

Styrene Expert Panel Report

Part A – Peer Review of the draft background document on styrene

The Report on Carcinogens (RoC) expert panel for styrene met at the Radisson Hotel, Research Triangle Park, North Carolina on July 21-22, 2008, to peer review the draft background document on styrene and make a recommendation for the listing status in the 12th Edition of the RoC. Members of the expert panel are as follows:

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One of the charges to this panel was to determine whether the information in the draft background document on styrene is presented in a clear and objective manner, to identify any missing information from the body of knowledge presented in the document, and to determine the utility of the body of knowledge in the background document for drawing conclusions about the carcinogenicity of a candidate substance and for applying the RoC criteria for listing. Following the discussion of all sections of the draft background document the expert panel reached a consensus concerning the critique of the draft background document, and voted 10 yes/0 no that the draft background with the

recommended changes in the expert panel report is adequate for drawing conclusions about the carcinogenicity of styrene and for applying the RoC listing criteria.

The expert panel proposed revisions for each section of the styrene background document are appended.

[Note: There was a typographical error in the initial vote for approval; it has been corrected from 11 yes/0 no to 10 yes/0 no (Dec. 2, 2010).]

Section 1: Introduction

1. General:

- There are two chemical structures that are considered to be styrene oxides; styrene-7,8-oxide and styrene 3,4-oxide (as shown in the metabolism section). Use the term styrene-7,8-oxide rather than styrene oxide throughout the background document.

2. Section 1.1 Chemical Identification

- Add a table for chemical identification for styrene-7,8-oxide in Section 1.
- Table 1-1: Add to Synonyms the following: phenylethene; styrole; NSC 62785, TTB 7302 (Scifinder Scholar 2008); cinnamenol, Stirolo; Styreen; Styren; Styrene Monomer; Styron; Styropol; Styropor; vinylbenzen; FEMA Number 3234; NCI-CO2200; UN 2055; IMO 3.3; 49072 65 1. (HSDB 2008).

3. Section 1.2 Physical-chemical properties

- Page 2, line 4: Insert after “It has a flash point of 34° (closed cup)”, lower explosive limit of 0.9-1.1% (v/v), upper explosive limit of 6.1-6.8% (v/v),” (HSDB 2008).
- Page 2, line 6: Insert after “...may form explosives mixtures with air” – “due to formation of an explosive peroxide” (HSDB 2008).
- Page 2, line 9: Insert after “Table 1-2”: “Usually styrene is stabilized for safe storage, transport, and use by an inhibitor, commonly 10-50 ppm (w/w) p-tert-butylcatechol (HSDB 2008). Typical impurities are ethylbenzene (85 ppm maximum), polymer content (10 ppm maximum); para-tert-butylcatechol (inhibitor) (10-15 ppm or 45-55 ppm), aldehydes (as benzaldehyde) (200 ppm maximum), peroxides (as H₂O₂) (0.0015 % by weight or 100 ppm maximum), benzene (1 ppm maximum); sulfur (1 ppm typical); chlorides (as chlorine) 1 ppm typical.”
- Table 1-2: Physical and chemical properties of styrene: Add at end: Hydroxyl radical reaction rate constant = 5.8×10^{-11} cu cm/molecule-sec @ 25 deg C (HSDB, 2008)

4. Section 1.3 Metabolites

- Recent evidence has been published that cytochrome P450-2A13, which is found mostly in the pulmonary tract is more efficient than cytochrome P450-2E1 in producing styrene-7,8-oxide and has overlapping specificity with the latter (Fukami *et al.* 2008). Add reference to Page 3, lines 1-2 and in the metabolism section.
- Page 3, line 10: Insert after “phenylglyoxylic acid” “and their conjugates.”
- Add information on stereochemistry of styrene-3,4-oxide to background document.
- Include IUPAC names for the *R*- and *S*-styrene-7,8-oxide isomers on page 4.

Section 2: Human Exposure

1. Introduction

- Page 7, line 5, Smoking is usually considered part of inhalation exposure, so change text to read as follows: “The primary sources of exposure to the general public include inhalation (indoor and outdoor ambient air, active smoking, and exposure from environmental tobacco smoke), dermal exposure, and ingestion of foods.”

2. Section 2.2 Production

- Page 9, line 10: The NTP should review the original source for use of the term “fractionally condensed.” Suggest deletion of “fractionally” [After the reaction, the products are cooled and the product stream, which contains styrene, toluene, benzene, and unreacted ethylbenzene, is fractionally condensed.]
- Page 11, line 8; The NTP should revise the language surrounding the 13 billion lb per year of styrene-resin production to clarify that it is a U.S. production figure.

3. Section 2.3 Biological indices of exposure

- Move subsection 2.3, Biological indices of exposure, after Section 2.6, Occupational exposures.
- The descriptive subsection 2.3 on Biological monitoring data needs to be more informative. NTP should provide an introductory paragraph on some of the limitations of the biological monitoring data for this document. These include: (1) the data are study specific and not generalizable across the body of literature, (2) the data are difficult to interpret without information on the kinetics of metabolism and clearance, and the intensity and duration of personal contact, and (3) metabolism and clearance parameters may vary across individuals, which might affect the internal dose.
- The introductory paragraph should also state that biomarkers of exposures reflect the summarization of exposures from all routes.
- NTP should review the available biomarkers studies and report any information on quantitative relationships. Studies that have calculated air exposure from urinary markers include the following: Godderis *et al.* 2004, Laffon *et al.* 2020a, and Maki-Paakkanen *et al.* 1991. NTP should report whether or not skin exposure was assessed in the studies (if the information is provided in the papers), and note if there was no skin component.
- The background document should note that biomarkers of effect (such as DNA repair and toxic endpoints) are discussed in Section 5.
- Page 12, line 11: Replace the sentence beginning with “These metabolites include. . .” with the following sentence: “These metabolites include Phase I intermediates and their conjugates (Phase II intermediates) of styrene glycol and styrene-7,8-oxide in blood; and the urinary biomarkers mandelic acid (MA) and phenylglyoxylic acid (PGA) (IARC 2002), 4-vinylphenol (Manini *et al.* 2003), and phenylhydroxymethylmercapturic acids (PHEMAs) from glutathione conjugation of styrene oxides (Ghittori *et al.* 1997).”
- Page 13, line 18: Insert after “...phenylglyoxylic acid”, “as the sum of free acid and conjugates.”

4. Section 2.4

- Add a note at the beginning of Section 2.4 that this section is intended to be a “semi-quantitative analysis,” noting that it only provides general information on exposure levels.
- Page 16, line 10: After “air”: Insert “Section 5 will discuss possible estrogenicity of styrene” (making note of the specific subsection).

- A new paragraph on analytical methods and the limitations of the quality of data should be added to the section and placed on page 16, right before Section 2.4.1. The paragraph should describe the types of analytical methods, including the limits of detection and interferences that might affect the quality of data. Differences in methods and detection limits over time also should be noted. It should also discuss the limitations in the quality of the data. For example, mean±SD, or GeoMean (GSD), or median (min-max) have not always been provided. Data obtained with low quality methods, such as detector tubes, should be noted. It should also discuss sampling. It should be noted that the purpose for collecting the data and sampling strategy are variable and thus lead to inconsistent data quality; convenience grab samples are much less informative than random samples gathered with a formal protocol. It is also recommended to note in Section 3 that some of the analytical methods for the exposure analyses are old.
5. Section 2.4.1 Environmental release fate, and occurrence: Air
 - Styrene is used to make many products, both as a solvent and as a monomer, and some may have residual styrene in them. Do they off gas styrene? NTP should research if there have been manufacturing changes to reduce residual styrene.
 - Page 18, line 23, add Uhde and Salthammer 2007 information on offgassing from cork flooring on concrete (emission rate in Table 4 of Uhde and Salthammer).
 - Section 2.4.1.2 (Indoor release), Page 18, lines 26-27. The review by Uhde and Salthammer 2007 should be reviewed and discussed for indoor air. Suggested text: “Uhde and Salthammer 2007 reviewed how styrene and its aldehyde degradation products fluctuated due to chemical surface interactions.”
 - Section 2.4.1.5 (Indoor occurrence), Page 24, after line 13. The review by Uhde and Salthammer 2007 should be used here for surface reactions in indoor air situations. Uhde and Salthammer reviewed the influence of surface interactions of styrene and its secondary byproducts in indoor air environments.
 6. Section 2.5 General Population Exposure
 - Page 37, line 8, “Sources of exposure to styrene include inhalation of outdoor air, smoking and inhalation of environmental tobacco smoke, and consumption of food”: add “especially due to” before “smoking . . .” Add “skin exposure,” before “and consumption of contaminated food. . .”
 - Page 42, line 19, After “... elevated levels in children were unexpected.” add the following new sentence, “Environmental tobacco smoke, automobile exhaust, and consumer products used in the home were noted as likely sources of styrene exposure.” NTP should review Sexton *et al.* to make sure that they stated this; if not, then the statement should be bracketed.
 7. Section 2.6.1 Occupational Exposure: The reinforced plastic industry
 - NTP should add the number of samples to all occupational exposure tables.
 - Page 46, In the discussion of temporal variation in exposure levels, add discussion of Kolstad *et al.* 2005 and add Figures 1 and 2 from the article.
 - Page 57, line 9, [Limaset et al. 1999 study]: Add as last sentence of paragraph “These results were similar to those of Brooks *et al.* 1980.”
 - Page 57 after line 24: Discuss the new skin references: Bogen *et al.* 1994; Brown 1985; Burkova *et al.* 1982; Dutkiewicz and Tyras 1968; Minamoto *et al.* 2002; Stewart *et al.* 1968, in this section and/or Section 5.1.1.1 (absorption) where appropriate.
 - Page 57, line 24: Add after the additional information on skin references noted above, information from Minamoto *et al.* 2002 which reported 1 case of occupational dermatitis caused by styrene in fiberglass reinforced plastics workers.

- Page 57 Add the following sentence to end of Section 2.6.1, “See Section 5.1.1.1 for more information on dermal exposure.”
 - Page 187, line 28: Review and cite Berode *et al.* 1985 rather than ATSDR.
8. Section 2.6.2 Occupational Exposure: The styrene-butadiene rubber (SBR) industry
- Page 57: Add brief discussion of basic SBR production process – raw materials to final products, with a diagram. Review Macaluso *et al.* 2004, Priddy 2007 (in Kirk-Othmer 5th edition) and Chen (Kirk-Othmer 5th edition) for process description for SBR and flow diagrams from Kirk-Othmer. Also add process description and process diagram for styrene monomer and polymer industry to appropriate places within Section 2.6.3.
9. Section 2.8 Summary
- Page 67, line 26: Need to mention skin exposure here.

Section 3: Human Cancer Studies

In general, the panel found that the approaches used to summarize the literature were consistent with standard epidemiological practices and provided a sound basis for evaluating the human carcinogenicity of styrene. Overall, the draft document provides the critical studies needed to evaluate the carcinogenicity in occupationally exposed populations, with the exception of the Delzell *et al.* 2006, which should be added. The use of several approaches in summarizing the literature, including summaries by industry type and by cancer site across studies, is appropriate.

The major comments recommended by the expert panel referred to the identification of new literature, and a more detailed discussion on the strengths and limitations of the human epidemiology studies (overall or for specific studies). In addition to these issues the expert panel identified changes related to specific statements in the draft background document (see below). The panel also made the recommendation that consistent language be used throughout in reporting risk estimates. In particular, non-statistically significant risk estimates should have the modifier “non-statistically significant elevated risks.”

Part A. Additional Literature

An additional report (Delzell *et al.* 2006, which is the complete investigators’ report) presents data from the mortality study of workers from the styrene-butadiene industry. In part, the data reported in Delzell *et al.* 2006 duplicate the reported findings of Sathiakumar *et al.* 2005 for the whole updated cohort (N = 17,924 men) followed from 1943 to 1998 for SMRs by all causes and by selected lymphohematopoietic malignancies and other cancers by hourly vs. not hourly, year since hire, years worked, and area of work (see below). This report also contains the most recent analyses of data of the cohort previously provided by Delzell *et al.* 2001 and Graff *et al.* 2005 and provides greater details on the statistical models for evaluating styrene, 1-3 butadiene and dimethyldithiocarbamate (DMDTC). The following text describes the additional information that is provided in Delzell *et al.* 2006 and this information and the findings from Tables 4, 6, 10, 12, 16, 17, 18 and 19 should be added to the draft background document.

Delzell *et al.* 2006 is more complete in details about methods, especially exposure measures, and specific subanalyses of results than is the Graff *et al.* 2005 paper. Most tables in Graff *et al.* 2005 have the same data that are in results tables from Delzell *et al.* 2006. Both papers include data on modeling, but Delzell *et al.* 2006 includes two-chemical models as well as the single- and three-chemical exposure adjusted models reported in Graff *et al.* 2005. This is important because the data from both reports indicate, with respect to the distribution of person-years among the chemicals, that the person-years for employees with no exposure to DMDTC is over two times higher than for the other chemicals. This observation explains further why the correlation between DMDTC and styrene and butadiene is not high and inclusion of this chemical in models is unnecessary, and could result in increased imprecision especially by dose group. [NOTE: The data on the two chemical models for styrene and butadiene as well as for these single agents, and the relationship with non-Hodgkin lymphoma (NHL) and with NHL and chronic lymphocytic leukemia (CLL) combined are shown in tables 18 and 19 of Delzell *et al.* 2006.]

With respect to the overall rates for workers without analysis of chemical exposures for subtypes of leukemia as well as an NHL-CLL group of lymphohematopoietic cancers [Tables 4 and 6], the risks for the combined NHL-CLL cancers are elevated for ever-hourly workers in the industry at borderline significance (SMR = 130; 95% CI = 99 to 167). This risk for ever-hourly workers is higher and

significant among those who have 10+ years of exposure and 20 to 29 years elapsed since first employment (SMR = 190; 95% CI = 101 to 325) and an SMR of 149 (95% CI = 102 to 210) for 10+ years worked and 30+ years since hire. Examination of these lymphohematopoietic cancers by work area (in Table 6) indicates that the risks of all (acute + chronic) lymphocytic leukemia are clearly associated with overall production area (SMR = 266; 95% CI = 122 to 505) and its subareas: polymerization (SMR = 497; 95% CI = 215 to 980), coagulation (SMR = 607; 95% CI = 197 to 1417), and finishing (SMR = 344; 95% CI = 138 to 709) as well as laboratories (SMR = 559; 95% CI = 152 to 1481). The combined category of CLL-NHL also was associated with work in the production area, including all 3 production subareas, and all these areas have statistically significant excesses of CLL-NHL except coagulation; however, the SMRs are lower than for all lymphocytic leukemia. The report also indicates that the highest risk of all leukemias is in ever-hourly workers hired in years 1950 to 1959 with 10+ years of work and 20 to 29 years since hire. No further analyses have looked at specific chemical exposures in the subset of employees with the highest risk.

The Delzell *et al.* 2006 report also analyzes leukemia, NHL and NHL-CLL data for three-chemical exposures, butadiene, styrene, and DMDTC. Both butadiene and styrene in single-agent models are associated with significantly increased risks for all leukemias in the two highest exposed groups and both show a dose response (although no trend information is provided). When both of these chemicals are in the model, both chemicals show increases in RR with increasing dose, but when DMDTC is added to the model as reported by Graff *et al.* 2005, the styrene risk disappears. Using a different exposure measure [in Table 12], namely number of styrene peaks, styrene in the single chemical model has RR values for all leukemia that are slightly higher than those of butadiene alone (except at the highest quartile). Both styrene and butadiene are associated with significant excesses of all leukemias at the highest quartile for number of peak exposures. Both have apparent positive dose responses for each chemical. Using a two-chemical model, an increasing frequency of peak styrene exposures in relation to the risk of all leukemias is associated with higher RR values for styrene than butadiene. The RRs remain significant only for styrene at high peak doses. The higher risks for styrene compared with butadiene remain even in the three-chemical (styrene+butadiene+DMDTC) model. The authors also provide trend data for the risk of leukemia for styrene unadjusted and adjusted for butadiene exposure, using three categories of cumulative exposure including 0 [Table 16]. This analysis indicates that for all leukemias, styrene is not a risk after adjusting for butadiene when using cumulative exposure, but no further analysis was done on exposures to styrene peak measures.

An examination of the subtypes of leukemia based on single-agent models or three-chemical models indicate that only chronic myelogenous leukemia (CML) is clearly associated with butadiene exposure, with no definite relationship to the other two chemicals (styrene and DMDTC) in three-chemical models [Table 17]. Acute myelogenous leukemia (AML) is not associated with exposure to any of the chemicals. The data for CLL indicate an association with both butadiene and styrene in single-agent models, as is the case for the category “other leukemias.”

Relative risks for NHL by dose were also compared [Table 18]. The relative risks in single-chemical models are slightly higher for styrene than for butadiene. When styrene exposures are adjusted for butadiene exposure in a two-chemical model, only styrene continues to show elevated RRs by dose that are similar to those of the single-agent model. Even with three chemicals in the model and some attenuation of the levels of the RRs, the association between NHL and styrene remains similar to the previous models. The data suggest a positive exposure response, but no analysis by trend is provided. Data for CLL and NHL are combined [in Table 19] since CLL and small B-cell NHL represent the same B-cell cancers. The relative risks for the single-chemical as well as the two-chemical models

both indicate elevated RRs for styrene by dose and the risks are similar for styrene alone as well as when adjusted for butadiene exposure. However, all elevated relative risks disappear for butadiene when adjusted for styrene.

The analysis methods restrict the ability to examine risks by year of hire since that was included along with age as a confounding variable when examining all models by exposure. The analysis is described as having included individuals in each exposure category through which they pass during employment. Thus, workers who survive to go from low to high categories will contribute many person-years to low-exposure groups as they accumulate exposure. While this is an accepted method of examining populations, the procedure could account for the very low SMRs seen in the “0” and lowest exposure categories. In addition, the SMR analysis is not an appropriate method of examining risks by exposure category. The highest exposure groups are likely to represent older workers compared to person-years in “0” exposure groups. This would mean that age groups are likely to be unbalanced by exposure. Use of SMRs will always allow adjustment for this age discrepancy and might conceal the problem of age differences in exposure categories. The preferable method is to adjust for age directly so that risks of having unbalanced age groups may be easier to detect, as has been done in this report. The age groups may have to be large to make such an adjustment (as is the case in the report analyses).

The Delzell *et al.* 2006 study has provided data on general disease risk in the styrene-butadiene rubber (SBR) industry. The data in Table 2 present SMRs for all commonly combined causes of death by calendar period of follow-up. There are no significantly increased SMRs for any single cause of death although several SMRs are above the SMR for all causes which is thought to represent the overall “healthy worker effect.” The authors have elected to examine in detail the risks for colorectal cancers and prostate cancers which, in addition to some lymphohematopoietic, exceed SMRs of 100 compared with the overall SMR of 86 for all causes of death. None of the models for styrene or butadiene exposures appears to show excess relative risks for these cancers in relation to styrene in single-, two- or three-chemical models.

Part B. Limitations of the literature

1. Limitations of the studies. The draft background document (Section 3.6.1) should expand on the discussion of the limitations of the studies in the different industries to evaluate cancer. The impact of the deficiencies of studies in these industries is not sufficiently pointed out. Although some of these limitations and strengths are partly described in the document, the draft document should cover all of the issues listed below.
 - In the reinforced plastic industry, there are low numbers of long-term workers, with the exception of the large combined multi-country cohort of Kogevinas *et al.* (1993, 1994), and small cohorts are each examined individually. In addition, workers had higher levels of exposure in the earliest years of employment, but only one study analyzed data by year of hire. Further, there are no quantitative measures of exposure. The impact of these limitations is that the power of these studies to show any risk is low, due mainly to small numbers and the fact that most of the workers are short-term workers, except for Kogevinas *et al.*
 - In the SBR industry, the strengths of the studies include the following: The studies include large populations with long-term exposures and long-term follow-up, so that cancers with longer latencies can be more readily identified. In addition, the SBR industry is unique because it was started in the 1940’s and plants were brought into operation during the same time period, so that exposures are likely to be quantitatively similar for the agents to which workers were exposed in different cohorts in the earlier years and have changed over time in a similar way.

The limitations include the following: Exposure misclassification is likely because there are no measurements for earlier time periods when exposures are likely to be higher, and analyses by duration of exposure do not reflect changes in precise exposure levels over time (few analyses look at data by year of hire as well as length of employment). In addition, length of follow-up may not be sufficient for long-latency cancers. The document needs to acknowledge the potential impact of exposure misclassification on dose-response analyses.

- In the styrene monomer/polymer industry, the main limitations of the studies are small numbers in the cohorts, many short-term workers, and few cancer outcomes. In addition, there are multiple chemical exposures.
- Page 133, lines 7-17. The panel does not agree that the reinforced plastics industry or the biomonitored populations are the best for evaluating carcinogenicity of styrene. As noted previously, the reinforced plastics industry has many short-term workers who have not had an adequate duration of exposure to carcinogenic agents, so the at-risk population is too small to adequately assess risks. There is only one study of biomonitored workers starting in the late 1970's [Antilla *et al.* 1998], but there is no information about duration or levels of exposure and a comparison of risks by exposure. The population is also quite small. In comparison, the SBR industry studies have several advantages for evaluating carcinogenicity due to long-term exposure, long follow-up and extensive analyses.
- Case Reports and Ecological Studies (Pages 122-125): The draft report is correct in the notation that the case-control study and the ecological study indicating a breast cancer risk in women exposed to styrene have problems related to exposure measures. However, the report might note that given the small number of women employed in these styrene industries, it is unlikely that this risk can be detected from cohort studies, and that large case-control studies may have some value for evaluating the risk of breast cancer in association with styrene exposure.

2. Limitations related to classification of disease and exposure. The following limitations and corrections should be discussed and integrated into Section 3.6.2 of the draft background document:

- *Limitations in classification of disease: NHL and leukemia overlap.*
 - * The report should provide more discussion on the overlap between NHL and CLL. Note that this is described in Delzell *et al.* 2006. For lymphohematopoietic malignancies the problem with diagnosis frequently stems from the overlap between non-Hodgkin lymphoma and CLL. CLL is the same disease as small-cell lymphocytic lymphoma and hence should be grouped where possible with NHL in data analyses.
 - * Page 134: the last paragraph states that a possible causal effect between styrene and leukemia is only expected for subgroups originating from a common stem cell. The panel suggests that this sentence be deleted and replaced by a statement that points out that AML, CML, and adult ALL arise from the same pluripotential stem cell based on observations of specific genetic rearrangements in these 3 subtypes of leukemia, which comprise about 80% of adult leukemia. (For example, the blast crisis of CML, 90% of which have the Philadelphia chromosome, cannot be distinguished from AML, and an estimated 10% of adult ALL cases have the Philadelphia chromosome, which suggests a common stem cell origin for these leukemias. See literature by Bloomfield *et al.* (1978), Jacobs (1989), and Yunis (1983)).
- *Limitations in evaluating exposure-response relationships based on duration of exposure:* The document should provide more detail on the issues of exposure assessment used for exposure-response analysis and adjustment for co-exposures in the SBR industry evaluation. The document should point out that exposure assessments are limited because there are no exposure data for periods when exposures were highest.

- * Subgroup 2 comment: It is also recommended to note that some of the analytical methods for the exposure analyses are old.
 - * Subgroup 2 comments: Page 135, L9, add “temporal variation in styrene exposure levels can be another source of misclassification over the study period (Macaluso *et al.* 2004, Kolstad *et al.* 2005). Also add note to see Figures 1 and 2 from Kolstad *et al.* 2005 that are recommended for addition to Section 2.6.1 page 46.
 - * Subgroup 2 comments: Page 135, line 9. A discussion of the temporal and job/task variation reported in Macaluso *et al.* 2004 should be provided, along with Table A-4 from the article.
 - * Page 135, lines 19-21: The document should note that in ecological studies measures of individual exposures are less precise than case-control studies by usual job titles.
 - * Page 135, lines 22-26: The draft document should clarify how the reduction in misclassification is affected by job titles and exposure ranks.
 - * Page 135, lines 25-28: If ranks and measurements of styrene correlate poorly, this tends to attenuate any apparent risk. Sometimes the investigation of reasons for a lack of correlation uncovers an explanation such as the introduction of controls for the substance prior to taking the measurements. This often means that exposure rank is a better measure of exposure in the past than any current measurement.
3. Limitations of adjustments for confounding exposures. (Create a new subsection to discuss the limitations of the statistical models)
- Limitations in the models for adjusting for butadiene, styrene, and DMDTC in the studies of the SBR industries present challenges. With respect to the Graff and Delzell studies, the panel is concerned that there is insufficient rationale for adjusting for DMDTC as a confounder because it is neither strongly correlated with styrene exposure nor associated with lymphohematopoietic cancers.
4. Limitations related to other possible biases and confounding.
- Page 137, lines 2-6: These lines regarding loss to follow-up need to be dropped. In any industrial study the population is established and then outcomes are sought in order to avoid the inefficiency of gathering deaths or cases on groups, which will not be analyzed. A few cases may be added or dropped during subsequent cleaning of records.
 - The possible confounding effects of benzene and ethylbenzene as contaminants of styrene should be discussed.

Part C: Specific Comments

1. Executive Summary and Section 3.9 summary
- Page vi: Include a statement in the limitations section (starting on line 18) that the majority of available studies on occupational exposures to styrene have been predominately on cohorts of male workers, and comparatively little information is available on cancer incidence or cancer mortality rates in female workers, thus limiting the ability to evaluate at tissue sites specific for females or for breast cancer.
 - Page vii, lines 10-12: Delete the statement related to a possible synergistic effect of styrene and butadiene.
 - Page vii, lines 9-10: Add after the “trend was attenuated somewhat after controlling for butadiene” “and was no longer present in models controlling for butadiene and DMDTC.”
 - Page vii, line 25: The executive and section summaries should clarify that the increases in pancreatic cancer were not all statistically significant increases – only one was statistically significant.

- Page viii, lines 8-10: Replace “An increase in breast cancer was observed in the ecological study and a case-control study, although the studies were limited by poor characterization of styrene exposure,” with “A significant increase in breast cancer mortality was observed in a case-control study of occupational exposures among adult females, though there was no evidence of increased risk between low- and high-exposure categories. An ecological study reported a significant increase in the risk of invasive breast cancer in the general population, but exposure estimates were based on environmental releases of styrene, which are the least precise measures of individual exposure.”
 - Add results for CLL and NHL combined and for NHL alone that are described in Delzell *et al.* 2006 (See Part A: Additional Information above). These studies found an exposure-response relationship with cumulative exposure to styrene for CLL and NHL combined or NHL, alone that was not attenuated when butadiene was added to the model. These results should also be added to Section 3.8 (Summary for selected cancer sites).
2. Introduction
 - Define in the introduction that the body of literature does not cover end users (tire manufacturing) except for the McMichael *et al.* 1976 study, because this appears to be the only study that evaluates risks specific for exposure to SBR latex. All U.S. plants produced multiple types of rubber but many had some of the same chemical components as SBR and always others were produced in lesser quantities.
 3. Section 3.1.4 Reinforced plastics industry: Kolstad *et al.* 1994, 1995
 - The expert panel for Section 2 recommended that a discussion of the data in Kolstad *et al.* 2005 be added to this section.
 4. Section 3.2.3 SBR industry: U.S. and Canada: Matanoski, Santos Burgoa, and co-workers:
 - Page 96, line 13: To be comparable to the presentation of data from other studies, the draft might note that the study showed increases in lymphohematopoietic cancers but the odds ratio was insignificant.
 5. Section 3.2.4 U.S. and Canada: Delzell, Sathiakumar, Macaluso, Graff:
 - Pages 97-102: The expansion of the Delzell *et al.* 2001 cohort needs to have a comment on caveats. Expanding populations to add more recent workers will add persons with lower exposures, shorter latency and duration worked which may reduce apparent risk
 - Page 100, lines 4-6: The subgroup analysis by latency and duration indicates that the leukemia subset with greatest risk was the group with 10 years exposure and 20 to 29 years since first employment, but no excess was seen for 30 years or more. The draft document should note that it may be important to do further specific analyses of this high-risk group.
 - Page 97: The Delzell *et al.* 1996, 2001 study population is described as having Canadian plant employees with one or more years of employment. However, Matanoski *et al.* 1993, 1994, 1997 found that Canadian follow-up systems for death did not permit identification of workers from the early years who had died and so had to rely on a pension system. This should be pointed out as a limitation.
 - Table 3-8 (Page 158): Add a footnote to Table 3-8 to note that caveats about pooling data across studies are described on Page 142.
 - The expert panel concurred that the Kolstad *et al.* 1994, 1995, 1996 cohort (i.e., workers in high-styrene exposure groups (laminators and others)) should be included in Table 3-8, but recommended that the definition of “high-styrene-exposure groups” in the Kolstad studies be clarified.
 6. Section 3.8

- Page 151: The discussion by cancer site for lymphohematopoietic malignancies should be separated into subtypes of lymphohematopoietic cancers. Overall lymphohematopoietic cancers should be discussed first due to overlap in the subtypes (and unspecified tumors).
- Page 154, lines 6-8: Rephrase/clarify the statement that Bond *et al.* 1992 found an increase in lymphohematopoietic cancers with duration of exposure. (See the description on page 114, lines 1-2 that Bond *et al.* analyzed lymphohematopoietic cancer mortality by < 1 year and > 1 year of exposure.
- Page 156: No significant associations have been noted in cohort occupational studies between styrene exposure and breast cancer risk or mortality. The critique on page 156 is accurate; these studies were limited by having small numbers of expected and observed cases of breast cancer, and women may have had low-exposure job tasks (though this is speculation). It should be noted that these cohort studies have not attempted to control for confounding factors that affect breast cancer risk (such as body mass index, family history of breast cancer, alcohol use, menopausal status, parity, hormone use, and age at first birth). Furthermore, there should be an additional comment that cohort occupational studies frequently do not have sufficient follow-up time to detect effects on breast cancer incidence or mortality because of the decades (sometimes in excess of 40 years) breast cancer may take to develop from initiation to detection of a tumor. Hence, a lack of an effect does not necessarily confirm a lack of risk if there was insufficient follow-up time.

Section 4: Studies of Cancer in Experimental Animals

1. General

- Most of the rodent cancer bioassays summarized in this section had design flaws and other limitations, including poor survivability in high-dose groups, small number of animals per dose group, inadequate duration, limited histological examination, incomplete reporting of study design and results, and/or lack of multiple dosing groups. Study limitations should be fully described in the narrative text, even when noted in the tables.
- Study descriptions do not consistently note when full vs. partial necropsies and histological examinations were performed, which is essential in fully interpreting the data in the animal cancer bioassays. If only some organ systems/tissues were examined upon necropsy, then other sites of tumorigenesis may have been missed that could have been observed on both gross and histological examination. Examination of gross lesions alone is also an insensitive method for discovering tumors for most sites.
- For some studies, results or interpretations are made by the authors and repeated in the draft document. This is done in some places without notation in the background document when data documenting the results or interpretation were not reported in the primary publication. In such cases, the background document should note when reporting the study authors' conclusion that data were not provided to support their interpretation.
- The purity of the styrene tested in each study should be noted to the extent possible because of the possible presence of contaminants as well as the addition of stabilizers added to styrene to prevent polymerization.
- There are two non-cancer studies that should not be included in this section and may be discussed in the toxicity section.
- A summary of the carcinogenicity of styrene-7,8-oxide by species, strains, routes, and tumor sites should be indicated in Section 4.
- The summary should emphasize the results of the well-conducted studies, and indicate important study limitations

2. Statistical analyses: Study authors did not report significance values with as great of precision as is of interest, or neglected to report analyses on all findings of interest. In some cases, statistical methods used were not reported by study authors. Pairwise comparisons using Fisher's exact tests and Cochran-Armitage tests for trend could be performed by the NTP. The draft background document reports some independent analyses by the NTP, but not for all findings of interest.

- All findings of interest should have both pairwise comparisons and trend tests performed and reported. If the study authors do not provide adequate analysis, NTP should. During the styrene review the contractor provided trend tests for several sites, which the Panel much appreciates. The important statistical findings should be included in the report, with suggestions for incorporation provided below in the commentary on the individual studies.
- Values should be reported at increased levels of precision. Several P -values are reported at the significance threshold: " $P < 0.05$." Thus the reader cannot discriminate easily between highly significant results, for example with a chance of one in 1,000 or one in 10,000 from those with a chance of 1 in 20. For the NCI studies where mortality is significant and could substantially impact the outcome of a statistical test, NTP should perform and report the results using poly-3 pairwise comparisons and trend tests, if individual animal data can be obtained. This is the standard for statistical analyses of animal data in NTP's current reports. It was not applied 30 years ago when the NCI bioassays for the styrene mixture and styrene alone were published.
- For cases where there are elevated findings of note that are not statistically significant but between the cut-off of $P = 0.05$ and $P = 0.1$, the exact P -value should be given.

3. Historical Controls. Because of small control groups in some of the studies and uncertainty regarding some findings, incidence values in treated animals are in a few cases compared with historical controls. In using historical control data, the panel recommends that the NTP use as a guide for the styrene review the general approach to historical controls used in current NTP reports and by the International Agency for Research on Cancer (IARC). Excerpts regarding use of historical control data from the IARC Preamble published in 2006 and a recently published NTP report from this year are given in Attachment I to this review.
4. Section 4.1.1 Mice Oral (NCI 1979)
 - Table 4-1 should include the data for hepatocellular carcinoma and hepatocellular adenoma and carcinoma combined. The text should note that there was a significant trend for hepatocellular adenoma in female mice, and that there were no hepatocellular carcinomas in any females and no significant hepatocellular tumor findings in male mice.
 - In response to a request during the peer review of the background document, NTP provided for the sites in Table 4-1 the exact Cochran-Armitage trend tests. The notable values should be given in Table 4-1, to one significant figure. Thus, i) For the trend value for the male lung carcinoma, $P = 0.08$. This should be included in the row indicating trend values in Table 4-1. ii) For the adenoma and carcinoma combined, the trend test value is $P = 0.02$. iii) For the female hepatocellular adenoma, the P -value is 0.03, iv) for the P -value for the female combined adenoma and carcinoma, the P -value (and incidence) is the same as for the female adenoma, $P = 0.03$.
 - Page 164, line 25: A limitation of the NCI studies is the small concurrent vehicle control group. Use of historical control data from NCI studies as reported in the background document provides perspective on the original study. A sentence should be added to indicate that the historical control animals were from the same source, same study protocol, the tests were performed in the same chronological window, and that the incidence was in the concurrent control group was not unusually low. However, the studies were performed in different laboratories. Statistical comparisons with these historical controls are not needed and the sentence on Page 165, lines 1-3 would therefore be deleted (“The combined lung tumor incidence...”). The studies performed at Litton Bionetics during the same chronological window provide the historical laboratory control data; these also indicate the findings of lung tumors are statistically significant. As noted above, the Panel recommends NTP and IARC guidance regarding historical controls be considered. Whether or not statistical comparisons are made, the data on the two types of historical controls indicate that the concurrent control group incidence was not unusually low and that the findings of lung tumors in male mice were significant.
 - The combined adenoma and carcinoma incidence for alveolar/bronchiolar tumors for the female should be given in Table 4-1. It will merely reflect the adenoma incidence since there were no carcinomas reported. This seems more correct than the “NAP” (not applicable) designation.
5. Section 4.1.1 Mice Oral (Ponomarkov and Tomatis 1978) – O20 mouse
 - To aid the reader the olive oil only group should be referred to as the vehicle control, at least where it first occurs.
 - Pages 165-166. Limitations of the study design and reporting should be more fully discussed in the text. There are several things to note: i) The rationale for choosing these doses was not discussed; the dose (1,350 mg/kg) for O20 mice was too high and shown to limit survival; ii) The substantial mortality rate, which should be emphasized to a greater extent; iii) The short duration of dosing is a study limitation; iv) The same effective number was applied to all tumor types — all styrene-treated mice surviving roughly 20 weeks. Because of the significant dose-

related decrease in mortality subsequent to 20 weeks in styrene-treated animals, there is strong bias in the study toward the negative. Only 4% (2/45) males and 15% (6/39) females treated with styrene survived to week 70 compared with 85% (17/20) male and 64% (14/22) female vehicle controls; v) Clearly this bioassay is inadequate for studying carcinogenicity, but given the low power, the finding of any significant elevation in a particular site is of interest.

- Page 166, lines 11 and 12. Sentence regarding earlier appearance of lung tumors in styrene-treated groups is problematic because the limited reporting does not enable conclusions regarding differences in tumor onset among the dose groups. A suggested revision is as follows: “Lung tumors were reported to occur at an earlier age in styrene-treated progeny than in control progeny, but this may be the result of higher mortality in the styrene-treated mice rather than an effect of styrene. Information necessary to interpret the significance of this observation (whether the lung tumors were incidental or fatal) was not reported.”
- Footnote c, Table 4-2a indicates that lung tumors in the male styrene-treated progeny do not significantly differ from the untreated controls. This is incorrect and inconsistent with the data presented in the table, which matches the data in the original paper ($P = 0.037$ by Fisher Exact for 34/53 vs. 20/23). The study authors found the tumor incidence to be non-significant ($P = 0.1$), but did not indicate test used. The Fisher Exact value should be reported. The authors’ finding of lack of significance could be noted parenthetically.

6. Section 4.1.1 Mice Oral (Ponomarkov and Tomatis 1978) – C57Bl

- Page 166, lines 16-23. More specifics regarding study findings should be given. A suggested rewrite for paragraph at lines 16–23 is the following:

“The predominant tumors occurring in C57Bl mice included lymphoma and lung or liver tumors (Table 4-2b). The incidences of these tumors in styrene-treated mice (dams or progeny) were not significantly higher than controls. While the authors reported that the higher incidence of liver tumors in styrene-treated male mice (3 carcinomas; 12.5%) was cause for some concern, one adenoma was also observed in a vehicle control male (8.3%) and one adenoma was observed in an untreated control male (2.1%). Therefore, no conclusions can be drawn regarding the low incidences of liver tumors in styrene-treated mice.”

- The following limitations should be noted: i) The rationale for choosing these doses was not discussed; no data were presented to indicate that the dose (300 mg/kg) for C57Bl mice was sufficiently high; ii) The study is lacking in power and this should be emphasized. The initial numbers in the vehicle reference groups were 12 males and 13 females, and dosed group size was 27.
- Page 167, Table 4-2b. Footnotes should indicate that the liver tumors in the untreated control male and vehicle control male mice were adenomas, while the 3 liver tumors in the styrene-treated males were carcinomas.

7. Section 4.1.2 Mice, Inhalation (Cruzan 2001)

- NTP should conduct statistical analyses of the Cruzan *et al.* data and report the P -values with greater precision than “ $P < 0.05$.” These values could be reported in Table 4-3. A possible replacement for Table 4-3 is given below:
- In the female results for lung carcinoma are highly significant: $P < 0.001$ for the pairwise comparison (Fisher exact), and $P < 0.001$ for trend (Cochran-Armitage).
- For females, combined incidence of adenoma and carcinoma at study end for the 20, 40, 80 and 160 ppm groups are $P = 0.01$, $P < 0.01$, $P = 0.1$, $P < 10^{-5}$. For trend, $P < 0.0001$.
- For the males, the combined incidence of adenoma and carcinoma P values for the 20, 40, 80 and 160 ppm groups are $P = 0.1$, $P = 0.0001$, $P < 0.01$, $P = 0.0001$. The trend is significant at $P < 0.001$.

- On page 168, at lines 16–20, the sentence regarding Cohen’s statistics should be replaced with NTP’s own statistical analysis and findings, as should statistics in Table 4-3.
- Table 4-3. Insert footnotes to indicate what the numerator (number mice with tumor) and denominator (number of animals examined for each tissue type) represent.

Suggested replacement for Table 4-3

Table 4-3. Lung tumor in CD-1 mice exposed to styrene by inhalation for 98–104 weeks

Sex	Exposure conc (ppm)	Alveolar/bronchiolar tumor incidence ^a (%)		
		Adenoma	Carcinoma	Combined
Male	0	15/50 (30)	4/50 (8)	17/50 (34)
	20	21/50 (42) [#]	5/50 (10)	24/50 (48)
	40	35/50 (70)****	3/50 (6)	36/50 (72)***
	80	30/50 (60)**	6/50 (12)	30/50 (60)**
	160	33/50 (66)***	7/50 (14)	36/50 (72)***
	Trend	$P < 0.001$	NS	$P < 0.001$
Female	0	6/50 (12)	0/50 (0)	6/50 (12)
	20	16/50 (32)**	0/50 (0)	16/50 (32)**
	40	16/50 (32)**	2/50 (4)	17/50 (34)**
	80	11/50 (22) [#]	0/50 (0)	11/50 (22) [#]
	160	24/50 (48)***	7/50 (14)***	27/50 (54)****
	Trend	$P < 0.001$	$P < 0.001$	$P < 0.0001$

Source: Cruzan *et al.* 1998.

^a(number mice with tumor) / (number of animals examined for each tissue type)

[#] $P = 0.1$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ [Fisher’s Exact test for pairwise comparison conducted by NTP. Exact Cochran-Armitage trend test by NTP]

- Page 168, line 10 and 11. Sentence regarding effect on body weight/weight gain needs to be clarified. The authors reported that males (80 and 160 ppm) and females (160 ppm) gained significantly less than controls. Thus, body weight *gain* was apparently analyzed statistically, and not body weight as indicated in the draft document.
- Section 4.1.3 Mice, Intraperitoneal (Brunnemann *et al.* 1992)
 - Page 170, lines 8–9. Strike sentence beginning with “Earlier studies indicated...”
 - Page 170, lines 12–13. Strike last sentence regarding the positive control group. Insert sentence as follows: “[The duration of the study, single sex, and small group size limit this study as a test for carcinogenic activity.]”
 - Section 4.2.1 Rats Oral (Wolfe *et al.* 1956)
 - The write-up as well as the tabulation on page 173 of this study should be deleted. Study duration was less than 25% of the lifespan of the animal. This study could be moved to toxicity section.
 - Section 4.2.1 Rats, Oral (Ponomarkov and Tomatis 1978)
 - Page 171, lines 8–17. Strike all sentences beginning with “No statistically increased...” and ending on line 17. Beginning on line 8, suggest replacing with the following: “The incidences of tumors in styrene-treated rats were not significantly higher than those of controls. Nevertheless, the authors reported that stomach tumors observed in one styrene-treated dam and two styrene-treated female progeny were of “some concern” because they were “rarely seen in controls.” While the histologic types of these stomach tumors were not specified in the table, they were described in the text as an adenoma, a fibrosarcoma, and a carcinosarcoma

without specific attribution to a particular dose group. A stomach fibrosarcoma was observed in one of the vehicle control female progeny. The low incidences of stomach tumors and inadequate reporting of tumor types preclude drawing any conclusion about their association with treatment.”

- Limitations (once per week dosing) of the study were not noted in text of draft document and should be.

11. Section 4.2.1 Rats, Oral (NCI 1979a)

- Some additional explanation regarding the low-dose group should be provided. The low-dose group was added at a later date with corresponding control, once the survival in the high-dose group plummeted. The size of the vehicle control groups should be noted.
- Page 171, line 24. Prior to the last sentence (lines 24 and 25) regarding the lack of treatment-related tumor increases, insert a statement to the effect that “survival of low- (44/50) and medium-dose rats (47/50) at week 90 was considered adequate.”

12. Section 4.2.1 Rats, Oral (Conti *et al.* 1988)

- Page 172 (top). The limitations of the oral study (low doses, short treatment duration) and limited reporting of the data (mortality and cause of higher mortality in females) by Conti *et al.* 1988 are not adequately noted in the text of the draft document. The less than lifetime dosing and the lack of reporting of time-dependent findings, make the ability to interpret the study findings difficult. That the reporting overall in Conti *et al.* is very limited should be highlighted.
- Effective numbers of treated animals are not given in the Conti report. This should be noted in the background document.
- Page 173, Table 4-4. The entry “decreased incidence of total benign and malignant tumors and total mammary tumors in females in the high-dose group” should be stricken from the Results/Comments column. The decreased incidence is likely related to the poor survival in that group and has no significance. The depression of mammary tumors in the high-dose group ($P = 0.040$) in this strain for which mammary tumors are common, highlights the problem of study interpretation in the absence of analysis that accounts for intercurrent mortality.

13. Beliles *et al.* 1985.

- Page 172, line 10. Note, as for IARC, that the solubility of styrene in water limited the dosing.
- Page 172. Limitations: The major limitations of the study (low doses) and failure to report actual tumor rates were not noted in the text.
- The incidences of mammary tumors as presented in the Huff report should be added in a table to the background document and the pairwise and trend statistics provided for fibroadenoma.

Suggested new table for Section 4.2.1

Exposure (mg/kg/d)	Mammary gland tumor incidence ^a (%)			
	Fibroadenoma	Adenoma	Adenocarcinoma	Combined
0	45/96 (49)	1/96 (1)	8/96 (8)	49/96 (51)
125	15/30 (50)	0/30 (0)	5/30 (17)	18/30 (60)
250	37/60 (62)*	0/60 (0)	8/60 (13)	40/60 (67)
Trend	$P = 0.046$	NS	NS	— ^a

^a Source: Huff *et al.* 1984. Statistics not reported for benign plus malignant because of lack of information on the histogenesis of the tumor.

* $P = 0.05$, [Exact Cochran-Armitage trend test P values provided by NTP]

14. Section 4.2.2 Rats, Inhalation (Conti *et al.* 1988)

- Page 174, lines 12-15. Strike the sentence beginning “The incidence of spontaneous benign mammary tumors...” No data were provided to support this statement.
- Remove the statistical analyses from the combination of benign and malignant, but leave the statistical analyses for the malignant.
- The significantly elevated tumor findings in Table 4-5 should be asterisked, according to NTP’s calculations, to make the presentation easier to follow.
- The NTP performed the tests for trend during the styrene review. The trend tests for females should be reported in Table 4-5.
- Page 174, lines 15–17. The sentence should add a clause indicating that there is a dose-related trend, e.g., “...incidences increased with increasing dose (Table 4-5).”

Suggested replacement for Table 4-5 (New text suggested by the expert panel is in red.)

Table 4-5. Mammary tumors in Sprague-Dawley rats exposed to styrene by inhalation for 52 weeks

Sex	Exposure conc (ppm)	Initial no. rats	Mammary tumor incidence ^a (%)	
			Malignant ^b	Benign + malignant ^c
Male	0	60	1/60 (1.7)	8/60 (13.3)
	25	30	1/30 (3.3)	6/30 (20.0)
	50	30	1/30 (3.3) ^d	3/30 (10.0)
	100	30	0/30 (0)	6/30 (20.0)
	200	30	1/30 (3.3)	4/30 (13.3)
	300	30	0/30 (0)	5/30 (16.7)
Female	0	60	6/60 (10.0)	34/60 (56.7)
	25	30	6/30 (20.0)	24/30 (80.0)
	50	30	4/30 (13.3)	21/30 (70.0)
	100	30	9/30 (30.0)*	23/30 (76.7)
	200	30	12/30 (40.0) ^{e***}	24/30 (80.0)
	300	30	9/30 (30.0)*	25/30 (83.3)
	Trend		<i>P</i> = 0.002	<i>P</i> = -^c

Source: Conti *et al.* 1988.

[Table provides significance values calculated by NTP: * $P \leq 0.05$, ** $P \leq 0.01$,

*** $P = 0.001$, Fisher Exact test for pairwise comparison. **Trend test: Exact Cochran-Armitage]**

^a (number mice with tumor)/(number of animals examined for each tissue type).

^b Authors reported to be treatment-related and statistically significant for females; however, no specific dosed group(s) was identified.

^c Authors noted higher incidence in all exposed groups of females compared with controls, but increases were not reported to be statistically significant and specific tumor types were not reported. **Statistics not reported for benign plus malignant because of lack of information on the histogenesis of the tumor.**

^d Reported as 3% by Conti.

^e Reported incidence may be in error because it exceeds the incidence reported for total malignant tumors of 10 of 30 (33.3%). If the true incidence is 10 of 30, the finding is significant at $P \leq 0.01$ level, and the trend is significant at the $P = 0.004$ level.

15. Section 4.2.2 Rats, Inhalation (Jersey *et al.* 1978)

- Exposures were terminated when the survival for any treatment group hit 50%. This was 18.3 months for the males and 20.7 months for the females. The animals were then allowed to live to the end of the study (24 months). This feature of study design should be part of the study description.

- The historical control data are available from the Jersey *et al.* 1978 report and are given in the attachment. The 6/85 incidence for females in each treatment group fell outside the range of the historical controls (see Attachment 2; 11/808 (1.36%) vs. 6/85 (7.1%)).
 - These tumors are not typically combined for statistical analysis in carcinogenicity studies unless the leukemias are lymphocytic (as opposed to granulocytic or other bone marrow cell types). The cell type of the leukemia(s) was not indicated.
 - The NTP performed a trend analysis for these tumors during the peer review. The *P*-value for trend should be included in the background document (*P* = 0.03).
 - Page 176, line 21. The background document should note that without the original data provided in the laboratory report (unpublished), data essential to the interpretation (survival data, appropriate statistical analyses, historical control data, leukemia cell type, site of origin of lymphoma) of this study are missing.
16. Section 4.2.2 Rats, Inhalation (Cruzan *et al.* 1988)
- Page 177, line 3. The sentence could be stricken since it does not contain protocol information critical for this section (“A battery of hematologic...”)
 - The footnote should indicate that histopathology findings for the pituitary reflect gross lesions and masses for the 50, 200, and 500 dose groups and not an analysis of all animals in the group.
 - During the review of the styrene document, NTP performed the trend tests for the testicular tumors. There was a significant dose-related trend, with *P* = 0.01. This should be included in Table 4-7 and noted in the text.
 - The benign mammary gland tumors need to be included in Table 4-7.
17. Section 4.2.2. Rats, Inhalation (Spencer *et al.* 1942 and Wolfe *et al.* 1956)
- Delete the write-up and tables on these studies (Page 178, L12 through Table 4-8), since these are not designed to be chronic carcinogenicity studies. Also, delete reference to these studies on page 176, line 22.
18. Section 4.2.3 Parental (Conti *et al.* 1988).
- Page 179, line 9. More detail is needed on the study limitations. Suggested revision to the last sentence is as follows: “No tumors were reported, but the studies were markedly limited by the low and infrequent doses, short duration of styrene exposure, and incomplete reporting.”
19. Section 4.3 Other experimental animals, Spencer *et al.* and Wolf *et al.* studies in guinea-pig, rabbit, and rhesus monkey.
- Delete section 4.3. These studies fall so far short of the animals’ lifetimes, they were not designed to be carcinogenicity studies, and they should not be included as carcinogenicity studies in the background document.
 - * The Rhesus monkey can live up to 30 years in captivity and the study duration was 7 to 12 months.
 - * Rabbits can live 5 years or longer and the study was terminated at 3 to 12 months.
 - * Guinea-pigs live 5 to 6 years, although some live as long as 8 years. The studies were terminated at 5 to 7 months for these animals.
 - * Thus, the studies in the three species were for 20% or less of the animals’ lifespans. It could be mentioned as reported in the literature but not included because of very short length in the introductory text on page 163, or, again, because these are not designed to be carcinogenicity studies, there is no reason to even do this.
20. Section 4.4 Mixtures containing styrene
- If NTP can obtain the individual animal data, it should conduct and report the results for the poly-3 test for pairwise comparisons and trend for lung tumors in male mice.

- Page 181, Table 4-10. The table should include a footnote to indicate what the denominator represents (i.e., the number of animals examined at site).
- Page 181, lines 14-15. The results of the trend test should also be included in the text. A suggested revision to the sentence is as follows: “The incidence in the high dose group was 2/36 (5.5%) and was not significant by pairwise comparison.” If the NTP conducts the poly-3 test, those results should be reported.
- Page 181, line 15. Following the revised sentence above, further context for the lung findings in relation to survival and other related lesions should be included. A suggested sentence is as follows: “It should be noted that adenomatous hyperplasia (a potentially preneoplastic lesion) was observed in the lung of one high-dose male, and because of poor survival of high-dose male mice there were substantially fewer animals at risk for the late occurring lung tumors.”

21. Section 4.6 Summary

- A point should be made in the review that lung tumors were induced in mice by both gavage and inhalation. On page 184, the review might also present the tumor sites that were seen in the styrene-7,8-oxide studies.
- The summary needs to be shaped more: to emphasize the better studies, to place in greater context the findings from the many marginal studies reported for the compound, and to give a more integrated impression of what the animal evidence indicates. The lung adenoma/carcinoma in the mouse is the compelling finding. It is seen in the two better studies, conducted at different times by different laboratories, under different protocols and by different routes. It has some additional support from the more marginal studies.
- There was no discussion in the summary regarding cases where the results of one study supported or failed to support the results of another study and what facts lend validity to the ultimate interpretation. This should occur in the Summary (Section 4.6).

The following is offered as a suggested revision to address these issues:

The carcinogenicity of styrene has been investigated in rats and mice by several routes of exposure and the results are summarized in Tables 4-11 and 4-12. Many of the studies were severely limited in their ability to detect carcinogenic effects because of inadequate study design (low doses, short treatment and/or short study duration, small group size) or intercurrent disease and high mortality (*e.g.*, pneumonia), or the studies were inconclusive because of limited reporting (tumor diagnosis, statistical methodology).

In mice, gavage studies in three strains and both sexes, inhalation studies in both sexes, and one i.p. study in females were found in the literature and reviewed. The oral gavage studies in B6C3F1 mice (NCI, 1979a) and the inhalation studies in CD-1 mice (Cruzan *et al.* 2001) were the most robust and most completely reported carcinogenicity studies.

Male B6C3F1 mice had a significant positive dose-response trend for alveolar/bronchiolar adenoma and carcinoma, combined, that was supported by a significantly increased incidence of these lung tumors in the high-dose group. While the authors questioned the significance of these lung tumors because the incidence in the control group was unusually low compared with historical untreated controls, the incidence in the high-dose group is significantly increased compared with historical vehicle controls from the same period (prior to 1979) with similar duration and from the same source. A dose-related trend in female B6C3F1 mice was also observed for lung adenoma. Significantly increased incidences of alveolar/bronchiolar adenoma and alveolar/bronchiolar adenoma or carcinoma combined were also observed in male and female CD-1 mice exposed to styrene by inhalation. In each sex, three different treatment groups showed increases in these tumors. The high-dose female mice had

an increased incidence of alveolar/bronchiolar carcinoma. In a short-term oral gavage study in O20 mice, a strain with a high spontaneous incidence of lung tumors, significantly higher incidences of lung tumors (adenomas and carcinomas combined) were observed in both males and females compared with vehicle controls. Thus, lung tumors in mice are clearly related to styrene exposure.

In rats, gavage studies in three strains, three inhalation studies in one strain (Sprague-Dawley), and a drinking water study in one strain were reviewed. The oral gavage studies in F344 rats (NCI, 1979a) and the inhalation studies in Sprague-Dawley rats (Cruzan *et al.* 1998) were the most robust and most completely reported carcinogenicity studies. Neither study showed an increase in tumor incidences in styrene treated rats, although Sprague-Dawley rats exhibited a dose-related reduction in pituitary and mammary gland tumors. In the inhalation study reported by Conti *et al.* 1988, there was a dose-related increase in the incidences of malignant mammary gland tumors; treatment-related and statistically significant incidences of these tumors were seen in the top three dose groups. The drinking water study in Sprague-Dawley rats did not report any dose-related carcinogenic effects; however, statistical analyses of study data indicated a marginal increase in mammary fibroadenoma in high-dose female rats and a significant dose-related trend. For the unpublished inhalation study by Jersey *et al.* 1978, a statistically significant increase in mammary adenocarcinoma in the low-dose, but not high-dose group was reported in several reviews of this study. The lack of consistent findings in the mammary gland of rats across studies and the negative findings in the study with the longest treatment duration (Cruzan *et al.*) prevents drawing a definitive conclusion about the association of mammary gland tumors with styrene treatment. Elevated leukemia/lymphosarcoma were observed in both treatment-related groups of female Sprague-Dawley rats in one inhalation study (Jersey *et al.* 1978).

No increase in tumor incidence was observed in rats exposed to a mixture of 70% styrene and 30% β -nitrostyrene. An increase in lung tumors (low-dose group only) was observed in male mice exposed to this styrene/ β -nitrostyrene mixture. Substantial mortality in the high-dose group precluded the observation of late occurring tumors, like the lung, in many animals.

Uncertain findings include the significant trend in hepatocellular adenoma in female mice (NCI 1979) and the findings of trend in interstitial testicular tumors in rats, both of which were statistically significant by trend but not by pairwise comparison between treated and control animals. Lack of information on whether the leukemia was lymphocytic in nature makes uncertain the finding of a small but significant number of female rats reported with “leukemia/lymphosarcoma” in the Jersey *et al.* (1978) study.

22. Table 4-11 (Summary table)

Replace table 4-11 with one for mice and one for rats with this suggested format. These tables will be at the end of the summary section.

Table 4-11. Summary of studies in mice

Studies	Design: dose, duration and initial group size	Comments on study	Results male	Results female
Oral				
B6C3F1 mice (NCI, 1979a)	150 or 300 mg/kg, 5 days/wk, 78 weeks 20/control group; 50/treatment group	Limited control group size	Significant increase and dose -related trend in lung adenoma and carcinoma combined	Dose related increase in hepatocellular adenoma
O20 mice (Ponomarkov and Tomatis, 1978)	1,350 mg/kg once prenatal day 17 & weekly postweaning for 16 weeks. Controls – 20 males, 22 females. Dosed – 45 males, 39 females	High mortality in treated animals; only one treatment group; short dosing duration; small control groups	Significant increase in lung tumors	Significant increase in lung tumors
C57Bl mice (Ponomarkov and Tomatis, 1978)	300 mg/kg once prenatal day 17 and weekly postweaning until death. Controls – 12 males, 13 females. Dosed – 27 males, 27 females	Only one treatment group; low dose; limited reporting; small group size, particularly controls	No significant increase in tumors	No significant increases in tumors
Inhalation				
CD-1 mice Cruzan <i>et al.</i> 2001	20, 40, 80 or 160 ppm, 6 hrs/day, 5 days/wk, 98-104 weeks 50 animals per group	No major limitations	Significant increase and dose-related trend in lung adenoma and combined adenoma and carcinoma	Significant increase and dose-related trend in lung adenoma, carcinoma, and combined adenoma and carcinoma
Intraperitoneal				
Female A/J mice (Brunnemann <i>et al.</i> 1992)	Total 100 mg/kg in divided doses, 3/wk, held for 20 weeks after last injection 25 animals per group	Only one treatment group, limited reporting, small group size	NA	No significant increase in lung tumors

Table 4-12. Summary of studies in rats.

Studies	Design: dose, duration and initial group size	Comments on study	Results male	Results female
Oral				
F344 rats (NCI, 1979a)	500, 1000 or 2000 mg/kg, 5 days/wk, 78 weeks (mid- & high dose) or 103 weeks (low dose) 20/control group; 50/treatment group	Poor survival of high dose groups, small control group	No significant increase in tumors	No significant increase in tumors
BD IV rats (Ponomarev and Tomatis, 1978)	1,350 mg/kg prenatally and 500 mg/kg weekly postweaning until death Controls: 36–39, Dosed: 71–73	Limited dosage regimen, once/wk dosing, limited reporting	No significant increase in tumors	No significant increase in tumors
Sprague Dawley rats (Conti <i>et al.</i> 1988)	50 or 250 mg/kg, 4–5 days/wk for 52 weeks and held until death 40/dose group	Mortality in high dose females, short treatment duration, low doses, limited reporting	No significant increase in tumors	No significant increase in tumors
Sprague Dawley rats (Beliles <i>et al.</i> 1985)	125 or 250 ppm in drinking water (7.7 – 14mg/kg/day in males and 12 – 20.5 mg/kg/day in females) for 2 years Controls: 76 (m), 106 (f) Dosed: 50 (m), 70 (f)	Low doses, limited reporting;	No significant increase in tumors	Small increase in mammary fibroadenoma
Inhalation				
Sprague Dawley rats (Conti <i>et al.</i> 1988)	25, 50, 100, 200 or 300 ppm, 4 hrs/day, 5 days/wk for 52 weeks and held until death	Limited dosing regimen, limited reporting	No significant increase in tumors	malignant mammary tumors increased in multiple groups, with significant trend
Sprague Dawley rats (Jersey <i>et al.</i> 1978)	600 or 1,200/1,000 ppm, 6 hrs/day, 5 days/wk, for 18.3 months (males) or 20.7 months (females)	Original report and data not available in published literature; limited reporting in reviews, high incidence of pneumonia	No significant increase in tumors	Small increase in leukemia/lymphosarcoma, with a significant trend
Sprague Dawley rats (Cruzan <i>et al.</i> 1998)	50, 200, 500 or 1000 ppm, 6 hrs/day, 5 days/wk for 104 weeks	No major limitations	Positive dose-related trend in interstitial testicular tumors	Pituitary and malignant mammary tumors decreased in all dose groups.

Section 5: Other Relevant Data

1. General

- Add information on pharmacokinetics of styrene-7,8-oxide (from RoC Background Document for), including route of administration and tumor sites. Note any apparent inconsistencies of tumor sites between routes.
- Mouse CYP2F2 should be Cyp2f2.

2. Section 5.1 Absorption, distribution, metabolism and excretion

- The statement that the “most important route of styrene exposure is by inhalation” (page xi, lines 2-3; Section 5.1, page 187, line 16), should be changed to “the predominant route of exposure in occupational settings is by inhalation.” Inhalation is not the predominant route of exposure in the general population. According to the table on page 39 (Table 2-9), the estimated daily intake of styrene in children younger than 12 years is predominantly through food sources, not through inhalation. Inhalation and food intake appears to make approximately equal contributions to daily intakes in adults according to these data.
- The NTP should review additional studies on dermal exposure supplied by the Section 2 subgroup and include information relevant to Section 5.1.1.1 (Sandell *et al.* 1978, Smith *et al.* 2006, Stewart *et al.* 1968).
- Page 188, line 14 [“The authors concluded that percutaneous absorption of styrene...”: Insert “vapor” after styrene.

3. Section 5.1.3 Metabolism

- Page 193, line 3: Change sentence as follows: Carlson *et al.* (2000) detected the metabolism of styrene to styrene-7,8-oxide in 6 out of 6 human liver microsomal preparations and 1 of 6 lung microsomal preparations collected from 12 individuals.
- Page 194 after line 20: Add a new paragraph that discusses evidence that formation of the 3,4-oxide includes the inability to form the 4-phenol with chemicals that block metabolism at that site. For instance, it has been reported that the 3- and 4-methylstyrene analogues (e.g., dihydrocoumarin, 4-methylstyrene, mixture of 3-, and 4-methylstyrene, styrene-7,8-oxide, and 1-phenylethanol) are not cytotoxic and do not form tumors in mice (confirm and add supportive references).

4. Section 5.1.3.3

- Page 195 after line 13: Add a description of Boers *et al.* 1999 as follows:
Human terminal airways don't have significant numbers of Clara cells; however, the contribution of Clara cells to the proliferation compartment of normal human tracheobronchial epithelium is substantial, demonstrating a role of the Clara cell in the maintenance of the normal epithelium of the distal conducting airways in humans. This concept was demonstrated in the study by Boers *et al.* (1999). These authors evaluated the number of Clara cells from normal tissue taken from seven lungs obtained by autopsy. The number of Clara cells in the terminal bronchioles was $11 \pm 3\%$ (mean \pm SD) and in respiratory bronchioles $22 \pm 5\%$, and was very low in the proximal airway. The overall proliferation compartment of the conducting airway epithelium was $0.83 \pm 0.47\%$; the contribution of Clara cells was 9%. In the terminal bronchioles 15% of proliferating airway epithelial cells were Clara cells, and in the respiratory bronchioles this percentage increased to 44%.
- Page 197, line 14 [“These results indicate that CYP2E1 is important for bioactivation of styrene in the liver.”: change “indicate” to “suggest.”

- Page 197, line 26: Begin sentence as follows: “In Table 2 of Carlson *et al.* (2003), the authors show that styrene metabolism by pulmonary microsomes in *CYP2E1*-knockout mice was about one-half that in wild-type mice.”
- Add a new subsection to discuss metabolic enzymes (P450s) in lung in general (Somers *et al.* 2007, Nichols *et al.* 2003). The other enzymes discussed should include human enzymes CYP2S1 and CYP2A13, CYP2F1, and CYP2F4 in rats. Also, add the numerical description of the catalytic activity of 2A13, 2E1, 2S1, and 2A6. Karlgren *et al.* (2005) describes high activity of 2S1 towards styrene, particularly the relative rate of styrene oxidation compared with other enzymes. This section should also describe studies that describe the use of primary human bronchial epithelial cells and immortalized cell lines (BEAS-2B) that are relevant to styrene metabolism and bioactivation to epoxides should also be discussed, e.g., Sheets *et al.* 2004. The document should also recognize that differing cellular compositions of lungs of different species makes metabolism studies using whole lung problematic. The following text addresses some of these issues:

Human lung has been reported to contain either mRNA or protein for the following P450 isozymes: CYP1A1, CYP1A2, CYP2A6, CYP2A13, CYP1B1, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2F1, CYP2J2, CYP2S1, CYP3A4, CYP3A5, CYP4B1, CYP5A1, CYP7B1, CYP8A1, CYP27, and CYP51 (Hukkanen *et al.* 2002, Zhang *et al.* 2006, Seliskar and Rozman. 2007, Ding and Kaminsky 2003, Pelkonen and Raunio 1997, Nishimura *et al.* 2003, Somers *et al.* 2007). Although levels of most P450 enzymes are reported to be lower in lung compared with liver (Somers *et al.* 2007), CYP2A13, CYP2F1, CYP2S1, CYP3A5, and CYP4B1 are preferentially expressed in the lung (Ding and Kaminsky 2003, Thum *et al.* 2006). Xenobiotic metabolism in human lung occurs primarily in bronchial epithelial cells, Clara cells, type II pneumocytes, and alveolar macrophages, while in rodents and rabbits metabolism is highest in Clara cells and type II pneumocytes. The CYP2F1 isoform of cytochrome P450 is a homolog of the CYP2F2 isoform expressed in mouse lung (see below) and the CYP2F4 isoform expressed in rat lung (Baldwin *et al.* 2005). The presence of a cDNA for CYP2F1 in a human lung library was first reported by Nhamburo *et al.* (1990). The mRNA for CYP2F1 has been shown by RT-PCR amplification to be present in human lung tissue and broncho-alveolar macrophages (Raunio *et al.* 1999) and in human bronchial biopsy trachea and lung (Thum *et al.* 2006, Bieche *et al.* 2007).

Sheets *et al.* (2004) reported that A549 human alveolar epithelial type II (adenocarcinoma) lung cells were capable of metabolizing benzene, and the activity decreased significantly (51%; $P < 0.05$) in the presence of 5-phenyl-1-pentyne (5P1P), a P450 inhibitor. 5-Phenyl-1-pentyne is an effective inactivator of CYP2E1 as well as CYP2F2 and CYP2F1 (Roberts *et al.* 1998, Simmonds *et al.* 2004). The authors concluded that CYP2F1 was important in benzene metabolism in this human lung cell line. BEAS-2B cells overexpressing CYP2F1 also were reported to have a significant ($P < 0.05$) increase in cytotoxicity resulting from bioactivation of 3-methylindole to 3-methylenindolenine (Nichols *et al.* 2003).

5. Add to Section 5.1.4.1, page 202, lines 7-11:

4-Vinylphenol in humans: Pfaffli *et al.* (1981) reported that they could detect 4-vinylphenol in the urine of styrene-exposed workers but not in nonexposed individuals by GC/MS, but the level detected was only 0.3% of the level of mandelic acid (MA) in the same individuals. Johanson *et al.* (2000) did not detect 4-vinylphenol in the urine of volunteers exposed to 50-ppm styrene for 2 hours, and they concluded that the level of 4-vinylphenol would have been below the limit of detection based on the level of (MA) in their study based on the results reported by Pfaffli *et al.* Manini *et al.* (2003) used liquid chromatography electrospray tandem

mass spectrometry to measure 4-vinylphenol conjugates in urine of workers exposed to styrene and in volunteers exposed to 50 mg/m³ styrene. Urinary 4-vinylphenol conjugates (glucuronates and sulfates) represented about 0.5% to 1% of the total excretion of styrene metabolites and were significantly correlated with airborne styrene ($r = 0.607$, $P < 0.0010$ and the sum of MA and phenylglyoxylic acid (PGA) ($r = 0.903$; $P < 0.001$) in end-of-shift samples for workers.

6. Section 5.2.1 Toxicity: Humans

- Page 205, Hearing loss: The most recent or comprehensive review should be added to the references already cited here (Johnson 2007, Hoet and Lison 2008).
- Page 206, line 26 to page 207, line 12. Add a description of the induction of pancreatic cancer via an inflammatory mechanism to Section 5.2, pages 206-7 and consider a paper by Michaud (2004) on the epidemiology and etiology of pancreatic cancer.
- Page 207 after line 22. Add the paper by Delzell *et al.* (2005) that suggested no overall risk of heart disease in workers, including those hired in later years.
- Add discussion of estrogenicity: Include a summary of the available scientific literature assessing the estrogenicity of polystyrene and related compounds. Suggested text as follows:

Estrogen can promote cell proliferation in estrogen receptor-positive tumors and in estrogen-responsive tissues. It has been hypothesized that polystyrene or styrene metabolites can mimic a physiological effect induced by estrogen (Colburn *et al.* 1996). The following studies investigate this hypothesis for polystyrene oligomers, which can leach from polystyrene food containers.

Polystyrene dimer and trimer extracts from food containers were tested *in vitro* for estrogen-like effects using estrogen-responsive element reporter and estrogen receptor binding assays, cell proliferation assays, and *in vivo* using a rat uterotrophic assay. Bachman *et al.* (1998) measured the effect of extracts from 23 polystyrenes in a rat uterotrophic assay at concentrations up to 0.75 mg/L [equivalent to 15 microgram/kg-b.w./day dosage]. None of the polystyrene extracts were positive in this assay. Fail *et al.* (1998) measured the estrogenicity of a polystyrene extract equivalent in dose per body weight to human consumption [amount not specified]. It was negative in a rat uterotrophic assay and in an estrogen-responsive element reporter assay [3 mM, approximate maximum concentration of polystyrene extract tested]. Azuma *et al.* (2000) and Date *et al.* (2002) reported a lack of estrogenicity of styrene monomers, dimers and trimers using *in vivo* and *in vitro* assay systems. Ohno *et al.* (2001) used high concentrations [up to 10⁻³ M *in vitro*] of specific oligomers in uterotrophic, estrogen-responsive element reporter and estrogen receptor binding assays and also obtained negative results. In this study, styrene monomer, three styrene dimers, and seven styrene trimers known to dissolve in small amounts from polystyrene cup noodle containers were tested. However, Ohyama *et al.* (2001) tested the same styrene dimers and trimers and obtained positive results (2 positives out of 4 dimers tested and 4 positives out of 7 trimers tested) at concentrations of 10⁻⁶ and 10⁻⁵ M [highest concentration tested] in a cell proliferation assay and in a binding affinity assay for human estrogen receptor alpha (9 oligomers were positive and 2 trimers were negative in this assay). These results were refuted by Ohno *et al.* (2003). This laboratory tested the same oligomers from the Ohyama *et al.* report using three different estrogen receptor binding assays. The results for all oligomers were negative in these assays. Further, the results of a rat uterotrophic assay and estrogen response element reporter assay were also negative using the same styrene oligomers. In a letter to Environmental Health Perspectives, Ohno and colleagues (Ohno *et al.* 2002) noted that in the assay system of Ohyama *et al.*, solubility was a problem at high concentrations leading to false positive results,

and the validity of the MCF-7 E-Screen assay was also questioned. Ohyama *et al.* replied that their results were valid, because they believed the insolubility of styrene oligomers observed in Ohno's studies appeared to be due to dissolving compounds in water rather than in DMSO, as was used in the Ohyama studies. Ohyama *et al.* also defended the use of the MCF-7 E-screen method as a well recognized method for estrogenic screening.

It is possible that metabolic activation of styrene oligomers may affect the estrogenicity of these compounds. Kitamura *et al.* (2003), using rat liver microsome system, found the activated form of *trans*-1,2 diphenylcyclobutane, a styrene dimer, to be estrogenic using a yeast estrogen screening assay and an estrogen-responsive element reporter assay. The active metabolite was a hydroxylated form called *trans*-1(4-hydroxyphenyl)2-phenylcyclobutane [activity at 10^{-5} M]. According to the authors, *cis*-1,2-diphenylcyclobutane, 1,3-diphenylpropane, and 2, 4-diphenyl-1-butene also exhibited estrogenic activity after metabolic activation, but the activity was lower than with *cis*-1,2-diphenylcyclobutane.

7. Section 5.3 Interspecies differences in metabolism, toxicity, and toxicokinetics

- Page 219, lines 29-30: The rate of metabolism in olfactory tissues was much higher in rats than mice.
- Page 222, 2nd paragraph: Validate and add disclaimer from Csanady *et al.* (2003) about the mistake in PBPK model used by Cohen *et al.* 2002. Also describe the differences in assumptions that the 3 models use regarding metabolism of styrene.

8. Section 5.1.3.4 Metabolic enzyme studies

- Page 196, line 15, Nakajima *et al.* 1994 study. Add the numerical values (see below) for the rates of formation of styrene glycol from styrene that were measured in cultured hepatoma G2 cells expressing cDNAs for human (12) mouse (2), rat (2) cytochromes P450 in recombinant vaccinia viruses (Nakajima *et al.* 1994a).

human CYP2B6 (119.6 nmol/(dish x 2 h))

human CYP2E1 (63.4)

human CYP2F1 (103.9)

rat CYP2B1 (198.8)

rat CYP2B2 (81.9)

mouse CYP1A1 (85.0)

mouse CYP1A2 (17.2).

- Add the following after the Nakajima study:

Fukami *et al.* (2008) reported that CYP2A13, a human cytochrome P450 expressed predominantly in the respiratory tract, had the highest catalytic activity for the formation of styrene-7,8-oxide from styrene when compared with CYP2A6 and CYP2E1. These enzymes have overlapping substrate selectivities. The CL_{int} values calculated from the initial slope of velocity plotted against the substrate concentration values were 46.8 for 2A13, 17.2 for 2A6, and 18.5 for 2E1.

9. Section 5.4.1 Adduct formation.

- Move first paragraph on page 226 (which describes *in vitro* studies of adducts and not chemical formation) to Section 5.4.2.1, page 227.
- Page 226. Matters of adduct persistence (in cells) should be differentiated from matters of chemical stability (Page 226). Here it is stated that N7 adducts are "efficiently removed" (line 3), which implies an active repair process, but a few lines later (line 8) it is said that they depurinate due to chemical instability, and thus may be lost by non-enzymatic processes. It is difficult to say that they are removed "efficiently" without some knowledge of repair fidelity.

(The word “efficiently” is used again in the Summary (Section 5.6.5) on page 336, line 1). Change text to say adducts are lost with half-life of 19 hrs. Removed implies an active process while lost is not as specific (repair or chemical instability).

10. Section 5.4.2 *in vitro* studies and 5.4.1 *in vitro*: DNA adducts

- Page 226, after line 22: add studies of styrene adduct structure in DNA which are available for N⁶-dA *R*- and *S*- isomers of styrene-7,8-oxide. Suggested text from these references as follows:
Structural data for both α - and β -N⁶-dA adducts of styrene-7,8-oxide in DNA are available. The α -N⁶-dA adducts locate in the major groove, with their orientation being dependent upon stereochemistry. The adducts with (*R*) stereochemistry orient in the 5' direction, whereas those with (*S*) stereochemistry orient in the 3' direction (Feng *et al.* 1995, 1996, Stone and Feng 1996). While the adducts with (*S*) stereochemistry induce a slight bend in the duplex, those with (*R*) stereochemistry do not (Le *et al.* 2000). The structures of the diastereomeric α -N⁶-dA adducts mispaired with dC have also been examined, both in the 5'-CXA-3' sequence and the 5'-AXG-3' sequence. This represents the putative intermediate leading to A to G transitions. In the former sequence, the adduct with (*S*) stereochemistry remains in the major groove and oriented in the 3'-direction, as observed for the corresponding adduct paired correctly with thymine. A shift of the modified adenine toward the minor groove results in the styrenyl ring stacking with the 5'-neighboring cytosine, which shifts toward the major groove. A wobble A•C base pair is not observed. In this mismatched duplex, the adduct of (*R*) stereochemistry is disordered (Painter *et al.* 1999). In the 5'-CXA-3' sequence, the thermodynamic stability of both the mismatched (*R*) and (*S*) adducts is dependent upon pH. At neutral pH, both exhibit significant structural perturbations and lower T_m values, as compared to the 5'-CXA-3' sequence. This is attributed to reorientation about the adenine C6-N⁶ bond. For the adduct of (*R*) stereochemistry, the styrenyl moiety remains oriented in the major groove but now orients in the 3'-direction. For the adduct with (*S*) stereochemistry, the styrene ring inserted into the duplex, approximately perpendicular to the helical axis of the DNA, but now in the 5'-direction (Simeonov *et al.* 2000). The increased tether length of the β -styrene-7,8-oxide N⁶-dA adducts results in two changes in structure as compared to the α -styrene adducts. First, less distortion is introduced into the duplex. For both the *R*- and *S*- β -N⁶-dA adducts, the styrenyl moiety is accommodated within the major groove of the duplex with little steric hindrance. Second, it mutes the influence of stereochemistry, such that in contrast to the α -N⁶-dA adducts, either the (*R*) or (*S*) stereoisomeric β -N⁶-dA adducts exhibit similar conformations within the major groove (Hennard *et al.* 2001).
- On page 227, lines 4-6: Change sentence to read- “...several *in vitro* studies on adduct formation in nucleosides, calf thymus or fish testis DNA, and in DNA in mammalian tissues and human cells...”

11. Section 5.4.3.1 *in vivo*: DNA adducts.

- Similar results for styrene (page 236, lines 2-7) and styrene-7,8-oxide (page 239, lines 12-20) are described in different sections, with studies from the same two groups compared. Add a cross-reference to the last sentence on page 239.
- In the description of the study of adducts in rats and mice by Boogaard *et al.* (2000b) given on pages 237-238, The use of metabolite standards, and the fact that some of these co-elute with radioactive peaks from the digests of rodent DNA, suggests that the peaks do not contain DNA adducts, so the material should not be described as an “adduct” on page 238, lines 12 and 15. The reference should be checked and “adduct” should be changed to “unidentified compound” on lines 8, 12, and 15 as appropriate. Also revise Table 5-7 as needed.

12. Section 5.4.4.1 Styrene exposed workers: DNA adducts.
- The phrase “oxidative DNA damage” is used on several occasions (see for example page 249, line 15; page 252, line 29; page 253, line 11; this list is not exhaustive). Although the literature contains many uses of this phrase, it is inappropriate because DNA is not oxidative. The correct term is “oxidative damage to DNA” or “oxidatively damaged DNA.” Conduct a global search and replace.
13. Section 5.4 Genetic and related effects (Sections 5.4.2.2, 5.4.3.2, 5.4.4.2): Single strand breaks and the comet assay
- There is a general failure to distinguish adequately between the different types of DNA damage detected by the comet assay. Under alkaline conditions (the vast majority of studies using the comet assay employ these conditions) the assay detects overt single-strand breaks and other lesions that are converted to single-strand breaks by alkali. These include apurinic and apyrimidinic (AP) sites. Strand breaks formed transiently during excision repair of AP sites and other lesions (including chemically stable adducts) can also be detected. Thus when the results of studies using the comet assay are described, it should be said that “alkali-labile lesions” were detected, not “strand breaks” or “single-strand breaks.” In only a few places have alkali-labile lesions been mentioned (e.g., page 241, line 6; page 242, line 15). The statement that single-strand breaks “may occur at apurinic/apyrimidinic sites” could also be explained or described better. The first time the comet assay is mentioned should note that it is conducted under alkali conditions which converts lesions to single-strand breaks.
 - Change heading of Section 5.4.3.2 to “Alkali-labile lesions and DNA damage and repair” (and other related headings that refer to single-strand breaks/DNA damage).
14. Section 5.4 Genetic and related effects: Mutations
- Where it is mentioned that the relative potencies of the styrene-7,8-oxide enantiomers in bacteria did not match their genotoxic activities *in vivo* (page 232, line 10), it might be helpful to mention the *in-vivo* endpoints (they are sister chromatid exchanges). Check Table 5-9 and confirm that first row results are the same as described on page 232 just before Table 5-4.
 - Page 288, line 4, change “first used by Mäki-Paakkanen” to “first used in studies on styrene exposure by Mäki-Paakkanen.” Line 5-6: change “cytochalasin B (Cyt-B) is added to the cell culture to block cells from dividing after they have undergone one round of replicative synthesis since mitogen stimulation; such cells are binucleate. Line 8, change “per 1,000 cells” to “per 1,000 binucleate cells.”
15. Section 5.5.1 Mechanistic studies and considerations: DNA or genetic damage
- Pages 326-327: Site-specific mutagenesis study of Latham *et al.* should be augmented with later study by Kanuri *et al.* Also, the Lloyd group has completed several studies of *trans* lesion synthesis by various DNA polymerases across site-specific styrene adducts. Suggested text for new studies as follows: (insert after codon 61 on line 3 on 327):
 The β -N⁶-dA styrene-7,8-oxide adducts have been examined as to site-specific mutagenesis in *E. coli*. These data indicate that the β -N⁶-dA adducts do not have significant deleterious effects on replication competence (Kanuri *et al.* 2001).

 The actions of native and various site-specific mutants of HIV-1 reverse transcriptase have been examined *in vitro* on DNA templates modified with α -N⁶-dA adducts. For the native enzyme, activity is dependent on both the chirality of the N⁶-dA adducts and their sequence contexts. Replication is possible but is terminated 3-5 nucleotides after translesion synthesis and before reaching the end of the template (Latham *et al.* 1994). Eight mutants of RT also terminated synthesis on these styrene-7,8-oxide-adducted templates. The sites of termination

occurred primarily 1 and 3 bases following adduct bypass, when the lesion is positioned in the major groove of the template-primer stem (Latham *et al.* 2000).

Similar replication assays have been performed using *E. coli* Klenow fragment, Sequenase 2.0, T4 polymerase holoenzyme, polymerase α , and polymerase β , *in vitro*. In all instances, lesion bypass is sensitive to both the local sequence context and the chirality of the α -N⁶-dA styrene-7,8-oxide adducts. For example, in the 5'-AXG-3' sequence, adducts having (*R*) stereochemistry are bypassed, whereas stereochemically-identical lesions in other sequence contexts are often poor substrates. Similarly, (*R*) vs. (*S*) α -N⁶-dA adducts introduced within identical sequences are often bypassed nonequivalently. The degree of adduct-directed termination and translesion synthesis during replication is also dependent on the choice of polymerase. Templates that are poor substrates for bypass synthesis with one enzyme often read through much more efficiently when a different polymerase is used (Latham *et al.* 1995). Similar studies have been conducted using reconstituted *E. coli* Pol III. Replication is poorly processive and strongly terminated by styrene-7,8-oxide lesions in 33-mer templates, although the same enzyme shows efficient bypass of the same adducts in M13 DNA (Latham *et al.* 1996). No data are available regarding replication by Y-family polymerases, *in vitro*.

- Some DNA adducts are xenobiotic specific (see page 326, line 2) but many are not (e.g., methylated adducts). Furthermore adducts formed by oxidative damage can be formed by many agents. In the same paragraph (line 6), 8-hydroxy-2'-deoxyguanosine is listed among the adducts in styrene-exposed individuals, and this most definitely is not styrene-specific. Delete first sentence (about adducts in general, lines 3-4), note and group the styrene-specific adducts together, and move 8-OH-dG to the end of the list of adducts.
- Page 326, lines 11 and 12 [“N7-guanine adducts are the predominant type, but are efficiently repaired.... O⁶-guanine adducts.... but are persistent...”]: Delete “efficiently” and add “more” before persistent.
- “adducts may result in depurination, may cause single-strand breaks, or may be repaired directly” (page 326, lines 13-14). Delete “or may be repaired directly.”
- Page 326, line 19 should be GC to TA instead of GC to AT.
- Replace “adducted in” on page 326, line 29 with “bonded at” or “bound at” and “adducted” on page 326, line 28 and page 327, line 2 with “bonded” or “bound.”
- Delete lines 3-7 on page 327 (This text mentions the geometry of the N⁶ adenine adducts and the GC to AT transition is not a major mutagenic lesion).

16. Section 5.5 Mechanistic studies and considerations (General)

- Add information in Section 5.5.3 on cytotoxicity/epigenetic mechanisms. Additional discussion could be included that relates the cytotoxicity of styrene in susceptible Clara cells or in human bronchial epithelial cells to cellular proliferation and mechanisms of clonal expansion of styrene-induced, or spontaneous mutants. Bogen *et al.* (2008) publication on naphthalene is a good example of this mechanism. Suggested text follows:

In a report of deliberations of a scientific panel at the Naphthalene State-of-the-Science Symposium on the pathogenesis of respiratory tumor formation in rodents (Bogen *et al.* 2008), the following hypothesis for naphthalene-induced neoplasia was presented. Rats chronically exposed to naphthalene by inhalation (NTP 2000) had chronic nasal tissue damage associated with regenerative hyperplasia, atypical hyperplasia, and in a few cases, adenoma formation. This association supports the hypothesis that an increase in tumor incidence after naphthalene exposure is occurring through a cytotoxic mechanism. Chronic cytotoxicity can result in a clonal expansion of cells and increased susceptibility to malignant transformation. Further, this panel reported that elevated incidences of mouse lung tumors occurred only with exposure to

cytotoxic concentrations of naphthalene. Metabolic activation was required and mouse Clara cells had the greatest capacity to metabolize naphthalene. Clara cells were also highly susceptible to naphthalene-induced cytotoxicity. Therefore, this section discusses cytotoxicity of styrene metabolites as a possible mechanism of styrene-induced carcinogenesis.

- Add discussion of styrene metabolism to 4-vinylphenols that are further oxidized to quinone reactive intermediates. Quinones participate in reactive oxygen mediated damage and cytotoxicity. Similar to naphthalene, this cytotoxicity could be a mechanism of clonal expansion of initiated cells.
- NTP to research relevance of cytotoxicity and tumorigenicity of ethylbenzene in mice and rats (Chan *et al.* 1998; Stott *et al.* 2003; Seghir *et al.* (submitted for publication) in regard to mechanistic considerations for styrene.
- Discuss Roder-Stolinski *et al.* 2008. Styrene induces an inflammatory response in human lung epithelial cells via oxidative stress and NF-κB activation. This paper characterized the inflammatory response of human alveolar epithelial cells to styrene. They reported that human lung epithelial cells (A549 cell line) exposed to styrene (10^{-1} to 10^6 $\mu\text{g}/\text{m}^3$) via the gas phase for 20 h had significantly increased (~ 1.4 fold; $P < 0.05$) release of the inflammatory mediator protein, monocyte chemoattractant protein (MCP-1) at 10^2 $\mu\text{g}/\text{m}^3$ and higher, which could be inhibited by antioxidants. Styrene exposure (at the same concentrations that caused an increased MCP-1 release) also increased GSTP1 mRNA expression, which was inhibited by pre-treatment with antioxidants and significantly ($P < 0.05$) increased phosphorylation of NF-κB (10^{-1} to 10^5 $\mu\text{g}/\text{m}^3$) and $\text{I}\kappa\text{B}$ (10^0 to 10^6 $\mu\text{g}/\text{m}^3$). The authors suggested that the activation of NF-κB signaling by styrene was mediated via a redox-sensitive mechanism.

17. Section 5.5.3

- Page 331, line 17: Add discussion of proposed mechanism that discusses a possible role of styrene-induced elevation in prolactin levels as a potential mechanism for the induction of breast cancer.
- Add a brief discussion of epoxides in mammary tissue (role of CYP1B1) in Section 5.5 on page 325. Several chemicals associated with induction of mammary tumors are epoxides or are metabolized to epoxides (includes glycidol, benzene, 1,3-butadiene, and ethylene oxide; see Dunnick *et al.* 1995), and it has been hypothesized that the mammary gland may efficiently metabolize chemicals to their epoxides (Bennett and Davis, 2002), and therefore, may be a target tissue for the induction of epoxide-induced tumors.
- Page 329: change subheading 5.5.3 to “Cytotoxic effects of styrene on mouse lung” (rather than styrene oxide)
- Section 5.5.3: Add additional text after line 17 on page 331 and to summary in Section 5.5.4. Suggested text as follows:

Similar to the first of the three factors put forward by Cohen *et al.* (2002) as possible mechanisms for the development of hyperplasia and lung tumors in mice (see above), Cruzan *et al.* (2002) proposed that interspecies differences in styrene toxicity are most likely explained through CYP2F-generated metabolites (2F2 in mice, 2F4 in rats, and 2F1 in humans). They noted that almost all of the effects of cytotoxicity and tumor formation were seen in tissues that are high in CYP2F isoforms and that CYP2F inhibitors prevented cytotoxicity (see Section 5.1.3.4). Ring-oxidation metabolites, including 4-vinylphenol, are ~ 6 -fold higher in mice compared with rats, and 4-vinylphenol is more potent than styrene-7,8-oxide as a pneumotoxicant (see Section 5.1.3.4). Also, styrene metabolism occurs primarily in Clara cells (see Sections 5.1.3.3 and 5.1.3.4), and mice produce higher levels of toxic metabolites (*R*-styrene-7,8-oxide, 4-vinylphenol, and oxidized reactive intermediates of 4-vinylphenol), and

have a lower level of epoxide hydrolase activity than rats or humans (see Sections 5.1.3.1 and 5.1.3.2). They stated that PBPK models predict that human do not generate sufficient levels of these metabolites in the terminal bronchioles to reach toxic levels. Cruzan stated that the tumor profile of styrene suggests a non-genotoxic mode of action since he felt that the tumors in animals, were common, reported in only one species and one site, did not occur at the 12 month sacrifice, and were associated with organ toxicity and cell turnover. Studies published after Cruzan's 2002 proposal that evaluated the role of CYP2F2, ring-oxidized metabolites, and cytotoxicity in the lung are discussed in Sections 5.1.3.4 and 5.2.2.2).

Report Approved Redacted Date Aug 26 2008
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Attachment 1 to Section 4 Subgroup Report. Excerpts on Comparisons with Historical Controls from the IARC Preamble and a Recent NTP 2008 Report

Preamble to the *IARC Monographs* (amended January 2006)

“It is generally not appropriate to discount a tumour response that is significantly increased compared with concurrent controls by arguing that it falls within the range of historical controls, particularly when historical controls show high between-study variability and are, thus, of little relevance to the current experiment. In analysing results for uncommon tumours, however, the analysis may be improved by considering historical control data, particularly when between-study variability is low. Historical controls should be selected to resemble the concurrent controls as closely as possible with respect to species, gender and strain, as well as other factors such as basal diet and general laboratory environment, which may affect tumour-response rates in control animals (Haseman *et al.* 1984; Fung *et al.* 1996; Greim *et al.* 2003).”

This may be found at the following link or obtained from WHO/IARC:
<http://monographs.iarc.fr/ENG/Preamble/currentb3studiesanimals0706.php>

From the May 2008 NTP Technical Report on the Toxicology and Carcinogenesis Studies of Methylene Blue Trihydrate (CAS No. 7220-79-3) in F344/N Rats and B6C3F1 Mice (Gavage)

“The concurrent control group represents the most valid comparison to the treated groups and is the only control group analyzed statistically in NTP bioassays. However, historical control data are often helpful in interpreting potential treatment-related effects, particularly for uncommon or rare neoplasm types. For meaningful comparisons, the conditions for studies in the historical database must be generally similar.”

Attachment 2 to Subgroup Section 4 Report: Laboratory historical control incidence of leukemia-lymphosarcoma for Jersey *et al.* 1978

Study number	Males	Females
1	3/80 (3.75%)	0/79 (0%)
2	0/25 (0%)	0/25 (0%)
3	None	2/159 (1.26%)
4	0/50 (0%)	None
5	2/90 (2.22%)	0/90 (0%)
6	5/85 (5.88%)	2/86 (2.32%)
7	4/112 (3.57%)	None
8	5/189 (2.64%)	5/189 (2.64%)
9	2/80 (2.5%)	2/80 (2.50%)
10	0/100 (0%)	0/100 (0%)
Total	21/811 (2.59%)	11/808 (1.36%)