain interfering materials that result in low sample recovery, or may contain interfering contaminating chemicals. Other sampling methods may be labor-intensive, or require tedious extraction and purification procedures (Green and Le Pape 1987; Miermans et al. 2000). ~~It is desirable to have~~ methods ~~to~~ that measure organic compounds such as styrene *in situ* in water and other environmental media without the need for sampling and extraction procedures to isolate the analyte prior to analysis are desirable. One such method has been patented, but no commercial products have been identified (see below).

In regard to methods for determining parent styrene and degradation products in environmental media, the following conclusions may be drawn: Because styrene can be detected instrumentally and determined in air and normal water samples with totally adequate selectivity and sensitivity, no additional data are needed at this time. A moderate need exists to improve methodologies to determine styrene in soil, sludges, and solid wastes. Styrene degradation products are a different matter in that little information is available on their determination in environmental samples. In air, these compounds should consist predominantly of photochemical oxidation products, whereas in water and soil samples, they are expected to be biodegradation products. Additional research is needed on the determination of these materials.

Figure 3-1 Levels of Significant Exposure to Styrene - Inhalation

Acute (≤14 days)

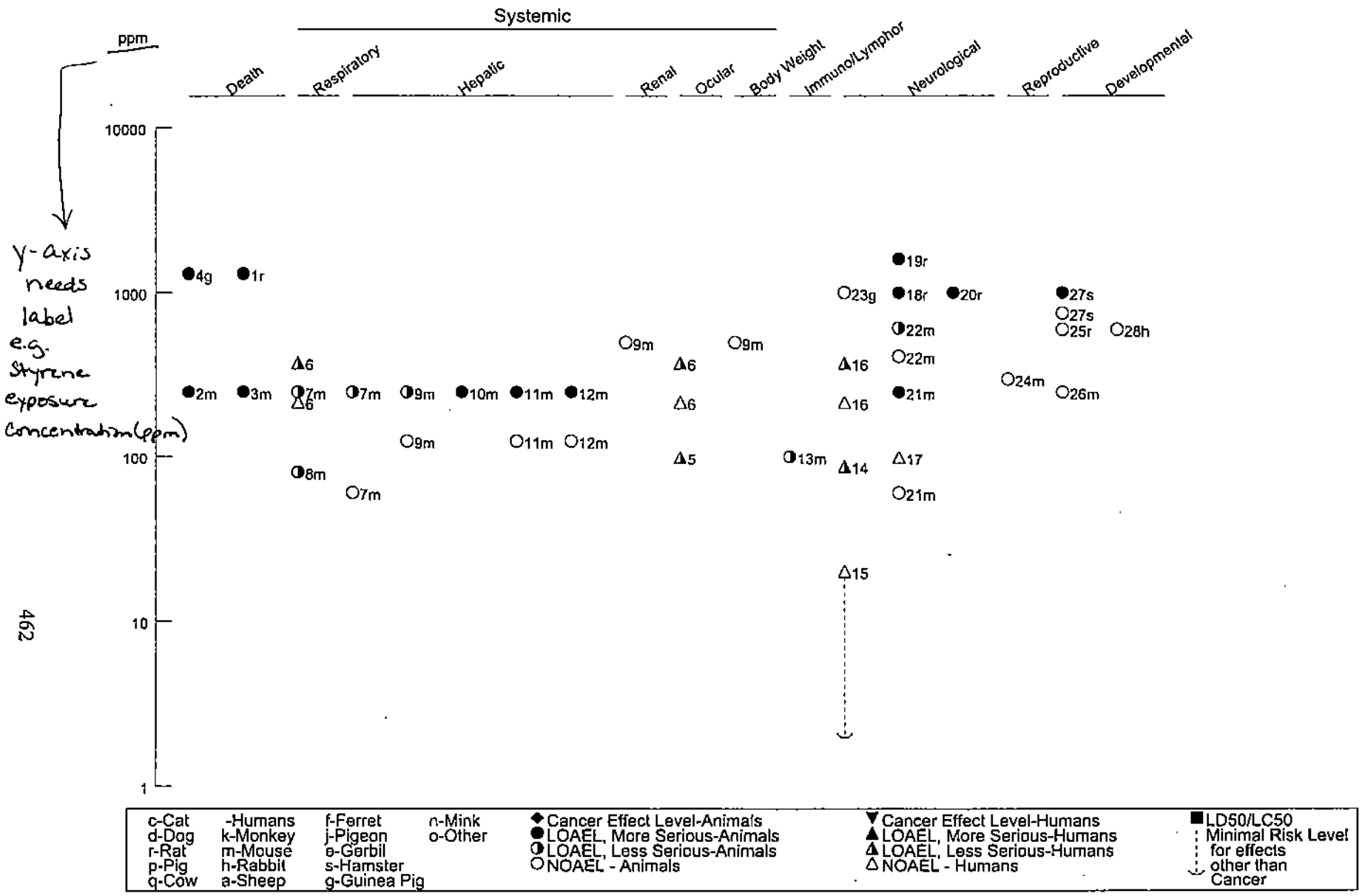
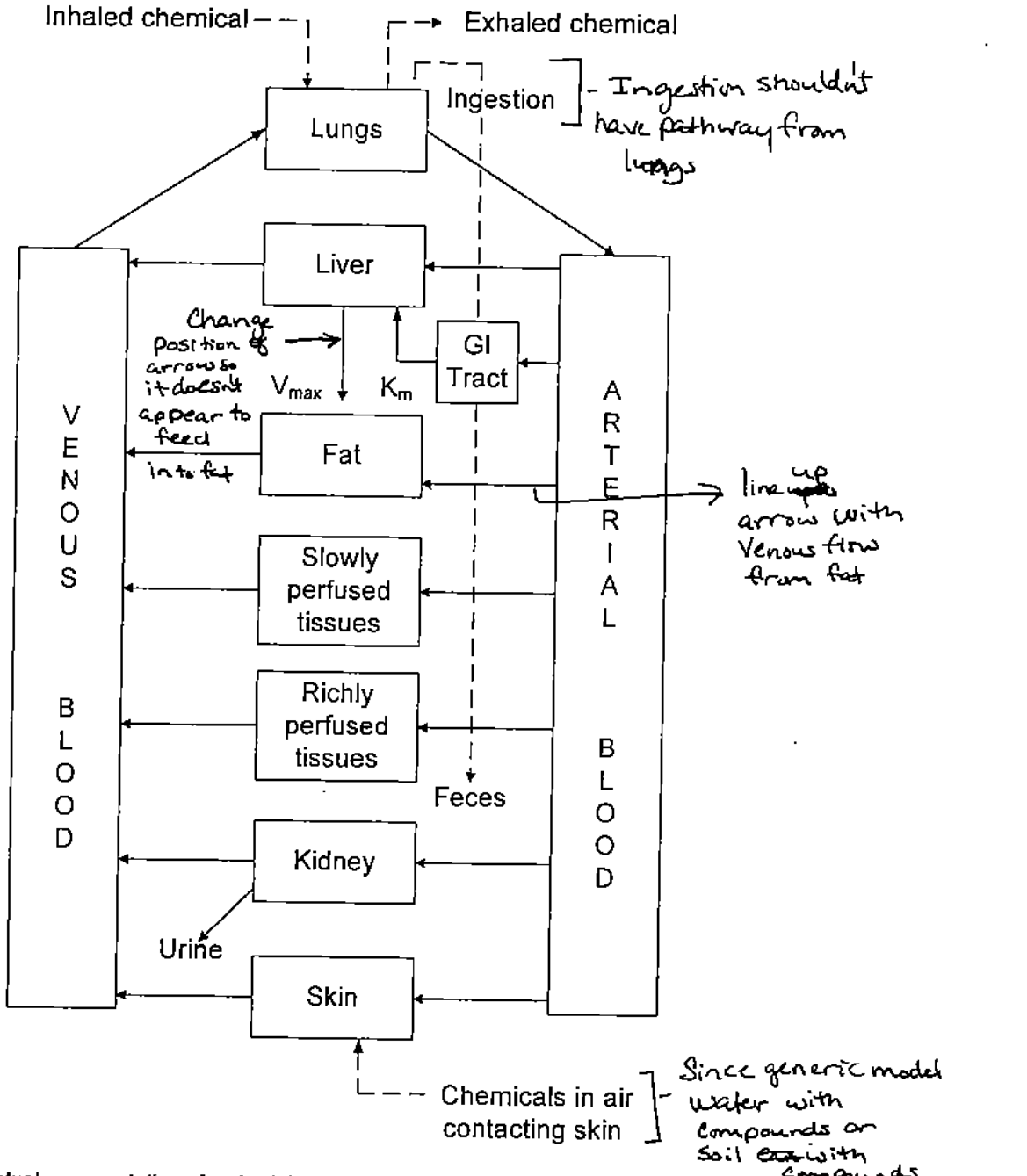


Figure 3-4. Conceptual Representation of a Physiologically Based Pharmacokinetic (PBPK) Model for a Hypothetical Chemical Substance



Note: This is a conceptual representation of a physiologically based pharmacokinetic (PBPK) model for a hypothetical chemical substance. The chemical substance is shown to be absorbed via the skin, by inhalation, or by ingestion, metabolized in the liver, and excreted in the urine or by exhalation.

Source: adapted from Krishnan et al. 1994

ANNOTATED PAGES SUBMITTED BY

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There are no reports of cancer resulting from styrene exposure by the oral or dermal routes in humans. Species differences in styrene carcinogenicity have been detected in animal studies. Inhalation (Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1982) and oral exposure (Beliles et al. 1985; Conti et al. 1988; Maltoni et al. 1982; NCI 1979b) studies in rats have not found significant increases in neoplastic lesions. However, increases in lung tumors have been found in mice following inhalation (Cruzan et al. 2001) and oral exposure (NCI 1979b). The increased production of styrene 7,8-oxide in lung Clara cells and the higher ratio of styrene oxide R- to S-enantiomers likely resulted in the increased sensitivity of mice. Overall, human and animal studies suggest that styrene may be a weak human carcinogen. The International Agency for Research on Cancer (IARC) has assigned styrene to Group 2B, possibly carcinogenic to humans (IARC 2006).

2.3 MINIMAL RISK LEVELS (MRLs)

Estimates of exposure levels posing minimal risk to humans (MRLs) have been made for styrene. An MRL is defined as an estimate of daily human exposure to a substance that is likely to be without an appreciable risk of adverse effects (noncarcinogenic) over a specified duration of exposure. MRLs are derived when reliable and sufficient data exist to identify the target organ(s) of effect or the most sensitive health effect(s) for a specific duration within a given route of exposure. MRLs are based on noncancerous health effects only and do not consider carcinogenic effects. MRLs can be derived for acute, intermediate, and chronic duration exposures for inhalation and oral routes. Appropriate methodology does not exist to develop MRLs for dermal exposure.

Although methods have been established to derive these levels (Barnes and Dourson 1988; EPA 1990), uncertainties are associated with these techniques. Furthermore, ATSDR acknowledges additional uncertainties inherent in the application of the procedures to derive less than lifetime MRLs. As an example, acute inhalation MRLs may not be protective for health effects that are delayed in development or are acquired following repeated acute insults, such as hypersensitivity reactions, asthma, or chronic bronchitis. As these kinds of health effects data become available and methods to assess levels of significant human exposure improve, these MRLs will be revised.

par. repeated

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MRLs. As an example, acute inhalation MRLs may not be protective for health effects that are delayed in development or are acquired following repeated acute insults, such as hypersensitivity reactions, asthma, or chronic bronchitis. As these kinds of health effects data become available and methods to assess levels of significant human exposure improve, these MRLs will be revised.

repeat MRLs

Inhalation MRLs

- An MRL of 2 ppm has been derived for acute-duration inhalation exposure (14 days or less) to styrene.

The acute-duration inhalation toxicity database for styrene consists of several human experimental studies primarily examining neurotoxicity (Odkvist et al. 1982; Seeber et al. 2004; Stewart et al. 1968), systemic toxicity studies in mice (Cruzan et al. 1997, 2001; Morgan et al. 1993a, 1993b, 1993c), neurotoxicity studies in rats (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003), mice (DeCeaurriz et al. 1983), and guinea pigs (Lataye et al. 2003), a reproductive toxicity study in mice (Salomaa et al. 1985), and developmental toxicity studies in rats (Murray et al. 1978), mice (Kankaanpää et al. 1980), hamsters (Kankaanpää et al. 1980), and rabbits (Murray et al. 1978). Exposure to 99 ppm for 7 hours or 376 ppm for 1 hour (Stewart et al. 1968) resulted in eye irritation; nasal irritation was also reported at 376 ppm. A significant inhibition of the vestibular-oculomotor system was observed in subjects exposed to 87 ppm for 1 hour (Odkvist et al. 1982). Studies by (Stewart et al. 1968) found alterations in tests of balance or coordination in subjects exposed to 376 ppm for 1 hour, but not after exposure to 99 ppm for 7 hours or 216 ppm for 1 hour; the test used in the Stewart et al. (1968) studies is probably less sensitive than those used by Odkvist et al. (1982). No significant alterations in performance on tests of reaction time were observed in subjects exposed to 20 ppm for 3 hours (Seeber et al. 2004).

In mice, the most sensitive target of styrene toxicity appears to be the nasal olfactory epithelium; single cell necrosis was observed following exposure to 80 ppm 6 hours/day for 3 days (Cruzan et al. 2001). At 250 ppm, hepatocellular necrosis and degeneration have been observed (Cruzan et al. 1997; Morgan et al. 1993a, 1993b, 1993c). The severity of this lesion appears to be inversely related to the duration of exposure, with more severe damage observed in mice killed within 3 days of exposure (Morgan et al. 1993a, 1993b, 1993c) compared to animals killed after 2 weeks of exposure (Cruzan et al. 1997; Morgan et al. 1993a). Exposure to 250 ppm 6 hours/day, 5 days/week for 2 weeks also resulted in lethargy and unsteady gait in mice (Cruzan et al. 1997). Impaired performance on a swimming test was observed in mice exposed to 610 ppm for 4 hours, but not in animals exposed to 413 ppm (DeCeaurriz et al. 1983). Exposure of rats to high concentrations (1,000 or 1,600 ppm) 6–8 hours/day for 5–14 days resulted in

auditory threshold shifts (indicative of hearing loss) and loss of outer hair cells (OHC) in the organ of Corti (Campo et al. 2001; Crofton et al. 1994; Lataye et al. 2003). No alterations in sperm morphology were observed in mice exposed to 300 ppm styrene 5 hours/day for 5 days (Salomaa et al. 1995) and no developmental effects were observed in rats or rabbits exposed to 600 ppm 7 hours/day on gestational days 6–15 or 6–18, respectively, (Murray et al. 1978) or mice exposed to 250 ppm 6 hours/day on gestational days 6–16 (Kankaanpää et al. 1980). An increase in fetal deaths or resorptions was observed in hamsters exposed to 1,000 ppm 6 hours/day on gestational days 6–18 (Kankaanpää et al. 1980).

These data suggest that the nervous system is the most sensitive target of styrene toxicity following acute-duration inhalation exposure. The lowest LOAEL for a relevant end point in humans is 87 ppm for vestibular impairment in subjects exposed to styrene for 1 hour (Odkvist et al. 1982). A similar LOAEL (80 ppm) was identified for nasal effects in mice exposed to styrene for 3 days (Cruzan et al. 2001); this effect was not considered suitable as the basis of an MRL. As stated previously, mice appear to have a greater capacity than humans to generate the reactive metabolite, styrene oxide, in the nasal cavity and a lower capacity to detoxify styrene oxide (Green et al. 2001a). The identification of the nervous system as the critical target of toxicity for styrene is supported by a large number of occupational exposure studies. Delays in reaction time have been observed in workers exposed to 21.9–92 ppm (Cherry et al. 1980; Fallas et al. 1992; Gamberale et al. 1976; Jegaden et al. 1993; Mutti et al. 1984a; Tsai and Chen 1996) and vestibular effects have been observed at 18–36 ppm (Calabrese et al. 1996; Möller et al. 1990; Toppila et al. 2006).

See letter pg1-2.

The Odkvist et al. (1982) study did not identify a NOAEL for vestibular effects; however, a NOAEL of 20 ppm for performance on several tests of reaction time and attention was identified by Seeber et al. (2004) in subjects exposed to styrene for 3 hours. Although there is some uncertainty whether deriving an MRL based on a 3-hour exposure study would be protective of continuous exposure to styrene for 2 weeks, the Seeber et al. (2004) study was selected as the basis on an acute duration inhalation MRL for styrene.

Groups of eight volunteers (gender not reported) were exposed to 0.5 or 20 ppm styrene for 3 hours (Seeber et al. 2004). The subjects were tested for simple reaction time, choice reaction time, and attention prior to exposure initiation, during the third hour of exposure, and 1.5 hours after exposure termination. The subjects were also asked to complete a symptom questionnaire before, during, and after exposure. The mean concentration of styrene in blood was 2.2 and 80 μg/L after 3 hours of exposure to 0.5 and 20 ppm, respectively. The blood levels of styrene were correlated with styrene levels in air (r_{xy}=0.98).

the absence of human neurotoxicity data, an intermediate-duration inhalation MRL is not recommended at this time.

- An MRL of 0.05 ppm has been derived for chronic-duration inhalation exposure (greater than 365 days) to styrene.

A large number of studies have examined the neurotoxicity of styrene in workers at reinforced plastic manufacturing facilities. These studies reported a variety of neurological effects, including impaired color vision, slowed reaction time, permanent hearing threshold shifts, vestibular effects, and increases in subjective symptoms. A summary of the results of studies for some of these neurological effects is presented in Table 2-1. The LOAELs for these effects range from 6 to 93 ppm.

Chronic-duration studies in animals identify the nasal olfactory epithelium as the most sensitive end point. Atrophic and/or degenerative changes were observed in rats exposed to 50 ppm styrene 6 hours/day, 5 days/week for 104 weeks (Cruzan et al. 1998) and respiratory metaplasia in the nasal olfactory epithelium has been observed in mice exposed to 20 ppm 6 hours/day, 5 days/week for 98–104 weeks (Cruzan et al. 2001). As noted previously, mice do not appear to be a good model for potential respiratory effects in humans.

Neuotoxicity observed in styrene workers was selected as the basis of the chronic-duration inhalation MRL for styrene. Two approaches to deriving the MRL were considered. In the first approach, the MRL is based on a single study identifying a sensitive LOAEL. In the study by Kishi et al. (2001), styrene workers were divided into three groups based on urinary mandelic acid excretion levels. A significant increase in color confusion index (CCI) was found in the workers exposed to equivalent styrene concentrations of 10 or 46 ppm, as compared to age-matched controls. No significant alterations were observed in workers exposed to 4 ppm. The second approach involves the use of a LOAEL estimated from a meta-analysis of occupational exposure studies finding effects on color vision and reaction time (Benignus et al. 2005). Benignus et al. (2005) used data color vision impairment data from the Campagna et al. (1996), Eguchi et al. (1995), Gobba et al. (1991), Gong et al. (2002), and Kishi et al. (2001) studies and choice reaction time data from the Jegaden et al. (1993), Mutti et al. (1984a), Triebig et al. (1989), and Tsai and Chen (1996) studies. Average styrene exposure concentrations were estimated from individual data reported in the papers; for studies reporting individual data as urinary mandelic acid levels, standardized methods for converting to styrene exposure levels were used. Cumulative styrene exposure was estimated by multiplying exposure level by length of employment. A common metric of effect magnitude (percentage of baseline) was calculated for the different neurological effects. The

see p. 17

on pg 16-17, emphasize serious nature of neurotoxic endpoints – see p. 3 of letter

analysis found a significant linear relationship between choice reaction time and cumulative styrene exposure; cumulative exposure accounted for 91% of the variance in reaction time. Similarly, a significant relationship between CCI and cumulative styrene exposure was found, with cumulative exposure accounting for 35% of the variance in CCI. Using the regression equations for these two effects, Benignus et al. (2005) estimated that exposure to 150 ppm for 8 work-years would result in a 50% increase in choice reaction time and a 17% increase in CCI score; exposure to 20 ppm for 8 work-years would result in a 6.5% increase in choice reaction time and a 2.23% increase in CCI score. As discussed in Benignus et al. (2005), a 7% decrease in reaction time would prevent 58,000–70,000 injuries per year from automobile accidents. The investigators also noted that CCI increases with age, the rate of increase is about 10% per 13 years of age; thus, a 2.23% decrease in color perception would be roughly equivalent to 1.7 additional years of age. Based on this analysis, 20 ppm is considered a LOAEL for neurological effects.

The LOAEL of 20 ppm is consistent with the LOAEL values identified in many of the individual studies. However, using the LOAEL identified from the Benignus et al. (2005) meta-analysis has several advantages over selecting a single study as the basis of the MRL. Because data were pooled from several studies, the relationships between styrene exposure and effects were examined in a large number of subjects (302 subjects for choice reaction time and 383 subjects for color vision). The use of standardized methods for estimating styrene exposure levels from urinary biomarker levels is also an advantage. Additionally, the biological relevance of the observed deficits in reaction time and color vision was estimated. The LOAEL of 20 ppm was adjusted for intermittent exposure (8 hours/day, 5 days/week) resulting in an adjusted LOAEL of 5 ppm, which was divided by an uncertainty factor of 100 (10 for use of a LOAEL and 10 for human variability) resulting in a chronic-duration inhalation MRL of 0.05 ppm.

Compare with other agencies

Oral MRLs

- An MRL of 0.1 mg/kg/day has been derived for acute-duration oral exposure (14 days or less) to styrene.

A limited number of studies have examined the acute toxicity of orally-administered styrene; these studies have examined potential neurotoxicity and developmental toxicity. No developmental effects were observed in rats administered a single dose of 300 mg/kg on gestational day 11 (Daston et al. 1991) or administered 300 mg/kg/day (administered as two daily doses of 150 mg/kg) on gestational days 6–15 (Murray et al. 1978). Impaired learning was observed in rats administered via gavage 100 or 200 mg/kg/day for 14 days; increases in serotonin levels were observed in the hypothalamus,

because it helps the users of the profiles to identify levels of exposure at which major health effects start to appear. LOAELs or NOAELs should also help in determining whether or not the effects vary with dose and/or duration, and place into perspective the possible significance of these effects to human health.

The significance of the exposure levels shown in the Levels of Significant Exposure (LSE) tables and figures may differ depending on the user's perspective. Public health officials and others concerned with appropriate actions to take at hazardous waste sites may want information on levels of exposure associated with more subtle effects in humans or animals (LOAELs) or exposure levels below which no adverse effects NOAELs) have been observed. Estimates of levels posing minimal risk to humans Minimal Risk Levels or MRLs) may be of interest to health professionals and citizens alike.

A User's Guide has been provided at the end of this profile see (Appendix B). This guide should aid in the interpretation of the tables and figures for Levels of Significant Exposure and the MRLs.

3.2.1 Inhalation Exposure

Most information on the effects of inhalation exposure to styrene in humans comes from studies of workers exposed to styrene vapors in the production and use of plastics and resins, especially polystyrene resins. In most cases, the studies involve workplace exposures such as fiberglass boat building factories where the actual levels of styrene are reported as a range of styrene air concentrations. However, there are a few human clinical studies in which exposures are better quantified. Provided below are descriptions of the known effects of inhalation exposure of humans and animals to styrene.

3.2.1.1 Death

There have been no reports of deaths in humans directly associated with exposure to styrene in the workplace (EPA 1988b; Gosselin et al. 1984; NIOSH 1983).

In animals, inhalation studies indicate that the acute toxicity of styrene is low to moderate. An LC_{50} of 2,770 ppm after 2 hours of exposure was reported in rats, and the LC_{50} for mice after exposure for 4 hours was 4,940 ppm (Shugaev 1969). All rats and guinea pigs survived after exposure to 1,300 ppm styrene for 30 hours and 16 hours, respectively (Spencer et al. 1942). However, all animals died after 40 hours of exposure. Gender differences in mortality were observed in repeated-exposure studies (Cruzan et al.

In an international cohort of styrene workers, a significant association between mortality from central nervous system disease and cumulative styrene exposure was found (Welp et al. 1996c). The rate ratio was 3.29 (95% confidence interval [CI] of 0.48–22.65) for workers exposed to 25–49 ppm-years and 16.32 (95% CI 3.47–76.73) for those exposed for 200–349 ppm-years. A similar relationship was found for shorter durations of styrene exposure. The rate ratio was 2.33 (95% CI 0.40–13.56) for workers exposed for 6–11 months and 8.80 (95% CI 1.87–41.33) for workers exposed for 7–9 months. A significant association between mortality from epilepsy and duration of styrene exposure was found; the rate ratio in workers exposed for ≥10 years was 28.4 (95% CI 2.11–381.5). Time since first exposure was also significantly associated with mortality from epilepsy. Significant associations between mental disorders and duration of exposure and between suicide and duration of exposure were also found; however, for both of these causes of death, the rate ratio decreased with increasing duration of exposure and the investigators noted that lifestyle factors, rather than a direct effect of styrene, appear to be the most likely cause of the higher mortality. discuss healthy worker effect in human studies - see p. 4 of letter

A variety of neurological effects have been reported in workers chronically exposed to styrene including altered vestibular function, impaired hearing, impaired color vision, impaired performance on neurobehavioral tests, and increased in clinical symptoms. In general, these occupational exposure studies have several limitations. In most cases, the exposure levels reflect current exposure conditions and do not take into consideration past exposure to higher styrene levels that may have resulted in permanent damage. Some workers, particularly laminators, wore respiratory masks with or without canisters; many investigators estimated exposure based on biomarker levels, particularly urinary mandelic acid levels, while others did not. Additionally, significant differences between workers and referents were reported as LOAELs; however, the magnitude of the alteration may have been subclinical. A summary of the neurological effects observed in styrene workers is presented in Table 3-2.

Color vision appears to be one of the more sensitive targets of styrene toxicity, with many studies reporting alterations. Color vision was typically measured using the Lanthony desaturated panel D-15 test in which the subjects were asked to arrange 15 painted caps in a line with definite chromatic sequence; the color confusion index (CCI) quantifies the number of types of mistake. A significant correlation between CCI and urinary mandelic acid concentration (after correction for age) was observed in workers at fiberglass reinforced plastic facilities (Kishi et al. 2001). When workers were divided into three groups based on end-of-shift urinary mandelic acid levels, there were significant differences between CCI in workers with a mean a mandelic acid level of 0.14 or 0.65 g/L and age-matched referents;

On pg. 30+31, explain significance of mandelic and phenylglyoxylic acids in terms of styrene exposure (styrene metabolites) - see p. 4 of letter

no difference was found for the third group with a mean mandelic acid level of 0.05 g/L. The investigators estimated that these urinary mandelic acid levels were equivalent to styrene exposure levels of 4, 10, and 46 ppm. Thus, this study identifies a NOAEL of 4 ppm and a LOAEL of 10 ppm for impaired color vision. Similarly, Gong et al. (2002) found significantly higher CCI values in workers at a fiberglass reinforced plastic boat facility with end-of-shift urinary mandelic acid and phenylglyoxylic acid levels of ≥0.24 g/g creatinine or <0.24 g/g creatinine; a mandelic acid plus phenylglyoxylic acid urine level of 0.24 g/g creatinine is equivalent to a styrene exposure level of 10 ppm. A significant increase in CCI was also observed in workers at fiberglass reinforced plastic facilities exposed to a geometric man concentration of 16 ppm, as compared to age-matched controls (Gobba et al. 1991). In contrast to other studies, Gobba et al. (1991) did not find a significant relationship between end-of-shift urinary mandelic acid levels and CCI; however, urinary styrene levels correlated with CCI values. Significantly higher CCI values were observed in fiberglass reinforced workers with a mean urinary mandelic acid levels of 1.06 g/L, which is roughly equivalent to a styrene exposure level of 93 ppm (Eguchi et al. 1995). This study did not find significant alteration in workers with a mean urinary mandelic acid level of 0.02 g/L, equivalent to 8 ppm. Another study of fiberglass reinforced plastic workers (some of this cohort was examined by Gobba et al. 1991 and Campagna et al. 1995) found a significant association between CCI and styrene exposure levels (Campagna et al. 1996). The investigators concluded that color vision impairment could be detected at styrene levels of 4 ppm with a 95% upper confidence limit of 26 ppm. Two other occupational exposure studies using different measures of color vision impairment also found significant alterations. Chia et al. (1994) found significantly poorer color discrimination, after adjusting for age, education, and alcohol consumption, in 21 workers at a fiber-reinforced plastic boat manufacturing facility; the styrene exposure level of 6 ppm was estimated from a mean end-of-shift urinary mandelic acid level of 84.0 mg/g creatinine. No relationship between the total color difference score and the urinary mandelic acid level was found. In 60 workers in the shipbuilding industry with a mean styrene exposure level of 24.3 ppm, a significantly higher incidence of workers with errors in the blue-yellow or red-green ranges, compared to a referent group, was found (Fallas et al. 1992). Total error score was significantly different in workers, with a lifetime weighted average exposure level of 22 ppm styrene, as compared to workers in a low exposure group (9 ppm) (Iregren et al. 2005). Several studies found improvements in color vision following an extended period of no styrene exposure or lower exposure. Triebig et al. (2001) reported a significant improvement in CCI scores following a 4-week period with no styrene exposure; in contrast, no improvement in CCI scores was found in another group of styrene workers following a 1-month period without styrene exposure (Gobba et al. 1991). Two studies found significant improvements in color vision (age-adjusted color confusion score or CCI score) were observed in styrene workers following a decrease in styrene air level (Castillo et al. 2001; Triebig et

al. 2001). However, one study found no change in age-adjusted near visual contrast sensitivity following a decrease in styrene exposure levels (Castillo et al. 2001).

A number of studies have found significant alterations in performance on a variety of neurobehavioral tests; among these studies, reaction time appears to be the most frequently examined end point. Significant increases in simple reaction time have been observed in styrene workers exposed to concentrations of 21.9, 22.68, 47, or 92 ppm (Cherry et al. 1980; Gamberale et al. 1976; Jegaden et al. 1993; Tsai and Chen 1996); tests for reaction time were measured in the morning before the work shift, suggesting that the effect was not due to acute exposure to styrene. The reaction times were 4–10% slower in the styrene workers as compared to the referent groups. No significant alterations in simple reaction time were observed in workers exposed to 8.6 ppm (Edling et al. 1993) or 24.3 ppm (Fallas et al. 1992). Similarly, complex reaction time was significantly increased among styrene workers exposed to 21.9, 22.68, or 25 ppm (Fallas et al. 1992; Jegaden et al. 1993; Mutti et al. 1984a); the variance from controls ranged from 7.5 to 20%. No alterations in complex reaction time were observed in workers exposed to 8.6 ppm (Edling et al. 1993).

Impaired performance on the digit span test, which measures attention/concentration, was observed in workers exposed to 6 ppm (Chia et al. 1994), 22.68 ppm (Jegaden et al. 1993), or 24.3 ppm (Fallas et al. 1992). Other neurobehavioral performance tests that may be altered by chronic exposure to styrene included digit symbol or symbol digit tests at 6 ppm (Chia et al. 1994), visuomotor at 50 ppm (Mutti et al 1984a) or 75 ppm (Lindstrom et al. 1976), and memory at 25 ppm (Mutti et al. 1984a). However, other studies have not found significant alterations in digit symbol at 8.6 ppm (Edling et al. 1993), 24.3 ppm (Fallas et 1992), or 25 ppm (Mutti et al. 1984a), or memory at 75 ppm (Lindstrom et al. 1976).

expand Mutti et al 1984 discussion to include CVS function as a domain or syndrome. see p. 9 of letter.

A number of clinical symptoms of neurotoxicity have been reported by styrene workers; commonly reported symptoms included headaches, dizziness, impaired memory, and feeling "drunk". At 6 or 12 ppm, abnormal tiredness and short memory were reported by most of the styrene workers examined by Flodin et al. (1989); problems concentrating and irritation were also reported by most workers exposed to 12 ppm. After a 7-month period without styrene exposure, there was a marked improvement in symptoms and the mean number of symptoms reported was 1.9, compared to 5.3 reported 7 months earlier. Fiberglass reinforced plastic industry workers exposed to 18.9 or 50.0 ppm reported a higher prevalence of headaches, dizziness, light headedness, fatigue, irritability, feeling "drunk", and memory loss (Checkoway et al. 1992); the prevalence of clinical signs was not significantly increased in workers exposed to 10.8 ppm. Increases in the incidence of headache, memory disturbances, forgetfulness,

these studies is the lack of exposure information, including levels of styrene and confounding exposure to other chemicals; thus, it is difficult to ascribe the increased cancer risks to styrene exposure. Loughlin et al. (1999) examined former students who attended a high school adjacent to synthetic styrene-butadiene rubber production facilities between 1963 and 1993 and found no significant alterations in deaths from lymphatic and hematopoietic cancer. Two studies have examined the possible association between styrene exposure and breast cancer. A case-control study by Cantor et al. (1995) found significant elevations in the risk of breast cancer among women possibly exposed to styrene in the workplace. Coyle et al. (2005) found a significant higher incidence of age-adjusted breast cancer rate in men and women, women, and women ≥50 years of age and living in counties with EPA toxics release inventory (TRI) facilities with on-site releases of styrene. As with the other population-based studies, these studies did not monitor styrene levels or exposure to other potentially carcinogenic chemicals and thus provided limited information on the carcinogenic potential of styrene.

See pg 5-7 of letter for discussion re pg 43-44 on bioassays

The carcinogenicity of styrene has been examined in three studies in rats (Conti et al. 1988; Cruzan et al. 1998; Jersey et al. 1978; Maltoni et al. 1982) and one study in mice (Cruzan et al. 2001). No significant increases in the incidence neoplastic lesions were observed in rats exposed to styrene concentrations as high as 1,000 ppm 6 hours/day, 5 days/week for 2 years (Cruzan et al. 1998). Similarly, exposure of female rats to 600 or 1,000 ppm styrene 6 hours/day, 5 days/week for 21 months did not result in styrene-related increases in the incidence neoplastic tumors (Jersey et al. 1978); a high incidence of chronic murine pneumonia in the control and 1,000 ppm male rats precludes the use of the male data for assessing the carcinogenic potential of styrene. A non-concentration-related increase in the incidence of malignant mammary tumors were observed in female rats exposed to 100, 200, or 300 ppm styrene 4 hours/day, 5 days/week for 52 weeks (Conti et al. 1988); the incidences were 6/60, 6/30, 4/30, 9/30, 12/30, and 9/30 in the 0, 25, 50, 100, 200, and 300 ppm groups, respectively. No other significant increases in specific tumors were observed in this study (Conti et al. 1988; Maltoni et al. 1982). The findings of the Conti et al. (1988) study conflict with those of Cruzan et al. (1998), who found a concentration-related decrease in mammary tumors in female rats exposed to similar or higher styrene concentrations for a longer duration. In contrast to the results in rat studies, significant increases in the incidence of bronchioalveolar carcinoma were observed in female mice exposed to 160 ppm 6 hours/day, 5 days/week for approximately 2 years (Cruzan et al. 2001). Significant trends for increasing incidences of bronchioalveolar ademona were also observed for the male and female mice.

no dose, no response see pg. 5-6 of letter

inconsistent results may be explained by high dose non-cancer toxicity - see p. 6-7 of letter

As reviewed by IARC (2002) and Cruzan et al. (2002), toxicokinetic differences in the metabolism of styrene in the lungs have been observed in humans, rats, and mice. In rats and mice, Clara cells have the

mouse lung tumors are relevant to humans - see p. 6 of letter
need information on cell type in tumor - see Cruzan et al (2001) and p. 6-7 of letter

ability to metabolize styrene to styrene 7,8-oxide in the lung; humans have limited ability to metabolize styrene to styrene 7,8-oxide in the lung. A physiologically based pharmacokinetic (PBPK) model predicts that the total amount of styrene oxide in the terminal bronchioles in mice is 10 times higher than in rats and 100-fold higher than in humans. In addition to these quantitative differences in the generation of styrene 7,8-oxide between rats and mice, there are qualitative differences in styrene metabolism. Mice produce a higher levels of the R-enantiomer of styrene oxide, as compared to rats; the R-enantiomer has been shown to be more potent pneumotoxic than the S-enantiomer. The ratio of R- to S-enantiomers ranges from 2.2 to 2.87 in mice exposed to 20–160 ppm styrene and from 0.7 to 0.73 in rats exposed to 50–1,000 ppm. Thus, mice appear to be very sensitive to the induction of lung tumors and the mechanism of inducing lung tumors is not likely to be relevant to humans. see p.6 of letter for more discussion on enantiomeric specificity.

3.2.2 Oral Exposure

No studies were located regarding health effects in humans after oral ingestion of styrene. Based on the animal data that follow, the oral toxicity of styrene in humans would be expected to be low to moderate.

3.2.2.1 Death

No deaths in humans from ingesting styrene have been reported in the evaluations of case studies (EPA 1989c; Gosselin et al. 1984; NIOSH 1983).

The approximate reported oral LD_{50} for male and female rats was 5,000 mg/kg (Wolf et al. 1956). A 100% survival rate and 100% mortality rate were reported in rats exposed to single oral doses of styrene (observation period 2 weeks) at 1,600 and 8,000 mg/kg, respectively (Spencer et al. 1942). Death in this study was mainly due to pronounced irritation of the esophagus and stomach. In another study, female mice were given a single oral dose of 1,350 mg/kg styrene on the 17th day of pregnancy (Ponomarkov and Tomatis 1978). After weaning, the progeny received the same dose once per week. The treatment was suspended after 16 weeks due to high mortality among the progeny (including both males and females). Fifty percent of the males and 20% of the females had died after 20 weeks, despite the suspension of treatment at week 16. The cause of death was liver necrosis and lung congestion. A high mortality rate was reported in 40 female rats exposed to 250 mg/kg/day styrene for 52 weeks (Conti et al. 1988). Mortality was significantly elevated in male and female rats administered styrene by gavage at a dosage level of 2,000 mg/kg/day for 78 weeks (NCI 1979b). In this study, mortality was unaffected at dosage levels of 500 and 1,000 mg/kg/day in male and female rats. Male mice administered styrene at

3.2.3.2 Systemic Effects

No studies were located regarding respiratory, cardiovascular, gastrointestinal, hematological, musculoskeletal, hepatic, endocrine, or renal effects in humans or animals after dermal exposure to styrene.

Dermal Effects. Marked irritation with denaturation of the skin was noted when styrene was applied in small amounts over a 4-week period to the shaved abdomen of rabbits at 20,000 mg/kg total dose) (Spencer et al. 1942).

Ocular Effects. Moderate conjunctival irritation and transient corneal injury of the eyes were observed when undiluted styrene was tested in rabbit eyes (Wolf et al. 1956). The effects were produced immediately (within 3 minutes) by a single administration of two drops (about 0.1 mL) and persisted throughout the 7-day observation period.

No studies were located regarding the following health effects in humans or animals after dermal exposure to styrene:

3.2.3.3 Immunological and Lymphoreticular Effects

3.2.3.4 Neurological Effects

3.2.3.5 Reproductive Effects

3.2.3.6 Developmental Effects

3.2.3.7 Cancer

3.3 GENOTOXICITY – see pg 7-8 of letter for fuller discussion on genotoxicity.

The genotoxicity of styrene has been examined in numerous *in vivo* studies of workers and laboratory animals; these data are summarized in Table 3-4. Chromosomal damage, DNA strand breaks, and mutagenic effects have frequently been studied in workers exposed to styrene in the production of reinforced plastic products and styrene/polystyrene production. In general, these studies are limited by the fact that workers in these industries are often exposed to chemicals other than styrene, such as methylene chloride and epoxide resins, and many studies did not control for potential confounding factors such as age, sex, and smoking status. Chromosomal aberrations have been reported in numerous studies of workers exposed to styrene for 1– 25 years in reinforced plastic operations (Anwar and Shamy 1995; Artuso et al. 1995; Hogstedt et al. 1979; Mäki-Paakkanen et al. 1991; Meretoja et al. 1977, 1978;

An oral dose of 20 mg/kg of ^{14}C styrene was administered to male and female rats (Plotnick and Weigel 1979). Tissue levels peaked at 4 hours or earlier after dosing. Less than 10% of the administered dose was found in the stomach, small intestine, and large intestine 8 hours after dosing. The kidney had the highest concentration of radioactivity at all time intervals, with decreasing amounts in the liver and pancreas. Fat tissue showed increased levels after 2 hours. All tissue levels were below 1 μg/g at 24 hours and at 48 and 72 hours were below the limit of detection. Excretion data from the Plotnick and Weigel (1979) study are presented in Section 3.4.4.2.

3.4.2.3 Dermal Exposure

No studies were located regarding distribution in humans after dermal exposure to styrene.

Immersion of rats' tails in pure liquid styrene for 1 hour resulted in styrene levels in the liver and brain that were estimated to be between 50 and 70% of the concentrations found in the same organs after 4-hour inhalation exposure to a vapor concentration of 11.8 g/m^3 (Shugaev 1969). A skin:air partition coefficient of 91.9 was calculated using rat skin (Mattie et al. 1994).

3.4.3 Metabolism

There have been numerous studies, conducted primarily via inhalation, that address the metabolism of styrene in humans and animals (Drummond et al. 1989; Engstrom et al. 1976; Korn et al. 1984, 1987; Leibman 1975; Lof et al. 1983; Withey and Collins 1979; Young et al. 1979). The proposed pathways of styrene metabolism are shown in Figure 3-3. Styrene is metabolized by the microsomal NADPH-cytochrome P-450 dependent mono-oxygenase to styrene oxide. The styrene oxide is then hydrated to phenylethylene glycol (styrene glycol). This transformation is catalyzed by microsomal epoxide hydratase. The styrene glycol is then metabolized directly to MA or to benzoic acid and then hippuric acid. Mandelic acid is also metabolized to PGA. The MA, hippuric acid, and PGA are excreted in the urine. In another pathway, styrene oxide is metabolized by cystolic glutathione-S-transferase to mercapturic acids appearing in the urine as hydroxyphenylethyl mercapturic acid. A minor metabolic pathway of styrene in rats involves the formation of 1- and 2-phenylethanol and ring hydroxlation to form vinyl phenol as urinary metabolites. The presence of 4-vinylphenol has been reported in the urine of workers exposed to styrene, but this may have been due to the contamination of the styrene to which the subjects were exposed (Pfaffli et al. 1981). The urinary metabolites that predominate in humans are MA and PGA. In rats, the predominant urinary metabolites are MA, PGA, hippuric acid, and glucuronide. Metabolic conversion to styrene-7,8-epoxide (styrene oxide) by the microsomal mixed function oxidase

CD - Reference list: Rabovsky et al (2001) missing. ~~Cited~~ in paper reference list although not cited - see 8 of letter and other letter discussions that cite this reference.

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Identify as USEPA. see p. 8 of letter.

Table 3-1 Levels of Significant Exposure to Styrene - Inhalation (continued)

Key to Figure[a]	Species (Strain)	Exposure/ Duration/ Frequency (Route)	System	NOAEL (ppm)	LOAEL Less Serious (ppm)	LOAEL Serious (ppm)	Reference Chemical Form	Comments
55	Mouse (CD-1)	6 hr/d 5 d/wk 98-104 wk	Resp		20 (respiratory metaplasia in nasal olfactory epithelium, bronchiolar epithelial hyperplasia)		Cruzan et al. 2001 Styrene	
			Cardio	160				
			Gastro	160				
			Hemato	160				
			Hepatic	160				
			Renal	160				
			Ocular	160				
			Bd Wt	80 M	160 M (11% decrease in body weight gain)			
Immuno/ Lymphoret								
56	Human	7 yr (Occup)			30 (alterations in lymphocyte subsets)		Bergamaschi et al. 1995b Styrene	
57	Human	13 yr (Occup)			26 (impaired immune response to concanavalin A)		Tulinska et al. 2000 Styrene	
Neurological								
58	Human	7.6 yr (Occup)			36 (altered performance vestibular tests)		Calabrese et al. 1996 Styrene	

Identified neurologic endpoints (LOAELs) are serious, not "less serious" see pg 4-5 of letter. applies to all neurologic endpoints.

Table 8-1. Regulations and Guidelines Applicable to Styrene

Agency	Description	Information	Reference
INTERNATIONAL			
Guidelines:			
IARC	Carcinogenicity classification	Group 2B[a]	IARC 2006
WHO	Air quality guidelines		WHO 2000
	TWA based on effects other than cancer or odor/annoyance using an averaging time of 1 week	0.26 mg/m^3	
	Based on sensory effects or annoyance reactions, using an averaging time of 30 minutes		
	Detection threshold	0.07 mg/m^3	
	Recognition threshold	0.21–0.28 mg/m^3	
	Guideline value	0.07 mg/m^3	
	Drinking water quality guidelines	0.02 mg/L[b]	WHO 2004
NATIONAL			
Regulations and Guidelines:			
a. Air			
ACGIH	TLV (8-hour TWA)	85 mg/m^3	ACGIH 2006
	STEL (15-minute TWA)	170 mg/m^3	
AIHA	ERPG-1[c]	213 mg/m^3	AIHA 1995
	ERPG-2[c]	1,065 mg/m^3	
	ERPG-3[c]	4,260 mg/m^3	
EPA	AEGL-1[d]		EPA 2007a
	10 minutes	85 mg/m^3	
	30 minutes	85 mg/m^3	
	60 minutes	85 mg/m^3	
	4 hours	85 mg/m^3	
	8 hours	85 mg/m^3	
	AEGL-2[d]		
	10 minutes	980 mg/m^3	
	30 minutes	682 mg/m^3	
	60 minutes	554 mg/m^3	
	4 hours	554 mg/m^3	
	8 hours	554 mg/m^3	

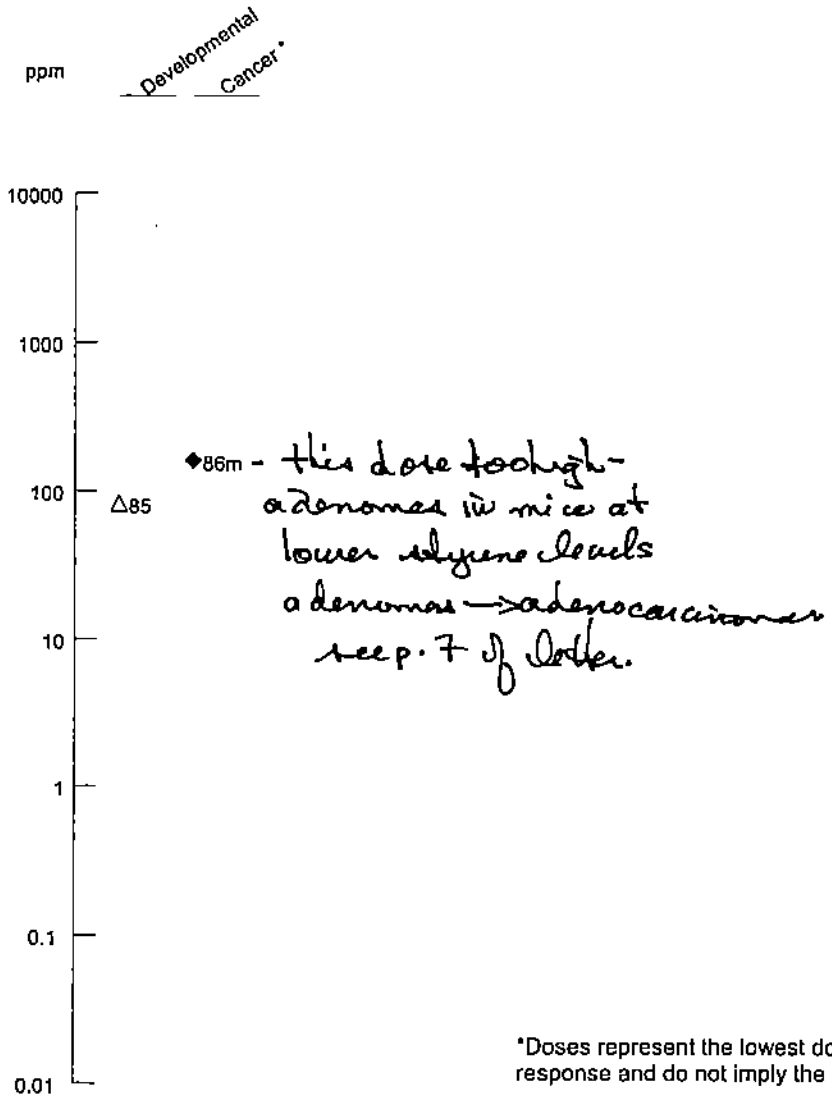
air level units not unified – mg/m^3 OR ppm.
See p. 5 of letter.

Table 8-1. Regulations and Guidelines Applicable to Styrene

Agency	Description	Information	Reference
NATIONAL *(cont.)*			
EPA	AEGL-3[d]		EPA 2007a
	10 minutes[e]	8,094 mg/m^3	
	30 minutes[e]	8,094 mg/m^3	
	60 minutes[e]	4,686 mg/m^3	
	4 hours	1,448 mg/m^3	
	8 hours	1,448 mg/m^3	
	Level of distinct odor awareness	2.3 mg/m^3	
EPA	Hazardous air pollutant	Yes	EPA 2007c 42 USC 7412
NIOSH	REL (10-hour TWA)	50 ppm	NIOSH 2005
	STEL (15-minute TWA)	100 ppm	
	IDLH	2,982 mg/m^3	
OSHA	PEL (8-hour TWA) for general industry	100 ppm	OSHA 2006c 29 CFR 1910.1000, Table Z-2
	Acceptable ceiling concentration	200 ppm	
	Acceptable maximum peak above the acceptable ceiling concentration for an 8-hour shift for a maximum duration of 5 minutes in any 3 hours	600 ppm	
	PEL (8-hour TWA) for shipyard industry	100 ppm	OSHA 2006a 29 CFR 1915.1000
	PEL (8-hour TWA) for construction industry	100 ppm	OSHA 2006b 29 CFR 1926.55, Appendix A
b. Water			
EPA	Designated as hazardous substances in accordance with Section 311(b)(2)(A) of the Clean Water Act	Yes	EPA 2007b 40 CFR 116.4

Figure 3-1 Levels of Significant Exposure to Styrene - Inhalation *(Continued)*

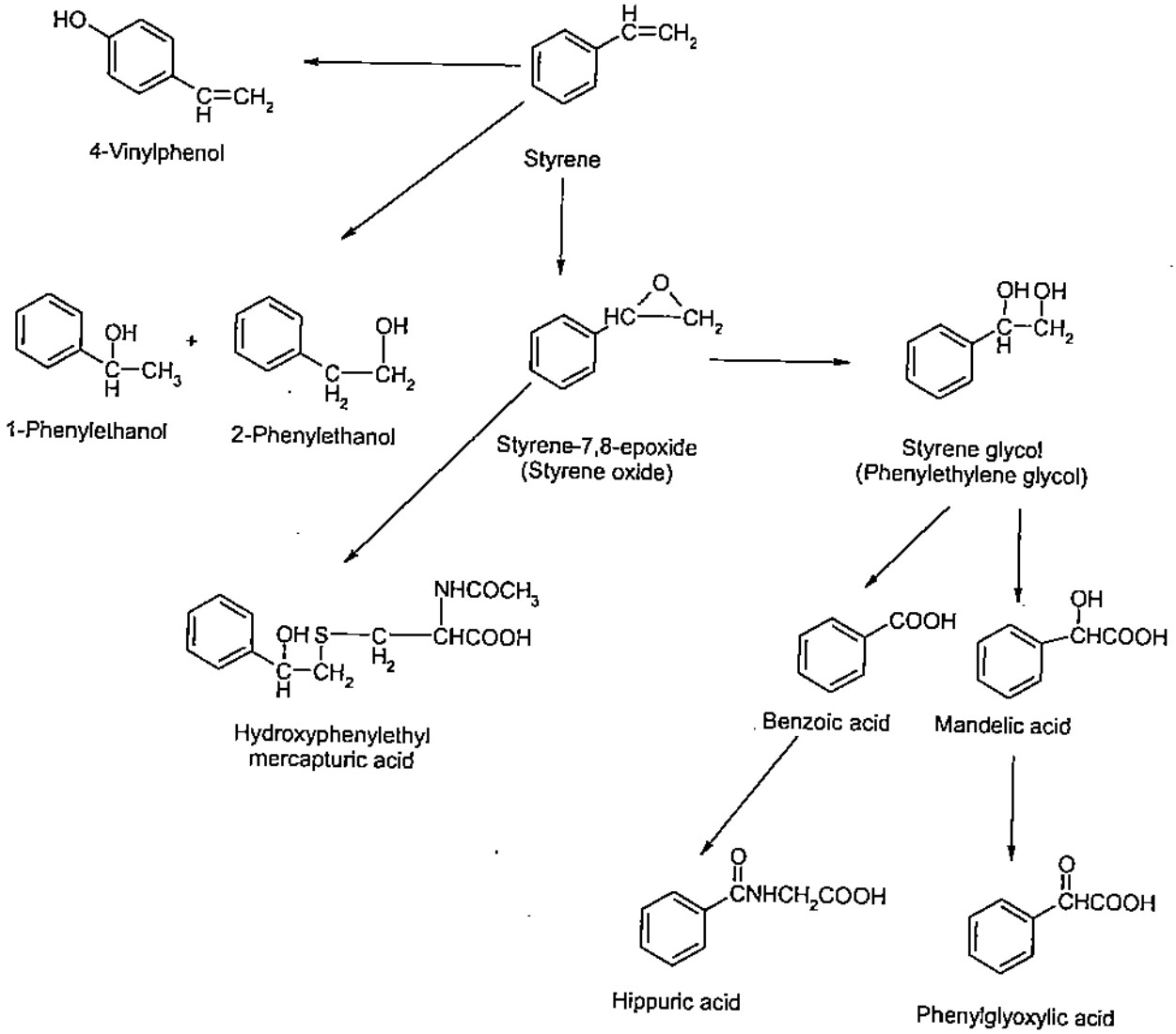
Chronic (≥365 days)



*Doses represent the lowest dose tested per study that produced a tumorigenic response and do not imply the existence of a threshold for the cancer endpoint.

c-Cat d-Dog r-Rat p-Pig q-Cow	-Humans k-Monkey m-Mouse h-Rabbit a-Sheep	f-Ferret j-Pigeon e-Gerbil s-Hamster g-Guinea Pig	n-Mink o-Other	◆ Cancer Effect Level-Animals ● LOAEL, More Serious-Animals ◑ LOAEL, Less Serious-Animals ○ NOAEL - Animals	▼ Cancer Effect Level-Humans ▲ LOAEL, More Serious-Humans ◭ LOAEL, Less Serious-Humans △ NOAEL - Humans	■ LD50/LC50 Minimal Risk Level for effects other than Cancer

Figure 3-3. Metabolic Pathways of Styrene



Source: Adapted from Bond 1989; EPA 1988b; Leibman 1975

only four bonds around carbon
see p. 8 of letter