NTP Response to Issues Raised in the Public Comments for Candidate Substances for the *12th Report on Carcinogens*

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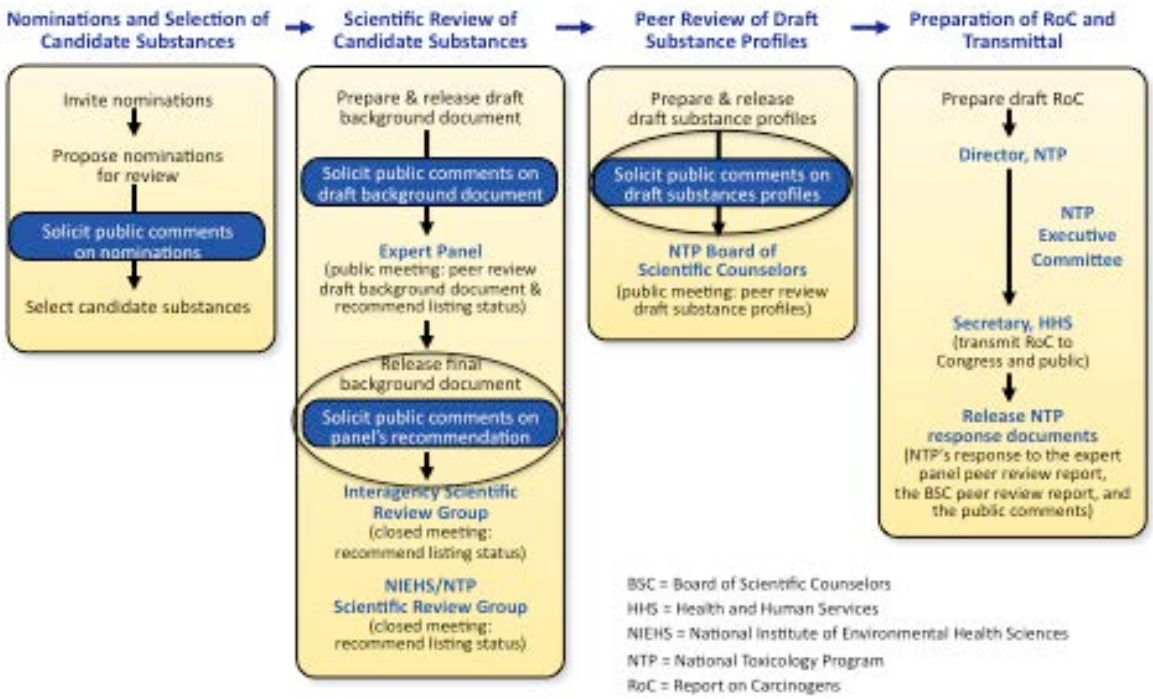
Introduction

The National Toxicology Program (NTP) followed a formal process for the review of candidate substances for the *Twelfth Report on Carcinogens* (*12th RoC*) that included multiple opportunities for public comments (see page 2 for a schematic of the review process).[1] The NTP shared the public comments[2] it received on candidate substances with the scientific review groups and also considered them in its hazard evaluation. As noted in the review process (part 4 in the schematic), the NTP prepares a report responding to written public comments received on candidate substances since issuance of the expert panel report (circled in the schematic) that is released at the time of publication of the *12th RoC*. However, the NTP also received comments for some substances, such as glass wool fibers, formaldehyde, and styrene, outside the designated public comment periods. In general, the public comments addressed scientific and technical comments (1) on the expert panel's recommendation for the listing status of a candidate substance and scientific justification for that recommendation and (2) on the draft substance profile[3] (parts 2 and 3 in the schematic).

This report, organized by candidate substances, includes a list of the organizations or persons submitting the public comments, scientific and technical issues raised in the public comments, and the NTP responses to those issues. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

[1] For a description of the NTP–RoC Review Process, see http://ntp.niehs.nih.gov/go/15208.
[2] To download the public comments submitted for candidate substances for the *12th RoC*, see http://ntp.niehs.nih.gov/go/9920; select candidate substance of interest.
[3] A draft substance profile provides the preliminary listing recommendation for a substance; the carcinogenicity studies that support the recommendation; information on human exposure, including data on use, production and occupational and environmental exposure; and current Federal regulations to limit exposure.

NTP Report on Carcinogens Review Process



Aristolochic Acids

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of aristolochic acids in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, April 30, 2008, 73 FR 23463)[4] and on the draft substance profile (December 22, 2008, 73 FR 78364).[5] The NTP received 1 public comment on aristolochic acids[6] since issuance of the expert panel report (see table). The relevant issues in the public comments and the NTP responses to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter
Expert Panel Report	0	
Draft Substance Profile/ February 2009	1	Arthur P. Grollman, M.D. Stony Brook University

NTP Response to Issues Raised by the Public Comments

- The substance profile should mention that exposure to aristolochic acids is a global human health concern: "AAN [aristolochic acid nephropathy] and its associated upper urothelial cancer represent a long-overlooked iatrogenic disease and an international public health problem of considerable magnitude (Grollman, February 11, 2009)."

  NTP Response: The profile discusses the sources of exposure to aristolochic acids (e.g., herbal remedies from either intentional or inadvertent use) and provides data on global exposure. Information on U.S. exposure is emphasized because that is the mandate of the Report on Carcinogens. It is outside the scope of a substance profile to provide opinion on the magnitude of the health problem from a specific exposure.

[4] See http://ntp.niehs.nih.gov/go/29682 (select "Aristolochic Acids and Riddelliine") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[5] See http://ntp.niehs.nih.gov/go/9741 (select "February 24, 2009") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[6] To download the public comments, see http://ntp.niehs.nih.gov/go/9920 (select "Aristolochic Acids").

Captafol

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of captafol in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, March 10, 2008, 72 FR 41755)[7] and on the draft substance profile (December 22, 2008, 73 FR 78364).[8] The NTP received no relevant public comments for response since issuance of the Expert Panel Report on Captafol.

[7] See http://ntp.niehs.nih.gov/go/29682 (select "Captafol and *ortho*–Nitrotoluene") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[8] See http://ntp.niehs.nih.gov/go/9741 (select "February 24, 2009") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").

Cobalt–Tungsten Carbide: Powders and Hard Metals

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of cobalt–tungsten carbide: powders and hard metals (hereafter referred to as cobalt–tungsten carbide) in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, February 11, 2009, 73 FR 60288)[9] and on the draft substance profile (April 22, 2010, 75 FR 21003),[10] The NTP received 4 public comments[11] on cobalt–tungsten carbide since issuance of the expert panel report (see table). The relevant issues in the public comments and the NTP responses to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter or Sponsored
Expert Panel Report/ March 2009	3	International Tungsten Industry Association (ITIA) (Michael Maby) Comments prepared by ARCADIS (Katherine Heim and Michael Pardus)
		U.S. Dept. of Defense (DOD) (Shannon Cunniff)
March 2010		Kennametal (Phil Weihl)
Draft Substance Profile/ June 2010	1	ITIA (Michael Maby)

NTP Response to Issues Raised by the Public Comments

Scope of the Nomination

The public comments (ITIA and Kennametal) conclude that the scope of the nomination should be specific for sintered hard metals. Specifically, they stated that:

- The hard metals of cobalt-tungsten carbide have a wide range of compositions that will likely affect toxicology and carcinogenicity, and most of the "*in vivo* and *in vitro* data almost exclusively cover unsintered cobalt-tungsten carbides with a narrow range of constituents."

  NTP Response: There are no toxicological data available that suggest differences between sintered and unsintered cobalt–tungsten carbide. Studies have shown that both materials (sintered and unsintered) release similar amounts of cobalt ions in artificial fluids

[9] See http://ntp.niehs.nih.gov/go/29682 (select "Cobalt–Tungsten Carbide Powders and Hard Metals") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[10] See http://ntp.niehs.nih.gov/go/9741 (select "June 21–22, 2010") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[11] To download the public comments, see http://ntp.niehs.nih.gov/go/9920 (select "Cobalt–Tungsten Carbide: Powders and Hard Metals").

(Stopford *et al.* 2003), and that both cause cytotoxicity and pathological changes in the lungs of rats and cytotoxicity and toxicity in experimental animals (Adamis *et al.* 1997).

- No increased risk of lung cancer from exposure to sintered materials was observed in the human epidemiological studies.

  NTP Response: The epidemiological evidence is inadequate to differentiate risks from sintered and unsintered cobalt–tungsten carbide. The effect estimates specific for the sintered and unsintered cobalt–tungsten carbide were imprecise, and it is not possible to determine from these studies whether the suggested differences in risk are due to differences in the levels of exposure or differences in the chemical and physical properties of the materials. In industrial settings, exposure levels to cobalt–tungsten carbide are higher before sintering than after sintering.

Human Cancer Studies

The public comments conclude that the "epidemiological data for listing are limited, weak, and inclusive" (ITIA and Kennametal) and "too weak for positive causal interpretation" (DOD). Specific comments followed by the NTP response to the comment are discussed below.

- The overall findings of the two multi-plant studies (Hogstedt and Alexandersson [1990] and Moulin *et al.* [1998]) are not statistically significant or just reach the threshold of significance and are weak (SMRs are ~1.3 for both studies).

  NTP Response: Overall findings from both the two multi-plant studies, as well as the studies of individual factories (Wild *et al.* 2000 and Lasfargues *et al.* 1994) within the French multi-plant study (Moulin *et al.* 1998), reported consistent increased risks of lung cancer among cobalt–tungsten carbide-exposed workers. The magnitude of the risk estimates in all studies increased when restricted to workers with the highest exposure. For example, risk estimates for individuals in the highest exposure categories were between 2 and 4 fold in the studies by Wild *et al.* (2000) and Moulin *et al.* (1998). The lower magnitudes observed in the overall populations are because they include workers with little or no exposure in addition to workers with moderate or high exposure.

- The data from the Moulin *et al.* study do not show clear exposure-response relationships; findings were only statistically significant for one of the sub-categories of the four exposure metrics (levels, duration, unweighted cumulative dose and weighted cumulative dose), and the unweighted cumulative estimates of exposure are flawed.

  NTP Response: The exposure-response relationships in the study by Moulin *et al.* were significant for two of the exposure metrics – duration ($P_{trend} = 0.03$) and unweighted cumulative dose ($P_{trend} = 0.01$) – and approached statistical significance for the other two metrics ($P_{trend} = 0.08$). In general, misclassification of exposure would bias towards the null hypothesis rather than cause a false positive.

- Several remarks were made concerning confounding from smoking or exposure to other occupational carcinogens. They criticized the use of "ever" vs. "never" to assess smoking and noted that the OR [Odds Ratio] for smoking was small in the Moulin *et al.* study.

  NTP Response: The NTP concurs that the use of "ever" vs. "never" is a less precise method for measuring smoking and the small OR for smoking in the Moulin *et al.* study

indicates some misclassification of smoking. Nevertheless, smoking is not considered to be a confounder in this study, because (1) the smoking-adjusted odds ratio for cobalt–tungsten carbide exposure (OR = 2.6, 95% CI = 1.16 to 5.82) was similar to the unadjusted risk (OR = 2.29, 95% CI = 1.08 to 4.88), (2) no increased risks of smoking-related diseases, such as chronic bronchitis and emphysema, were found, and (3) adjustment for smoking or exposure to other occupational carcinogens did not change the findings in the exposure-response analyses (Moulin *et al.* 1998).

Toxicology and Mechanistic Studies

The public comments (ITIA) conclude that the "genotoxicity and carcinogenicity data are primarily for soluble cobalt and tungsten compounds and do not support the listing of cobalt–tungsten carbide powders and hard metals." Specific comments followed by the NTP response to the comment are discussed below.

- Cobalt–tungsten carbide has unique properties and release rates of cobalt from metal may not occur at the same rate as water-soluble compounds. There are no long-term chronic studies in experimental animals on cobalt–tungsten carbide.

  NTP Response: As stated above, both sintered and unsintered cobalt–tungsten carbide release similar amounts of cobalt ions in artificial fluids (Stopford *et al.* 2003).

- The NTP did not consider the NTP sub-chronic study (13 weeks) on cobalt–tungsten carbide.

  NTP Response: As per the NTP review process,[12] the NTP uses data from the publicly available peer-reviewed literature. The 13-week study conducted by Brookhaven National Laboratories on cobalt–tungsten carbide is not peer-reviewed or publicly available. The NTP does not believe that this represents a deficiency in the scientific review, because 13-week studies are generally not informative for evaluating carcinogenicity in rodents.

"Studies In Progress"

The public comments (ITIA and Kennametal) state that there is ongoing research (listed below) that will address data gaps, and the NTP should defer its review until the data from these studies are available.

- University of Pittsburgh epidemiology study of hard-metal workers.
- The NTP sub–chronic and chronic drinking water studies for tungsten.
- The NTP two–year chronic inhalation study for cobalt metal powder.

NTP Response: The NTP believes that the available evidence clearly meets the criteria for listing of cobalt–tungsten carbide: powders and hard metals in the *12th RoC*.

[12]See http://ntp.niehs.nih.gov/go/29353, Scientific Review of Candidate Substances

References

Adamis Z, Tatrai E, Honma K, Karpati J, Ungvary G. 1997. A study on lung toxicity of respirable hard metal dusts in rats. *Ann Occup Hyg* 41(5): 515-526.

Hogstedt C, Alexandersson R. 1990. Dödsorsaker hos Hardmetallarbetare. *Arbete och Hälsa* 21: 1-26.

Lasfargues G, Wild P, Moulin JJ, Hammon B, Rosmorduc B, Rondeau du Noyer C, Lavandier M, Moline J. 1994. Lung cancer mortality in a French cohort of hard-metal workers. *Am J Ind Med* 26(5): 585-595.

Moulin JJ, Wild P, Romazini S, Lasfargues G, Peltier A, Bozec C, Deguerry P, Pellet F, Perdrix A. 1998. Lung cancer risk in hard-metal workers. *Am J Epidemiol* 148(3): 241-248.

Stopford W, Turner J, Cappellini D, Brock T. 2003. Bioaccessibility testing of cobalt compounds. *J Environ Monit* 5(4): 675-680.

Wild P, Perdrix A, Romazini S, Moulin JJ, Pellet F. 2000. Lung cancer mortality in a site producing hard metals. *Occup Environ Med* 57(8): 568-573.

Formaldehyde

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of formaldehyde in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, February 11, 2009, 74 FR 44845)[13] and on the draft substance profile (April 22, 2010, 75 FR 21003).[14] The NTP received 20 public comments[15] on formaldehyde since issuance of the expert panel report (see table). The relevant issues in the public comments and the NTP responses to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
Expert Panel Report/ December 2009 to February 2010	14	Public Citizens–4 independent comments Jean Public, Bob Kerr, Mr. Stachowski, Evan Craig
		Office of Environmental Health Hazard Assessment, CA EPA (Joan Denton)
		Scientists from academia or research institutes 4 independent comments Drs. Gary Marsh (University of Pittsburg), James Swenberg (University of North Carolina), Kenneth Sloan (University of Florida), Mel Anderson (Hamner Institutes of Health Sciences)
		Personal Care Products Council (John Bailey)
		Georgia-Pacific, LLC (Traylor Champion)
		Hexion Specialty Chemicals, Inc. (2) Comments prepared by ENVIRON International Corporation (Joseph Rodricks, Annette Shipp, Robinan Gentry and Duncan Turnbull)
		Formaldehyde Council, Inc. (FCI) (Betsy Natz)
Draft Substance Profile/ May to June 2010	4	Formaldehyde Council, Inc. (Betsy Natz)
		Hexion Specialty Chemicals, Inc. (3) Comments prepared by ENVIRON International Corporation

[13] See http://ntp.niehs.nih.gov/go/29682 (select "Formaldehyde") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[14] See http://ntp.niehs.nih.gov/go/9741 (select "June 21–22, 2010") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[15] To download the public comments, see http://ntp.niehs.nih.gov/go/9920 (select "Formaldehyde").

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
Other Comments/ November 12, 2010	2	Momentive Specialty Chemicals Comments prepared by ENVIRON International Corporation and scientific experts including: Drs. Kenneth Mundt, Philip Cole, Richard Irons, Gary Marsh, and Jack Mandel
April 22, 2011		American Chemistry Council Comments prepared by ENVIRON International Corporation and scientific experts including: Drs. Richard Albertini, Philip Cole, Richard Irons, Jack Mandel, Gary Marsh, James Swenberg, and Michael Thirman

NTP Response to Issues Raised by the Public Comments

Lymphohematopoietic Cancer (LHC), Primarily Myeloid Leukemia

Human Cancer Studies

Some of the comments opine that the epidemiological data are not sufficient to support a causal relationship between formaldehyde exposure and myeloid leukemia. The NTP responses are grouped by scientific issues raised in the comments.

- Three of the four major studies (Coggon *et al.* 2003, Pinkerton *et al.* 2004, and Beane Freeman *et al.* 2009) relied on by the NTP did not find a statistical association or dose-response relationship between formaldehyde exposure and myeloid leukemia. The findings from these large cohorts (greater than 50,000 workers) outweigh the results of the fourth study (Hauptmann *et al.* 2009).

  NTP Response: Statistically significant risk estimates for myeloid leukemia were found among workers with the highest indices of exposure in all three of the major studies that specifically evaluated myeloid leukemia. With respect to the National Cancer Institute (NCI) cohort study, a statistically significant association with myeloid leukemia was found for peak exposure; the relative risk (RR) among workers with the highest peak exposure (compared with the lowest peak exposure) was RR = 2.79 (95% CI = 1.08 to 7.21) and a significant dose-response relationship (P_{trend} = 0.02) was found with peak exposure in the 1994 update (Beane Freeman *et al.* 2009). In the 2004 follow-up study, elevated risk estimates were still observed, but the magnitude of the association between formaldehyde exposure and myeloid leukemia decreased as time since the last known exposure increased to at least 24 years. As stated in the draft substance profile, this type of pattern is consistent with a follow-up period longer than the optimal latency period for cancer, as has been seen with other leukemia-inducing agents (Silver *et al.* 2002). With respect to the NIOSH study (Pinkerton *et al.* 2004), statistically significant risks for myeloid leukemia were observed in analyses that included all causes of death listed on the death certificate (rather than just the underlying cause) among workers who had been exposed for at least 10 years (standard mortality ratio [SMR] = 2.24, 95% CI = 1.02 to

4.25, 9 deaths). The study by Coggon *et al.* (2003) did not report risk estimates specifically for myeloid leukemia.

- There is no excess risk of leukemia when the combined observed deaths are compared to the combined expected deaths in the four major studies. The NTP also did not consider the meta-analyses results and relied on just four studies. Meta-analyses show no consistent or collective effect regarding leukemia. Excess risk only found among "low-exposure" industries such as embalmers and pathologist and anatomists. The Zhang *et al.* 2009 analyses combined different measures of exposure (such as peak, average) across studies. They also criticize NTP for not using the findings from the Bachand *et al.* (2009) meta-analysis.

  NTP Response: As discussed above, the excess risks for myeloid leukemia in the major studies were found among workers with the highest exposure. The combined analyses (comparing total observed vs. total expected deaths) or meta-analyses (such as Bachand *et al.* 2009) using ever exposure (that is, combining the data from individuals with low and high exposure into a single exposure group) are not very sensitive for detecting a real association. Recently,[16] Schwilk *et al.* (2010) published a meta-analysis, which is the only meta-analysis to date that includes the findings from Hauptmann *et al.* (2009) and uses risk estimates (similar to the earlier meta-analysis reported by this research group [Zhang *et al*. 2009]) for workers with the highest measures of exposure. The exposure metric chosen for the study was determined *a priori.* They found a RR of 1.53 (95% CI = 1.18 to 1.98) for leukemia and 2.47 (95% CI = 1.57 to 3.86) for myeloid leukemia.

  The NTP disagrees with the statement that embalmers and pathologists represent a low-exposure group. Hauptmann *et al.* (2009) stated that embalmers in their study tended to have longer duration of exposure, higher cumulative exposure, and were more likely to be exposed to peak exposure levels greater than 4 ppm than industrial workers.

- Comments expressing concerns about the NCI Cohort of Industrial Workers (Hauptmann *et al.* 2003, Beane Freeman *et al.* 2009) are as follows:
  - The 2003 publication missed over 1000 deaths among cohort members with proportionally more deaths in the unexposed groups.

    NTP Response: Although the statement about the missed deaths (all causes) is accurate, the NTP believes the NCI study is well conducted and one of the most informative studies because it uses internal analyses (using the low-exposure group as the referent) to evaluate exposure-response relationships and is large in size. The authors (Beane Freeman *et al.* 2009) stated that they do not believe the missed deaths are due to a systematic liberalization of matching criteria, because there was no apparent change in National Death Index (NDI) procedures for assigning matches, and the matching scores provided by NDI for those 1006 deaths not included in the previous analyses were not different from those that were included. The re-analyses using the additional deaths did not substantively change the findings for lymphohematopoietic cancers. The NTP used the risk estimates (provided in the

[16] Since the draft substance profile was peer reviewed at the June 21-22, 2010 NTP Board of Scientific Counselors.

comment above) based on the "corrected data," which include the missing deaths in their evaluation.

- The exposure-response associations (primarily peak exposure) for leukemia originally reported by Hauptmann *et al.* (2003) are due to statistically significant deficits in deaths among the low-exposed (peak exposure) and unexposed subgroups and were found using SMR analyses (Marsh and Youk 2004).

  NTP Response: Internal analyses, which use unexposed or low-exposure groups (the low-exposure group was used as the referent in the NCI study), are more informative than SMR analyses, which use the general population as the reference group, because internal analyses help reduce potential confounding and "healthy worker effects." Deficits observed in the low-exposure group may be because workers in general are healthier than the general public because the general public includes individuals who cannot work as a result of health conditions including cancer. In addition, the Marsh and Youk (2004) reanalysis is no longer relevant to the evaluation because it uses the "uncorrected data," i.e., data that did not include additional cancer deaths that contributed to the missed 1006 deaths (see above) in the 1994 follow-up. SMRs for myeloid leukemia or leukemia using the "corrected data," (i.e., that include the 1006 additional deaths) for the 1994 follow-up were not identified in any publications for the low-peak-exposure group (reference group).

- Concerns about Hauptmann *et al.* (2009)
  - "The study evaluated deaths occurring between 1960 and 1986 as reported on death certificates, but as late as 1992 NCI Surveillance, Epidemiology and End results (SEER) Program did not report study results on cancer types being evaluated because SEER questioned the validity of these diagnoses as reported on death certificates." (FCI comments on the draft substance profile.)

    NTP Response: The case-control study by Hauptmann *et al.* is informative for evaluating cancer because it looks at exposure-response relationships using internal analyses and has adequate statistical power to detect effects. Mortality data from myeloid leukemia can be found in the SEER website from 1973 to 2007 (http://seer.cancer.gov/faststats/selections.php?series=cancer). Hauptmann *et al*. provided documentation that indicate that the identification of cases was conducted using acceptable epidemiological methods, e.g., they state that "deaths were coded for underlying and contributing cause of deaths according to the rules at the time of death and assigned codes according to the International Classification of Disease, Eighth Revision (ICD-8)."

  - Comments related to statistical analyses are as follows:
    - Statistical analyses were unreliable because there was only one unexposed myeloid leukemia case.
    - Incomplete or misleading interpretation of statistical analyses because the odds ratios were not tested for statistical significance; the tests for trend were calculated from continuous model analyses (Cole *et al*. 2010, letter to the editor).

- New proportional mortality ratio analysis does not find an association with myeloid leukemia (PMR = 108, 95% CI = 70 to 106, 29 deaths) (Cole *et al*. 2010, letter to the editor).

NTP Response: Effect estimates – although of high magnitude (estimates for myeloid leukemia and high indices of exposure were often greater than 10) and statistically significant – were imprecise because of the very small number (one case) of unexposed cases. Therefore, the authors conducted analyses that used individuals with < 500 embalmings as the reference group. These analyses improved the precision of the effect estimates, and statistically significant elevated risks for myeloid leukemia were found among individuals with the highest cumulative exposure to formaldehyde, that performed the largest number of embalmings, and that performed embalming for the greatest number of years. As expected, the magnitude of the risk estimates decreased using the larger reference group because this group included exposed (low exposure) individuals.

Concerning the statistical issues raised in the letter to the editor by Cole and colleagues, we find these issues were adequately addressed by the authors' response (Hauptmann *et al.* 2010). The tests for trends were based on the slope of the original continuous variable (Wald test), which is an accepted statistical method and was selected *a priori*. The new proportional mortality ratio (PMR) analysis is not informative; the internal analyses used by the study authors are generally accepted in the epidemiological community to be more useful for calculating relative risks. The calculated PMR is not consistent with the PMR reported in the literature for the three studies (Walrath and Fraumeni 1983, 1984, Hayes *et al.* 1990) whose populations were encompassed by the nested case-control study; each of those studies reported a PMR of ~ 1.5.

* Excess risk of myeloid leukemia could be due to another component in embalming.

NTP Response: In addition to finding an association between myeloid leukemia and embalming, the study also found associations with exposure to formaldehyde; an increased risk for myeloid leukemia was found among individuals with the highest level of cumulative exposure to formaldehyde (in analyses using individuals with < 500 embalmings as the reference group) and positive exposure-response relationships (trends) were found for peak (P_{trend} = 0.036) and average exposure to formaldehyde (P_{trend} = 0.058). The findings of increased risks of myeloid leukemia among industrial workers exposed to formaldehyde who are not exposed to embalming fluids argue against the hypothesis that other components in embalming caused the myeloid leukemia in embalmers. In addition, none of the components in embalming fluid (such as isopropanol, ethylene glycol, methanol, phenol, and glutaraldehyde) have identified leukemogenic properties.

* Myeloid leukemia cases and controls had almost the same estimated values for 8-hour time-weighted average and peak exposure.

NTP Response: The public comment is referring to data reported in Table 2 of Hauptmann *et al.* (2009). These data find similar 8-hour time-weighted average and peak exposure for myeloid cases and controls that embalmed (not all controls);

however, the percentage of individuals who embalmed was greater in cases than controls.

- There are important differences (earlier year of death, all white race, longer time employed, and age first employed) between the myeloid cases and controls and several time-dependent co-factors appear not to have been adequately considered or controlled in the analyses. For example, myeloid cases were more likely (50%) to have begun employment in the funeral industry prior to 1942, suggesting they were an older and earlier source population than the controls, which may explain why they performed more embalmings.

  NTP Response: The authors of the study state that "control subjects were stratified to be similar to the case subjects with respect to the data source, sex, and dates of birth and death (5 year intervals)," and the data provided in Table 1 support this statement. With respect to age, the mean age of death of controls was 64 compared with 65 for cases (Table 1). With respect to year of first employment, Table 1 does provide data suggesting that in general myeloid cases first worked in embalming at earlier calendar years than controls, although the authors do not state there were any significant differences between the cases and control. One of the main findings of the paper is that duration of working in jobs with embalmings is associated with myeloid cancer risk (P_{trend} = 0.020), and it is not clear why the calendar year(s) that the duration of embalming occurred is important. There do not appear to be data on whether formaldehyde levels were higher in earlier calendar years, but if this were the case, it would strengthen the association between formaldehyde exposure from embalming and myeloid leukemia risk.

- There were significant uncertainties in the estimates of exposure, information on exposure variables was missing for up to 35% to 45% of the subjects, and the data were imputed. Sensitivity analyses, excluding subjects with missing data for 30% of their work history, resulted in lower risk estimates, and the findings for peak exposure and myeloid were no longer significant.

  NTP Response: The completeness and quality of the data and the sensitivity analyses were adequately discussed in the original publication. Information on duration of working in jobs with embalming was available for all subjects, and reported work histories were available for 97% of the person-years from the first to the last reported job. However, information was often missing for one of several characteristics (such as spill frequency) used to calculate average, cumulative, and peak exposure to formaldehyde. The missing data did not differ substantially between case and controls. The author attributed the decrease in risk estimates in the sensitivity analyses to chance and small numbers. The specificity of the association with myeloid leukemia argues against a systematic bias in exposure assessment because that would likely affect all types of cancer.

- The following comments were on issues related to combined lymphohematopoietic cancers.
  - There is no biological basis for combining all lymphohematopoietic cancers since these diseases have different etiologies.

- In the NCI study, no association with average exposure with all LHC was observed when results were reanalyzed by Marsh and Youk (2004) or after 10 years of additional follow-up (Beane Freeman *et al.* 2009).

  NTP Response: Blood cells are derived from a common multipotential progenitor cell in the bone marrow. If a carcinogenic agent affects this progenitor cell, it may give rise to more than one type of lymphatic and/or hematopoietic cancer. In the case of formaldehyde, the mechanism of carcinogenicity is not known. Most of the epidemiological studies that evaluated subtypes of lymphohematopoietic cancers and formaldehyde found the strongest evidence for myeloid leukemia. Although increases have been reported for other types of lymphohematopoietic cancers such as Hodgkin's lymphoma and multiple myeloma (Beane Freeman *et al.* 2009), at this time a causal relationship has not been established. The comments are correct that there is not a statistically significant association with average exposure and all lymphohematopoietic cancers in the 2004-year update of the NCI cohort; however, a statistically significant association was found with peak exposure.

Toxicology and Mechanistic Studies

Some of the public comments question the plausibility of formaldehyde to cause leukemia. Comments and responses on similar topics are discussed together.

- The public comments state that "the vast majority of more credible data show essentially no reported adverse hematological effects in humans or animals following either oral or inhalation exposure to formaldehyde" (FCI, February 11, 2010 comments). Their specific comments are as follows:
  - No adverse effects on hematological parameters, dose-related lymphoma in lymph nodes, or myeloid leukemia were observed in rats chronically exposed to formaldehyde via drinking water (Til *et al.* 1989).
  - No adverse effects on red or white blood cell counts or on the bone marrow were observed in rats in a subchronic inhalation study (personal communication by Mel Anderson to FCI, February 11, 2010 comments), or in long-term inhalation studies (Appelman *et al*. 1988, Kamata *et al.* 1997, Kerns *et al.* 1983, Woustersen *et al.* 1987).
  - The Chinese studies reviewed by Tang *et al.* (2009) are questionable because it is not possible to know what additional exposures were present, and the study by Kuo *et al.* (1997) is not credible because the levels of formaldehyde were implausibly low.
  - Studies of accidental ingestion of large quantities of formaldehyde have not shown any effects on the blood or blood-forming organs (Eells *et al.* 1981, Freestone and Bentley 1989, Koppel *et al.* 1990).

  NTP Response: The NTP believes that there is credible data showing adverse hematological effects in humans following inhalation exposure to formaldehyde. Zhang *et al.* (2010) found that formaldehyde-exposed workers had lower counts of total white blood cells, granulocytes, platelets, red blood cells, and lymphocytes than did non-exposed workers. The authors stated that controlling for exposure to other agents did not change their findings. Although the review (Tang *et al.* 2009) of the Chinese studies has limited documentation about the original studies, the studies do provide supporting

information for the findings reported by Zhang *et al.* The review of eight studies of formaldehyde-exposed workers found decreased white blood cell counts in most studies, and exposed workers had higher percentages of blood abnormalities (decreased white blood cell and platelet counts and abnormal hemoglobin levels) than non-exposed workers in the largest study (Tang *et al.* 2009). The study by Kuo *et al*. (1997) (which was included in the review) found significant correlations of decreased white blood cells, airborne formaldehyde concentrations, and symptoms (such as nasal discharge) potentially related to formaldehyde exposure.

The NTP agrees with the statement that adverse hematological effects have not been reported in subchronic or chronic studies in the available studies in experimental animals, and this is acknowledged in the substance profile. However, increased proliferation and hyperplasia of nasopharyngeal lymphoid tissue (NALT), and lack of germinal center development in the superficial cervical lymph nodes were observed in rats exposed by inhalation to high doses of formaldehyde over a four-week period (Kuper *et al.* 2011). This study suggests that adverse effects on the rat lymphohematopoietic system occur with high-dose (15 ppm) exposure to formaldehyde. Although leukemia has not been reported in experimental rats exposed to formaldehyde by inhalation, the studies are limited. Only a small number of available studies comprehensively evaluated multiple tissue sites because earlier studies focused primarily on the nasal cavity.

- Several comments questioned whether formaldehyde could cause myeloid leukemia. They cite the following studies or comments in support of their opinion.
  - States that formaldehyde cannot reach distal sites and propose that exogenous formaldehyde would not increase endogenous levels of free formaldehyde. In addition, formaldehyde-DNA adducts were not observed at distal sites in rats exposed to formaldehyde by inhalation for 1 and 5 days; in contrast, formaldehyde-DNA adducts were observed in nasal tissue (Lu *et al.* 2010).

    NTP Response: The NTP acknowledges in the substance profile the arguments questioning the plausibility of formaldehyde causing leukemia (and other cancers at distal sites), including the recent study of formaldehyde-DNA adducts in experimental animals (Lu *et al.* 2010), and other issues related to the complex chemistry and biochemistry of formaldehyde. The NTP is also aware of the recent study that did not find formaldehyde-DNA adducts in the bone marrow of monkeys (Moeller *et al*. 2011). However, as stated in the substance profile, recent hypotheses and studies supporting the human epidemiological data finding of increased risks of myeloid leukemia among highly exposed workers have been advanced. Several mechanisms have been proposed for how formaldehyde could reach hematopoietic stem cells; exposure of stem cells to formaldehyde may occur through contact with circulating stem cells, through contact with nasal-associated lymphoid organs (NALT), or through contact with stem cells at sites distal to the exposure site, such as in the bone marrow (Zhang *et al.* 2009).

  - Questions the statement in the substance profile that formaldehyde may be transported to distal sites via its equilibrium with its hydrated form methanediol because (1) the equilibrium findings are based on tissue fixation (Fox 1985), which involves much higher than endogenous concentrations, and (2) it is unclear how the

equilibrium changes to release free formaldehyde at distal sites because the equilibrium constant for acetal (methanediol) vs. free formaldehyde is somewhere between 5,000 and 10,000. Also, the small amounts of formaldehyde, which are in equilibrium with methanediol and S-hydroxymethyl glutathione moving from contact site to distal sites, will have no appreciable influence on the total levels of endogenous formaldehyde at distal sites.

NTP response: In an aqueous solution formaldehyde is in equilibrium with methanediol; the equilibrium constant in solution at room temperature is approximately 1,000 with the majority of the chemical present as methanediol (approximately 5% by weight aqueous solution of formaldehyde is made up of 80% methanediol) (Mugnai *et al.* 2007, Kent *et al.* 2003). Since these chemicals are in equilibrium, as free formaldehyde levels are depleted, free formaldehyde is released from methanediol and the rate of release increases with temperature (Fox 1985). Although present in limited amounts, formaldehyde, a strong electrophile, can rapidly bind to macromolecules. Exogenous formaldehyde has a plasma half-life of approximately 1.5 minutes and readily binds cellular macromolecules and glutathione, a ubiquitous cellular antioxidant. Glutathione concentrations in liver tissue significantly decrease after inhalation exposure to formaldehyde (Söğut *et al.* 2004). The metabolism of formaldehyde is dependent upon the intracellular concentration of glutathione, and high levels of formaldehyde have been shown to overwhelm this antioxidant pathway and increase formaldehyde toxicity without increasing blood levels of free formaldehyde (Bolt 1987).

* States that toxicity noted at distal sites in experimental animals is not related to the potential for formaldehyde to cause myeloid leukemia because the exposures were high in these studies (5 to 20 ppm).

  NTP Response: Workers in the highest peak exposure groups were exposed to > 4 ppm formaldehyde in the NCI cohort of industrial workers (Beane Freeman *et al.* 2009), and to > 9 ppm in the nested case-control study of embalmers. These levels are in the range of 5 to 20 ppm stated by the comment.

* States that the higher formaldehyde-albumin adducts observed among individuals with higher exposure to formaldehyde do not provide direct evidence that formaldehyde enters the blood stream since the assay measuring adducts does not distinguish between endogenous and exogenous adducts. Another limitation of this study is the low levels of formaldehyde (Pala *et al.* 2008).

  NTP Response: The study by Pala *et al.* (2008) despite having low statistical power because of its small size, found that individuals with higher exposure to formaldehyde had higher levels of hemoglobin adducts compared with individuals with low exposure. The effects remained after adjusting for sex, age, and exposure to paints.

* The proposed indirect methods (Zhang *et al.* 2009) by which formaldehyde could cause leukemia require additional studies to determine whether they are biologically plausible. Endogenous concentrations of formaldehyde in the blood do not increase after exogenous exposure, thus it is not plausible that exogenous exposure would result in adverse effects (Heck *et al.* 1985). In addition, the study by Murrell *et al.*

(2005) does not provide evidence that olfactory stem cells can be transported back to the bone marrow and repopulate the bone marrow in a normal biological setting since this study involved repopulating the bone marrow after the destruction of normal progenitor tissues (from treatment with radiation). In addition, Neuss *et al.* (2010) reported that formaldehyde acts on cells at first contact but is not released from these cells and able to damage other cells.

NTP Response: The findings of genetic damage in nasal tissue and lymphocytes of formaldehyde-exposed workers provide some support for the hypotheses by Zhang *et al.* (2009) concerning damage to circulating stem cells.

- The following comments were on genotoxicity studies:
  - Criticizes the study by Zhang *et al.* 2010 showing aneuploidy in human chromosomes 7 and 8 in myeloid progenitor cells of formaldehyde-exposed workers because (1) these chromosomes are minimally related to leukemia, and (2) the methods to evaluate them are not credible.

    NTP Response: As stated in the substance profile, the associations of monosomy 7 and trisomy 8 with myeloid leukemia are reported in the published literature (Johnson and Cotter 1997, Paulsson and Johansson 2007). Speit and colleagues raised concerns about this study in a letter to the editor, which were addressed by the study authors (Speit *et al.* 2010). The methods used in the Zhang *et al.* (2010) study are similar to methods published in the peer-reviewed literature: (1) the method to evaluate chromosomal aneuploidy, OctoChrome FISH, was used in a study evaluating aneuploidy (including monosomy 7 and trisomy 8) in lymphocytes of benzene-exposed workers (Zhang *et al.* 2005), and (2) methods used to culture myeloid progenitor cells were also used in a study evaluating hematotoxicity in workers exposed to low levels of benzene (Lan *et al.* 2004). In addition, a similar strategy to measure chromosomal damage in hematopoietic stem cells from exposed people to radiation has been reported (Kreja *et al*. 1999).

  - The relevance of cytogenetic effects in circulating lymphocytes to the mechanism of myeloid leukemia has not been demonstrated. While some studies have been positive, others did not observe differences between formaldehyde-exposed and control subjects. The evaluation of studies needs to consider the effects from potential confounders, such as genetic or environmental (diet, exposure to other compounds) factors. DNA-protein cross-links are rapidly repaired and thus it is improbable that they would accumulate in lymphocytes after inhalation exposure in sufficient amounts to cause measurable effects (Schmid and Speit 2007).

    NTP Response: There is consistent evidence that formaldehyde causes increased micronuclei formation in the lymphocytes (positive findings in 5 of 7 studies), nasal epithelium (positive findings in all 5 studies), and buccal epithelium (positive findings in 4 of 5 studies) from formaldehyde-exposed subjects. The consistent findings among different types of subjects (e.g., industrial workers or laboratory workers) argue against attributing increases in myeloid leukemia to confounding. Moreover, in most studies, exposed subjects were matched to controls on variables such as age, sex, smoking, and diet. As discussed above, these findings provide support for the hypotheses proposed by Zhang *et al.* (2009).

- Criticizes citing the study by Bonassi *et al.* 2008, which found that chromosomal aberrations (CA) are associated with cancer risks, because the only specific site statistically associated with CA was stomach cancer.

  NTP Response: The large, multi-country pooled cohort study published by Bonassi *et al.* (2008) found that CA frequency measured in peripheral blood lymphocytes of healthy subjects is associated with all cancer risk, which supports mechanistic studies linking CA to early stages of cancer. Elevated, non-statistically significant risks for other cancers, including lymphohematopoietic cancers, were found among individuals with medium or high levels of CA. Increased micronuclei formation, which is clearly associated with formaldehyde exposure, also was found to predict cancer risk in humans (Bonassi *et al.* 2007).

- Speit *et al.* (2009) did not report systemic genotoxic effects in experimental animals exposed to formaldehyde by inhalation; this study and other studies by Speit and colleagues were not cited in the substance profile.

  NTP Response: The negative genotoxic findings in experimental animals reported by Speit and colleagues are acknowledged in the substance profile, but are cited using a secondary source, the NTP background document (NTP 2010), rather than from the primary literature.

Nasopharyngeal Cancer

Human Cancer Studies

- Five of the nine deaths from nasopharyngeal cancer in the NCI cohort study, which was the only informative cohort study for evaluating nasopharyngeal cancer, occurred in Plant 1 (Hauptmann *et al.* 2004). Marsh *et al.* (2007) provided evidence that the observed mortality from nasopharyngeal cancer may not be due to formaldehyde exposure because five of the six cases had previously worked in occupations with exposures, such as sulfuric acid mists and metal dust, that are potential risk factors for upper respiratory cancer.

  NTP Response: In the NCI cohort study, formaldehyde exposure was significantly correlated with an increased risk of nasopharyngeal cancer. Relative risk increased with increasing cumulative exposure ($P_{trend} = 0.025$ across exposed subjects), peak exposure ($P_{trend} < 0.001$), and average exposure ($P_{trend} = 0.066$) (Hauptmann *et al*. 2004). Of the 7 exposed workers who died of nasopharyngeal cancer, all were in the highest peak-exposure category, and 6 were in the highest average-exposure category. Plant 1 was the plant with the largest numbers of workers in the highest formaldehyde-exposure category; 46% of workers at Plant 1 were in the highest peak-exposure category, compared with 20.1% of workers in all other plants (Stewart *et al.* 1990, Marsh and Youk 2005). As mentioned in the comments, a nested case-control study of nasopharyngeal cancer among workers in Plant 1 found a significantly elevated risk for ever having worked in silversmithing jobs before or after employment at Plant 1; however, silversmithing was not correlated with formaldehyde exposure levels at this plant, and there no studies have been identified linking silversmithing and cancer.

Toxicology and Mechanistic Studies

- Nasal tumors only occurred in experimental animals at formaldehyde concentrations that are sufficient to produce cytotoxicity and regenerative proliferation (greater than 6 ppm) and that are in excess of tolerable irritant levels in humans. It is unlikely that anyone could be exposed to high concentrations where nasopharyngeal cancer effects in experimental animals have been observed.
- There is no evidence that genotoxicity is initially involved early in the tumorigenic process, and genetic changes are secondary to formaldehyde-induced cytotoxicity, metaplasia, and hyperplasia. Meng *et al.* (2010) reported that exposure to formaldehyde for 13 weeks caused cell proliferation, but did not increase *p53* mutations. In addition, studies by Andersen *et al*. (2008) showed that significant changes in genes do not occur at concentrations of 2 ppm or less.

  NTP Response: In humans, excess risks of nasopharyngeal cancer have been found among workers exposed to formaldehyde. In the NCI cohort study of industrial workers, exposure-response relationships were observed for average, cumulative, and peak exposures. The median exposure levels for the 10 plants ranged from 0.1 to 3.5 ppm (Stewart *et al.* 1990) although higher levels of peak exposure occurred; the highest category of peak exposure was ≥ 4 ppm (Hauptmann *et al.* 2004). In a case-control study, an excess of nasopharyngeal cancer was found among workers with cumulative exposure of > 1.0 ppm-year (Vaughan *et al.* 2002). Formaldehyde has been shown to cause genetic damage (micronuclei) in nasal tissue of people without cancer (Ye *et al.* 2005, Burgaz *et al.* 2001, Ying *et al.* 1997, Ballarin *et al.* 1992, Titenko *et al.* 1996); exposure levels in these studies ranged from < 1 ppm to 4 ppm.

  Studies evaluating formaldehyde-induced nasal tumors in rats have identified genotoxicity and cytotoxicity as important mechanisms, which may or may not be independent of each other. In rats, DNA crosslinks were found to correlate with tumor incidence (Liteplo and Meek 2003), and N^2-hydroxymethyl-deoxyguanosine (dG) DNA monoadducts were found in the nasal mucosa in short-term studies (Lu *et al.* 2010). Formaldehyde causes genetic damage in rodents; inhalation of formaldehyde caused dominant lethal mutation in rats (Kitaeva *et al.* 1990) and heritable mutations in mice (Liu *et al.* 2009).

Sinonasal Cancer

- The meta-analysis did not find an increased risk in the cohort studies (Bosetti *et al*. 2008, Collins *et al.* 1997), and no statistically significant risk was found in the NCI cohort studies of industrial workers (Hauptmann *et al.* 2004).

  NTP response: Cohort studies, including the NCI study, do not have adequate statistical power to detect statistically significant risks for rare cancers such as sinonasal cancer. The meta-analyses of case-control studies by Collins *et al.* found a statistically significant risk for sinonasal cancer (mRR = 2.9, 95% CI = 2.2 to 4.0, 582 deaths). Although the meta-analysis was limited in its ability to separate the effects of formaldehyde from exposure to wood dust, several individual studies have found increased risk of sinonasal cancer associated with formaldehyde exposure among individuals with little or no

exposure to wood dust or after adjustment for wood-dust exposure (Olsen *et al.* 1984, Hayes *et al.* 1986, Olsen and Asnaes 1986).

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Certain Glass Wool Fibers (Inhalable)

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of glass wool fibers in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, 74 FR 67883)[17] and comments to the draft substance profile (April 22, 2010, 75 FR 21003).[18] The NTP received eleven public comments[19] on glass wool fibers since issuance of the expert panel report (see table). The relevant issues in the public comments and the NTP responses to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
Expert Panel Report/ September 28, 2009	3	North American Insulation Manufacturers Association (NAIMA) (Angus Crane) Unifax I LLC (Dean Venturin) Johns Manville (Bruce Ray) Also includes comments prepared by Bruce Ray and John Bauer
Draft Substance Profile/ May to June 2010	8	NAIMA (6 comments) Submitted by Angus Crane (2) Prepared by John Hadley Prepared by Kenneth Donaldson Prepared by Thomas Hesterberg Prepared by Toxicology and Human Health Risk Analysis (Roger McClellen) and Hahn Consulting (Fletcher Hahn) Owens Corning Science and Technology Center (John Hadley) Johns Manville (Bruce Ray)

NTP Response to Issues Raised by the Public Comments

Scope of the Nomination

Some of the public comments criticized the proposed listing, glass wool fibers (respirable as a class), in the draft substance profile. Specifically, they stated:

[17] See http://ntp.niehs.nih.gov/go/29682 (select "Glass Wool Fibers") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[18] See http://ntp.niehs.nih.gov/go/9741 (select "June 21–22, 2010") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[19] To download the public comments, see http://ntp.niehs.nih.gov/go/9920 (select "Glass Wool Fibers").

- Classifying all glass wool fibers as carcinogenic is scientifically incorrect because biosoluble fibers do not have the same hazard as biodurable fibers.

  NTP Response: NTP concurs that biosolubility and biodurablility (i.e., biopersistence) are important factors in predicting carcinogenicity and has revised the title of the listing to "certain glass wool fibers (inhalable)" to take into account that not all glass wool fibers are potential carcinogens. The revised profile states that evidence from studies of fiber properties indicates that only certain fibers — specifically, fibers that are biopersistent in the lung or tracheobronchial region — are reasonably anticipated to be human carcinogens. However, the physical chemical properties that predict fiber biopersistence and carcinogenicity are not fully established.

- Glass wool fibers should be distinguished into two categories of biological potency based on their *in vitro* solubility as measured by the dissolution rate K_{dis}.

  NTP Response: Although K_{dis}, the *in vitro* dissolution rate of a fiber, is a useful parameter for designing fibers and is an important determinant of fiber clearance, there are limitations in its use for predicting carcinogenicity. Dissolution rate is a measure of the biosolubility of a fiber and does not account for the removal of fibers from the lungs by macrophages, the deposition of fibers within the respiratory tract, or species differences in clearance rate (Bellmann *et al.* 2010). Further, the dissolution rate, K_{dis}, has not been standardized nor has it been recognized by regulatory agencies in the United States or European Union.

- The experimental animal data for insulation wools are insufficient to support the RoC listing.

  NTP Response: The listing is not based on use of the material but on physical and chemical composition — although used for different purposes, both insulation and special-purpose wools are glass wools and there is overlap in the size and chemical composition of these fibers. Commercial products are produced in bulk and have a range of fiber dimensions. The listing of certain (e.g., more biopersistent fibers) glass wool fibers (inhalable) is supported by studies showing that these fibers caused tumors by multiple routes (inhalation, intratracheal instillation, intrathoracic implantation, and intraperitoneal injection) of exposure, in multiple species (rats and hamsters), and at multiple tissues sites (such as lung, mesothelium and the lymphohematopoietic cells).

Cancer Studies in Experimental Animals

- Reporting studies by authors who used non-physiologic routes of administration as equally informative as the chronic inhalation data is misleading. There is a broad consensus among fiber toxicity experts that a much greater weight should be given to more recent, quality chronic inhalation studies than studies using non-physiologic routes of administration.

  NTP Response: In general, chronic inhalation studies are more informative than non-physiologic routes of administration for risk assessment; however, the latter studies are informative for identifying hazards, which is the objective of the Report on Carcinogens. Intracavity administration (such as intraperitoneal injection) of fibers provides information on biodurability and hazard assessment; hazard ranking of fibers by

intraperitoneal injection has been shown to be equivalent to that observed by inhalation (Bernstein 2007). Furthermore, fibers can migrate to the pleural and peritoneal cavities from the lung upon inhalation, so these areas can be exposed through a physiologic route.

- In the Mitchell *et al.* (1986) and Moorman *et al.* (1988) reports, the lung burden of fibers in the rats exposed by inhalation to insulation wool was the same as the lung burden in the control animals. Therefore, these data do not support a conclusion that insulation wool exposure caused the observed increase in mononuclear-cell leukemia. Further, the statistical tests used show an absence of significance based on the FDA Guidance (2001), which is $P < 0.01$ for pairwise comparison and $P < 0.05$ for trend test; the guidance also states that the effects in males and females should be evaluated independently.

  NTP Response: Mitchell *et al*. (1986) notes that "fibrous glass-related changes were found in the lungs and thymic and tracheobronchial lymph nodes," which is evidence that insulation fibers were inhaled. Furthermore, there was translocation of fibers to pulmonary lymph nodes in the insulation wool treatment group. Results of tests for lung burden noted in the above comment are from the final unpublished report from Battelle Laboratories; this test measures representative amounts of fiber in the lower respiratory tract. Information provided in the Battelle report was not cited, because per the RoC process (http://ntp.niehs.nih.gov/go/29353) only peer-reviewed data can be used in the evaluation. Data from males and females were combined because their control rates for mononuclear-cell leukemia were similar. The combined incidence of leukemia (males plus females) was significant for insulation wool by Fisher's exact test ($P = 0.0095$) and chi-square test ($P = 0.0127$) as calculated by NTP. According to the authors, the combined incidence (males plus females) of mononuclear-cell leukemia was significant using chi-square analysis ($P < 0.05$) with respect to the concurrent controls, and they noted that the incidences in those controls were "essentially the same" as those observed in historical controls.

- A special-purpose fiber used in the Mitchell *et al*. (1986) study was mislabeled as an insulation wool fiber in the draft substance profile.

  NTP Response: This error was corrected on the draft profile (see glass wool fibers, draft substance profile revised 6/4/10 at http://ntp.niehs.nih.gov/go/10091).

Fibers Properties Related to Carcinogenicity

There were several comments related to the discussion of *in vitro* fiber dissolution rate (K_{dis}) in the section of the draft substance profile titled, "Fiber Properties Related to Carcinogenicity."

- The Miller *et al. (*1999a) paper reported that the incidence of mesothelioma in rats exposed by intraperitoneal injection was higher for the insulation fiber than for the special-purpose fiber, although the K_{dis} for the insulation fiber was greater than for the special-purpose fiber. However, the dose in the intraperitoneal study for insulation wool was 17.4 times greater than for the special-purpose fiber. It is inappropriate to suggest that dissolution rate is not reliable while failing to mention the difference in dose between the two groups.

  NTP Response: The NTP concurs with this comment. The statement "…the incidence of mesothelioma in rats exposed by intraperitoneal injection was higher for the insulation

fiber (59%) than for the special-purpose fiber (33%) (Miller *et al*. 1999a)" has been removed from the profile because there was a large difference between the doses used for insulation and special-purpose fibers.

- Another study by Miller (1999b), which supports the utility of K_{dis}, was not cited or mentioned in the draft substance profile. The profile should add that the dissolution rate is primarily a function of fiber composition.

  NTP Response: The key strengths and weaknesses of the utility of K_{dis} are briefly discussed in the final substance profile, which summarizes the data supporting the listing. The profile is not a comprehensive review of the literature of all studies published on a specific issue. The emphasis of the profile is on biopersistence, which includes *in vivo* dissolution rate as well as physiological clearance by the lung. The profile states that fiber dimensions (length and width) and chemical composition determine biodurability (Muhle and Bellman 1997).

Mechanistic Studies

- The draft substance profile's discussion of *in vitro* studies on genotoxicity and cytotoxicity is flawed because it does not take into account the impact of biopersistence. Long non-biopersistent fibers, such as insulation glass wools, are cleared from the airspaces and do not cause persistent inflammation, fibrosis, and genotoxic effects. Since biopersistence is not a factor in short-term *in vitro* assays, false positive results can occur with *in vitro* genotoxicity testing of non-biopersistent fibers such as insulation glass wools.

  NTP Response: Because the fibers have a range of sizes, not all insulation fibers may be rapidly cleared. Positive genotoxicity tests performed *in vitro* are an indication that further testing should be conducted *in vivo*. *In vivo* testing of insulation fibers by inhalation has shown granulomatous inflammation (Mitchell *et al.* 1986, Moorman *et al.* 1988), and by intratracheal instillation has shown an increase in lipid peroxidation (indicating production of reactive oxygen species) and strand breaks in rat alveolar macrophages and lung epithelial cells (Topinka *et al.* 2006).

- The final substance profile should address two issues that are sometimes raised regarding biosoluble fibers: (1) potential systemic toxicity from inhaled biosoluble fibers should not be a concern considering the very small mass of an inhaled fiber, and (2) the effect of a constantly replaced fiber with newly deposited fibers in the lungs is not a realistic concern due to the anatomy of the human lung.

  NTP Response: These two issues are speculations and thus outside the scope of the substance profile.

References

Bernstein DM. 2007. Synthetic vitreous fibers: a review toxicology, epidemiology and regulations. *Crit Rev Toxicol* 37(10): 839-886.

Bellman B, Schaeffer HA, Muhle H. 2010. Impact of variations in the chemical composition of vitreous mineral fibers on biopersistence in rat lungs and consequences for regulation. *Inhal Toxicol* 22(10): 817-827.

FDA Guidance of Industry assessed on 9/16/10 at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079272.pdf.

Miller BG, Searl A, Davis JM, Donaldson K, Cullen RT, Bolton RE, Buchanan D, Soutar CA. 1999a. Influence of fibre length, dissolution and biopersistence on the production of mesothelioma in the rat peritoneal cavity. *Ann Occup Hyg* 43(3): 155-166.

Miller BG, Jones AD, Searl A, Buchanan D, Cullen RT, Soutar CA, Davis JMG, Donaldson K. 1999b. Influence of characteristics of inhaled fibres on development of tumours in the rat lung. *Ann Occup Hyg* 43(3): 167-179.

Mitchell RI, Donofrio DJ, Moorman WJ. 1986. Chronic inhalation toxicity of fibrous glass in rats and monkeys. *J Am Coll Toxicol* 5(6): 545-575.

Moorman WJ, Mitchell RT, Mosberg AT, Donofrio DJ. 1988. Chronic inhalation toxicology of fibrous glass in rats and monkeys. *Ann Occup Hyg* 32(Suppl 1): 757-767.

Muhle H, Bellmann B. 1997. Significance of the biodurability of man-made vitreous fibers to risk assessment. *Environ Health Perspect* 105(Suppl 5): 1045-1047.

Topinka J, Loli P, Dušinská M, Hurbánková M, Kováciková Z, Volkovová K, *et al.* 2006. Mutagenesis by man-made mineral fibres in the lung of rats. *Mutat Res* 595: 174-183.

ortho-Nitrotoluene

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of *ortho*-nitrotoluene in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, 73 FR 12736)[20] and on the draft substance profile (December 22, 2008, 73 FR 78364).[21] The NTP received one public comment[22] on *ortho*-nitrotoluene since issuance of the expert panel report (see table). The relevant issues in the public comments and the NTP responses to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
Expert Panel Report/ April 24, 2008	1	U.S. Department of Defense, Shannon E. Cunniff
Draft Substance Profile	0	

NTP Response to Issues Raised by the Public Comments

Comments Related to Mechanistic or Toxicology Studies

- The comments support the recommendation for listing *ortho*-nitrotoluene as reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals, but stated there are weaknesses in the extrapolation of carcinogenicity in animals to humans and it is unclear whether humans metabolize *ortho*-nitrotoluene to an active/carcinogenic metabolite.

  NTP Response: The NTP believes that the cancer findings in laboratory animals are relevant to humans. As stated in the substance profile, studies of *ortho*-nitrotoluene-exposed workers found *ortho*-nitrotoluene–hemoglobin adducts in the blood (Jones *et al.* 2005a) and *ortho*-nitrobenzoic acid and *ortho*-nitrobenzyl alcohol in the urine (Jones *et al.* 2005b), providing evidence that exposure to *ortho*-nitrotoluene produces a reactive metabolite(s) in humans. In addition, adducts between hemoglobin and 2-methylaniline (a metabolite of *ortho*-nitrotoluene) were identified in both exposed workers and exposed rats, and the level of 2-methylaniline–hemoglobin adducts in the blood of rats was proportional to the level of 2-methylaniline. *Ortho*-nitrotoluene has been shown to be metabolized in rats to *ortho*-nitrobenzylglucuronide and can be excreted in the bile where it is deconjugated and reduced by intestinal microflora to *ortho*-aminobenzyl alcohol, reabsorbed, and metabolized to reactive metabolites in the liver. Neither

[20] See http://ntp.niehs.nih.gov/go/29682 (select "*ortho*-Nitrotoluene") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[21] See http://ntp.niehs.nih.gov/go/9741 (select "February 24, 2009") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[22] To download the public comments, see http://ntp.niehs.nih.gov/go/9920 (select "*ortho*-Nitrotoluene").

ortho-aminobenzyl alcohol nor its metabolites has been detected in mouse urine after exposure to *ortho*-nitrotoluene (NTP 2002); therefore, other unidentified biochemical pathways leading to tumor formation most likely are involved. Sills *et al.* (2004) have shown that similar genetic alterations occur in human and mouse large intestinal carcinomas, suggesting that *ortho*-nitrotoluene-induced tumors in mice are likely relevant to humans.

References

Jones CR, Sepai O, Liu YY, Yan H, Sabbioni G. 2005a. Hemoglobin adducts in workers exposed to nitrotoluenes. *Carcinogenesis* 26(1): 133-143.

Jones CR, Sepai O, Liu YY, Yan H, Sabbioni G. 2005b. Urinary metabolites of workers exposed to nitrotoluenes. *Biomarkers* 10(1): 10-28.

Sills RC, Hong HL, Flake G, Moomaw C, Clayton N, Boorman GA, Dunnick J, Devereux TR. 2004. *o*-Nitrotoluene-induced large intestinal tumors in B6C3F1 mice model human colon cancer in their molecular pathogenesis. *Carcinogenesis* 25(4): 605-612.

Riddelliine

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for the listing status of Riddelliine in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, April 30, 2008)[23] and on the draft substance profile (December 22, 2008, 73 FR 78364).[24] The NTP received no relevant public comments since issuance of the Expert Panel Report on Riddelliine.

[23] See http://ntp.niehs.nih.gov/go/29682 (select "Aristolochic Acids and Riddelliine") for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[24] See http://ntp.niehs.nih.gov/go/9741 (select "February 24, 2009") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").

Styrene

The NTP solicited public comments through the *Federal Register* on the expert panel's recommendation for listing status of styrene in the *12th RoC* and the scientific justification for that recommendation (Expert Panel Report Part B, May 20, 2008, 73 FR 29139)[25] and on the draft substance profile (December 22, 2008, 73 FR 78364).[26] The NTP received 19 public comments[27] on styrene since issuance of the expert panel report (see table). The relevant issues raised by public comments and the NTP response to those issues are provided below. The NTP did not respond to comments on (1) the final background document, (2) the review process, or (3) non-technical or non-scientific issues, and only responded to specific issues for the expert panel report that are applicable to the final substance profile.

Public Comments since Issuance of the Expert Panel Report

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
Expert Panel Report/ September to October 2008	3	Private Citizen (Anonymous)
		Styrene Information and Research Center (SIRC) (Jack Snyder); also includes Comments prepared by Elizabeth Delzell Unpublished manuscript by Cruzan *et al.* 2009[28]
		Styrenic Steering Committee and Styrene Producers Association (Oliver Sloan)
Draft Substance Profile/ February 2009	10	American Chemistry Council (ACC) (Michael Walls) Comments prepared by Cantox Health Sciences International
		American Composites Manufacturers Association (ACMA) Comments prepared by Gradient Corporation (Julie Goodman)
		Elizabeth Delzell, University of Alabama Research supported by ACC, IISR, Health Effects Institute, and SIRC
		Emulsion Polymers Council (Andrew Jaques)
		Industrial Dielectrics (Jay Merrell)
		International Institute of Synthetic Rubber Producer (IISR) (Jim McGraw)
		National Marine Manufacturers Association

[25] Go to http://ntp.niehs.nih.gov/go/29682 (see Styrene) for the *Federal Register* soliciting public comments and for the Expert Panel Report Part B.
[26] See http://ntp.niehs.nih.gov/go/9741 (select "February 24, 2009") for the *Federal Register* soliciting public comments and for the draft substance profile (select "Meeting Materials").
[27] To download the public comments, go to http://ntp.niehs.nih.gov/go/9920 (see Styrene).
[28] Manuscript published electronically on July 7, 2009.

Comment on/ Date Received	Number of Comments	Submitter or Sponsor
		Comments prepared by Gradient Corporation (Lorenz Rhomberg) Private Citizen, Jean Rabovsky SIRC (2 sets of comments) (1) Comments prepared by Philip Cole (2) Comments prepared by SIRC, include unpublished manuscript by Cruzan *et al.* 2009[29]
Additional Comments/ December 2008 April 2009 May 2009 October 2009 October 2010 May 2011	6	SIRC Comments prepared by Boffetta and Colleagues[30] Comments prepared by James Bus and George Cruzan Comments submitted by Jack Snyder Comments submitted by Jack Snyder Comments submitted by Marcy Banton, George Cruzan and James Bus Comments submitted by Marcy Banton

NTP Response to Issues Raised by the Public Comments

Human Cancer Studies

Some of the public comments disagreed that there is limited evidence of carcinogenicity from studies in humans. They argued that the findings of increased risk of lymphohematopoietic cancer and styrene exposure are not consistent across studies. Other public comments stated that the association between styrene exposure and any form of cancer is not causal or that the evidence that styrene causes cancer is not sufficient; however, they did not voice an opinion whether the evidence is inadequate, or limited.

The NTP responses to specific comments are organized by the major scientific issues raised in the comments.

- Comments relating to the evaluation of different types of lymphohematopoietic cancers.
  - The types of lymphohematopoietic cancers varied across studies, and lymphohematopoietic cancers are different diseases with different etiologies.

    NTP Response: Blood cells are derived from a common multipotential progenitor cell in the bone marrow. If a carcinogenic agent affects this progenitor cell, it may give rise to more than one type of lymphatic and/or hematopoietic cancer. Several types of lymphohematopoietic cancers are associated with other epoxides or epoxide-forming substances, such as 1,3-butadiene and ethylene oxide. With respect to the styrene epidemiology studies, it is difficult to compare the risks for specific types of lymphohematopoietic cancers across cohort studies, because (1) these cancers have

[29] Manuscript published electronically on July 7, 2009.
[30] Report published on November 5, 2009.

been grouped differently among studies, or in the same study between different types of analyses (e.g., external and internal analyses in the study by Wong *et al.* 1994); (2) diagnoses based on death certificates may be inaccurate; and
(3) lymphohematopoietic cancer classification and groupings have changed over time. In general, these limitations make it more difficult to document consistent associations between styrene exposure and specific types of lymphohematopoietic cancer across studies.

- "On balance the UAB [University of Alabama] study results suggest a positive but statistically imprecise relation between styrene and NHL [non-Hodgkin's lymphoma] but no association between styrene and CLL [chronic lymphocytic leukemia]. Thus if the association with NHL is real, it may be limited to forms of NHL other than small lymphocytic lymphoma. No study has examined this possibility, as it has not been feasible to obtain systematic retrospective data on histopathologic subtypes of NHL in the occupational cohort studies."

  NTP Response: The NTP agrees with the comment that the University of Alabama study of styrene-butadiene rubber (SBR) workers suggests a positive association between styrene and NHL. A recent case-control study found exposure to styrene was associated with statistically significant increased risks for B-cell NHL (all) and follicular cell lymphoma (Cocco *et al.* 2010). This study used incidence data, which is less likely to have misclassification of diagnoses, and it had adequate statistical power (due to larger numbers) to be able to look at subtypes of lymphoma.

- Comments related to the utility of studies from the styrene-butadiene rubber (SBR) industry.
  - Studies of reinforced plastics workers should have the greatest weight since the workers have the highest exposures. Studies of SBR workers are also limited by the high correlation between exposure to styrene and exposure to butadiene.
  - Short-term workers do not make studies of the reinforced plastic workers less useful than studies of SBR workers.

  NTP Response: The substance profile discusses the strengths and limitations of the studies of styrene-exposed workers for evaluating cancer risks. The profile notes that the strength of the studies in the reinforced plastics industry is that the workers have higher exposures to styrene, and a limitation of the studies in the styrene-butadiene industry is that workers are also exposed to butadiene. However, studies of the reinforced plastics industry also have some major limitations; many workers were only employed for short durations of time (approximately 60% of the workers worked for less than two years in the European cohort study [Kogevinas *et al.*1994] and less than one year in the Danish cohort study [Kolstad *et al*. 1994]) and were only followed for a short period of time (average follow-up was 13 years in the Kogevinas study and ~11 years in the Kolstad study). In contrast the median time since hire was 33 years in the multi-plant study of styrene-butadiene workers (Delzell *et al*. 2006).

- Comments related to concerns about the Danish cohort study (Kolstad *et al*. 1994, 1995).

- The low- and high-exposure groups in the Danish cohort (Kolstad *et al*. 1994, 1995) do not represent individuals with low and high exposure but represent individuals at companies with fewer (low) or greater (high) than 50% employees involved in reinforced plastics manufacture.

  NTP Response: The comments are correct in that probable high and low exposures in the publication on solid tumors (e.g., pancreas and esophagus) were based on the number of employees at a company involved in reinforced plastics manufacture; employees of companies with 50% of the workforce involved in the production of reinforced plastics were classified as probable high exposure (Kolstad *et al.*1995). The authors stated that there were few opportunities for workers to avoid styrene exposure even when they were not directly involved in its production due to the highly volatile nature of styrene, which was the dominant exposure in the industry, and the fact that most of the companies were small with few workers. They also stated that most of the companies were boat yards or manufacturers of containers produced by lamination, which is associated with high exposure to styrene. We acknowledge that non-differential misclassification of exposure (low vs. high) is a concern because some workers in the probable low exposure group (employees at companies with less than 50% workers involved in reinforced plastics production) may actually have higher exposure than some workers in the probable high exposure group, but this would bias findings towards the null hypothesis, rather than result in a false positive. In the publication on lymphohematopoietic cancers, exposure level was primarily assessed via first year of employment; employment during the 1960s implied higher exposure to styrene. A statistically significant association was found for leukemia among workers with earlier dates of first employment (1964 to 1970).

- The draft substance profile states that higher risks of leukemia were found in short-term workers than long-term workers in the Danish cohort (Kolstad *et al.* 1994), and mentioned that many of those classified as short-term workers were long-term workers; however, it does not provide a rationale for assuming that such misclassification could explain the null results for longer term workers.

  NTP Response: The NTP agrees that this statement was unclear and has removed it from the substance profile.

- Concerns about the multi-plant European cohort study by Kogevinas *et al.* (1994).
  - The draft substance profile states that Kogevinas *et al.* (1994) did not control for duration of exposure in analyzing cumulative exposure.

    NTP Response: NTP agrees that the draft substance profile was in error and has deleted this statement from the final profile.

  - Table 3 lists 74 SMRs, of which only 6 were statistically significant (2 increases and 4 decreases), which is consistent with 5% probability of false positives.

    NTP Response: Table 3 in Kogevinas *et al*. (1994) provides SMRs (Standard Mortality Ratios) (using external references) for all cancers, all lymphohematopoietic cancers, and specific lymphohematopoietic cancers stratified by two categories of duration of exposure (less than two years and 2 years or greater), and three categories

of time since first exposure (< 10 years, 10 to 19 years, and 20 years or greater). The table provides some evidence that the risk of all lymphohematopoietic cancers and non-Hodgkin's lymphoma increased with increasing time since first exposure. These external analyses are consistent with internal analyses (using unexposed workers in the same study as the reference group) reported in Table 4, which found that the risk of lymphohematopoietic cancers (P_{trend} = 0.012) and NHL (P_{trend} = 0.072) increased with increasing time since first exposure, which supports that the findings observed in Table 3 are not due to chance.

- The draft profile does not include the findings from two other studies in the reinforced plastics industry (Wong *et al.* 1994 and Ruder *et al.* 2004), which had refined exposure analyses and found no increases in any type of lymphohematopoietic cancer.

  NTP Response: Although the Wong and Ruder studies were not specifically mentioned, the draft substance profile acknowledges that findings were not consistent in all studies. The final profile mentions the findings from Wong *et al*. (1994) and Ruder *et al*. (2004), although these studies were not considered to be informative in the evaluation because the Ruder *et al.* (2004) study had limited statistical power to detect positive associations for lymphohematopoietic cancers and the Wong *et al.* (1994) study was limited because the internal analyses only evaluated exposure duration and cumulative exposure.

- Comments about the appropriate analyses and exposure metric and consistency across exposure measures.
  - Overall findings (SMR analyses) do not provide evidence for considering styrene as a human carcinogen.

    NTP Response: SMR analyses of "ever-exposure," or entire cohorts, which combine the data from individuals with no, low, and high exposure into a single exposure group, are not very sensitive for detecting a real association. Internal analyses using unexposed workers as the referent are considered more informative for evaluating cancer risks because they help diminish confounding and bias, such as the "healthy worker" effect.

  - The appropriate exposure metric should not be chosen based on statistically significant findings. Positive associations between styrene exposure and leukemia or lymphoma cancers were found for only some of the exposure metrics evaluated in internal analyses. The evidence for a carcinogenic effect of styrene is based mainly on a borderline significant finding of average exposure and NHL in the large European study of reinforced plastics workers, and is not supported by findings of duration in this study or results of average exposure in other studies.

    NTP response: In the reinforced plastics industry, the evaluation of the relationship between duration of exposure and cumulative exposure (which is dependent on duration of exposure) is complicated by the high percentage of short-term workers who may have experienced higher levels of exposure than long-term workers. Average exposure would be a better metric in these particular studies. Positive exposure-response associations were found for average exposure and lymphohematopoietic cancers (P_{trend} = 0.019), and lymphoma (P_{trend} = 0.052) (Kogevinas *et al*. 1994). No other cohort studies of reinforced plastics workers

evaluated average exposure in internal analyses. In the styrene-butadiene industry, which did not have the problem of a high percentage of very short-term workers, a positive association was found between cumulative exposure to styrene and NHL in models that accounted for butadiene exposure (Delzell *et al*. 2006). Average exposure was not evaluated in the Delzell study, but a nested case-control study from the Matanoski cohort (which includes many of the workers in the Delzell study) found significantly increased risks of all lymphohematopoietic cancers ($P = 0.001$) and of lymphoma ($P = 0.020$) (International Classification of Disease codes 200 and 202, which are the same codes as for NHL) with exposure to styrene (1-ppm time-weighted average compared with 0 ppm) in a statistical model that accounted for exposure to butadiene (Matanoski *et al.* 1997). Since peer review of the draft substance profile,[31] a case-control study was published that found statistically significant associations for B-cell NHL ($P_{trend} = 1 \times 10^{-5}$) and follicular lymphoma ($P_{trend} = 1 \times 10^{-5}$) and three independent measures of exposure (intensity, frequency, and confidence) (Cocco *et al.* 2010).

- Comments about the findings for non-Hodgkin's lymphoma (NHL) and exposure to styrene among styrene-butadiene workers (Delzell *et al*. 2006).
  - Statistics for exposure-response relationships were not reported.
  - "The elevated RR for NHL and CLL-NHL among workers with nonzero exposure to styrene reflect, to some extent, unexplained and substantial deficits of deaths from NHL and CLL-NHL in the styrene-unexposed workers," which were observed in "external" analyses that compare the workers to the general population. The deficit could be due to chance or confounding, and it is not known whether the "internal" analyses (using unexposed workers as controls) removed confounding.
  - Residual confounding by butadiene, especially of associations seen for styrene and NHL-CLL and all leukemia, and statistical problems stemming from the inclusion of both styrene and butadiene, highly correlated variables, in regression models cannot be ruled out as explanations of these results.

  NTP response. In models controlling for butadiene exposure and using the unexposed group as the referent group, the relative risks (RR) for NHL increased from 1.7 in the lowest cumulative styrene-exposure group to 3.2 for the highest cumulative styrene-exposure group, which suggests a positive exposure-response relationship (a similar pattern is observed for NHL-CLL) (Delzell *et al*. 2006). However, the RRs are imprecise as a consequence of small numbers of exposed cases, and it is unclear whether the overall exposure-response trends are statistically significant because the authors did not report trend values.

  Internal analyses, which use the unexposed workers as the referent, are more informative than SMR analyses, which use external reference populations, because internal analyses help reduce potential confounding and the "healthy worker effect." Deficits observed in the no exposure group may result from the fact that workers in general are healthier than the public because the latter includes individuals who cannot work as a consequence of adverse health conditions such as cancer. In external analyses (Delzell *et al.* 2006), SMRs

[31] The draft substance profile was peer reviewed by the NTP Board of Scientific Counselors on February 24, 2009.

for NHL also increased with increasing cumulative exposure to styrene, supporting the positive exposure-response relationship observed in the internal analyses.

- Comments about conclusions on the evaluation of the effects of styrene exposure and the risk of cancer of the esophagus and pancreas.
  - Risks for esophageal and pancreatic cancer were not consistently increased across studies, and risks did not increase with increasing exposure in the Wong *et al.* (1994) study.
  - There is no common mode of action between the solid tumors and lymphohematopoietic tumors.

  NTP Response: As stated in the substance profile, mortality from esophageal cancer was increased in two of the four studies (Ruder *et al*. 2004, Wong *et al.* 1994), and a third study found a statistically nonsignificant increased risk among reinforced plastics workers with higher cumulative exposure (Kogevinas *et al.* 1994). Wong *et al.* did not find a positive association with cumulative exposure for esophageal cancer. For pancreatic cancer, increased risks were suggested in most of the cohort studies. Internal analyses of the Danish cohort found a significant risk of pancreatic cancer (incidence) among workers classified as having "probable high exposure" (Kolstad *et al.* 1995). Statistically nonsignificant increased risks (SMRs or RRs) of pancreatic cancer mortality were reported by the two U.S. cohort studies of reinforced plastics workers (Ruder *et al*. 2004, Wong *et al.* 1994) and for workers with higher cumulative exposure in the European study (Kogevinas *et al.* 1994). Cancer risk increased with increasing cumulative exposure in the European multi-plant cohort (P_{trend} = 0.068) (Kogevinas *et al.* 1993, 1994).

  The mechanisms by which styrene causes cancer are not known.

Cancer Studies in Experimental Animals

Some comments proposed that the evidence from cancer studies in experimental animals does not meet NTP criteria for sufficient evidence because there is only suggestive evidence for tumors by oral administration. They state that:

- The NTP used historical control data from Hazleton laboratories to conclude that the incidence of lung tumors in the current controls (by corn oil gavage) in the NCI bioassay of styrene (Litton laboratories) is not unusually low. The NTP's use of historical controls from a different laboratory (Hazleton laboratories) is not scientifically supported because of inter-laboratory variability. There is a laboratory difference in the incidence of lung tumors at 91 weeks between the historical controls at Hazleton (3% based on 28 studies by corn oil gavage or diet) compared to Litton (9% based on 13 studies by corn oil gavage or diet).

  NTP Response: The NCI report (NCI 1979) stated that the incidence of alveolar/bronchiolar carcinomas in historical *vehicle* control male $B6C3F_1$ mice (i.e., 0/40) was based on too small a number of animals for meaningful use as historical controls. Therefore, the NTP looked at appropriately matched vehicle historical controls (4% incidence, 11/273) from another laboratory. When compared with the vehicle controls from the Hazleton laboratories, the incidence of lung tumors in vehicle control male mice (i.e., 0/20) in the NCI study was not found to be unusually low. This

observation supports the NCI finding of a statistically significant higher incidence of lung tumors in styrene-exposed male mice compared with the concurrent vehicle controls (Litton laboratories) by Fisher exact pairwise comparison and by Cochran-Armitage trend test. For Litton laboratories, the incidence of lung tumors (12%) in the untreated controls is statistically different ($P < 0.05$) from the incidence in the vehicle (corn oil) and, therefore, the two incidences cannot be combined.

- In the NCI study, the incidence of lung tumors in the high-dose styrene group of exposed animals (18%) was lower than in 2 of the 15 Litton studies (20%) during this period.

  NTP Response: In the NCI study, the incidence of lung tumors (combined alveolar/bronchiolar adenoma and carcinoma) is 21% for the high-dose group of mice exposed to styrene by gavage (corn oil) for 78 weeks and surviving at least 52 weeks (NCI technical report, Table 5). The incidence in the high-dose animals is higher than the upper range (20%) in the Litton untreated controls.

- Increased tumors observed in O20 mice by Ponomarkov and Tomatis (1978) occurred only in the presence of high mortality.

  NTP Response: The NTP agrees that the high mortality is a concern in this study, and it was considered in NTP's evaluation.

Mechanistic Studies

Some of the public comments state that the mode-of-action for styrene-induced lung tumors in mice is species-specific and not relevant to humans. The NTP responses to specific comments are grouped by scientific issues.

- Many of the comments opine that styrene-7,8-oxide is not likely involved in mouse lung tumors because of the following observations:
  - Styrene oxide (gavage) did not cause lung tumors in mice even though the lung level of styrene oxide from gavage administration was the same as that metabolically generated from inhalation of styrene (Sarangapani *et al.* 2002); the only tumors observed were at the site of contact (Conti *et al.* 1988, Lijinsky, 1986). The mechanism of action was attributed to increased cell proliferation resulting from the cellular damage induced by styrene oxide.
  - Concentrations of styrene-7,8-oxide in the rat lung are higher than in the mouse lung.
  - Similar cytotoxicity and tumors in mouse lungs, but not in rat lungs, have been demonstrated for at least two similar chemicals that are not metabolized to epoxides like styrene oxide, namely ethylbenzene and cumene.

  NTP Response: Inhalation of epoxide or epoxide-forming chemicals such as ethylene oxide, butadiene, and isoprene induced lung tumors in mice but not in rats (Melnick and Huff [1993], Melnick [2002], also reviewed by Cohen *et al*. [2002]). Species differences may be due to a greater susceptibility of the mouse lung to epoxide-induced carcinogenesis. Studies of butadiene have found a similar pattern as styrene. Predicted (based on a physiologically based pharmacokinetic model) levels of the epoxide metabolite (epoxybutene) in the blood and lungs of rats are higher than mice, suggesting that factors other than tissue dosimetry are important in explaining species differences (Melnick and Kohn 1995). Although mice have a higher rate of epoxide formation than

the rat or human, mice use the glutathione detoxification pathway to a significant extent and so more readily deplete glutathione stores, as compared with rats and humans. An exposure to 20-ppm styrene for six hours resulted in a higher concentration of styrene oxide in the terminal bronchi of mice than rats and in a large depression of tissue glutathione in mice versus only a marginal decrease in rats (Csanády *et al*. 2003). Oxidative damage as a result of styrene metabolism to styrene-7,8-oxide or to styrene-3,4-oxide can result in genotoxicity and cytotoxicity, which would be exacerbated with glutathione depletion (Hofmann *et al.* 2006) and could contribute to pulmonary hyperplasia and potentially neoplasia.

- The comments opine that a genotoxic mode of action for styrene is unlikely because of the following statements:
  - Styrene is only positive for genotoxicity *in vitro* where any styrene oxide formed is not readily detoxified. The relevance of studies with human lymphocytes using high styrene concentrations is questionable.

    NTP Response: In *vitro* genotoxicity studies are useful for identifying whether substances have the ability to damage DNA or chromosomes. In these assays, styrene induced (1) mutations in *Salmonella typhimurium* strains (when assayed under gaseous conditions to minimize loss from volatilization [deMeester *et al.* 1981]), yeast, insects, and Chinese hamster cells; (2) single strand breaks in human lymphocytes and lung and testicular cells, and in mammalian cells; and (3) chromosomal damage–sister chromatid exchanges, chromosomal aberrations, micronuclei, and aneuploidy (NTP 2008). Most of the positive genotoxicity studies with styrene occurred in the presence of, or were enhanced by, added metabolic activation, which is consistent with the hypothesis that styrene causes genotoxicity primarily via its metabolic conversion to the epoxide styrene-7,8-oxide, although styrene-3,4-oxide and other metabolites may also contribute to styrene's genotoxicity. Human tissues, including lung and blood, have the capacity to metabolize styrene to styrene-7,8-oxide, and styrene-7,8-oxide is found in the blood of styrene-exposed workers, so the mutagenicity and genotoxicity of styrene-7,8-oxide is relevant for evaluating health hazards caused by styrene. While some studies (Norppa and Tursi 1984, de Raat 1978) reported that induction of sister chromatid exchanges in mammalian cells required either high concentrations of styrene or the presence of epoxide hydrolase inhibitor, styrene-induced cytogenetic effects occurred at lower concentrations in experimental systems with metabolic activation (such as S9 or whole blood), and without requiring inhibition of epoxide hydrolase (NTP 2008). Erythrocytes also convert styrene to styrene-7,8-oxide by the action of erythrocytic oxyhemoglobin (Vainio *et al.* 1985, Tursi *et al.* 1983). Single strand breaks, mutations (weak evidence), and chromosomal aberrations were also detected in lymphocytes of workers exposed to styrene (see below) (NTP 2008).

  - DNA adducts and single-strand DNA breaks, the only endpoints well-established to be associated with styrene exposure, are markers of exposure and do not necessarily represent genotoxic risk. DNA adduct levels are similar in target and non-target (liver) tissues and similar in rats and mice, thus there is not a correlation between adduct formation and development of tumors. The levels of adducts found in

lymphocytes of humans exposed to styrene are low, and it is difficult to extrapolate the quantitative mutagenic risk.

NTP Response: DNA adducts are not only biomarkers for exposure, but are also markers of DNA reactivity in that they measure direct damage of a xenobiotic to DNA bases. Adducts investigated after styrene exposure are most likely from metabolism to styrene-7,8-oxide; no studies on DNA adduct formation by other styrene metabolites have been located although other metabolites may also be genotoxic. A number of studies have identified promutagenic DNA adducts (adducts at base-pairing sites) formed as a result of metabolism of styrene to styrene oxide in the lymphocytes of styrene-exposed workers: betaN1-adenine, O^6-guanine, and N^2-guanine adducts have been observed and could result in AT > GC, GC > AT, and GC > TA mutations, respectively (Vodicka *et al.* 2006). Levels of betaN1 adenine adducts in workers were significantly correlated with measures of styrene exposure (Koskinen *et al.* 2001, Vodicka *et al.* 2003). Moreover, N^2-guanine and O^6-guanine adducts are persistent (Cohen *et al.* 2002). Examination of mutations from styrene oxide-exposed human lymphocytes resulted in predominantly base substitution mutations: AT > GC, GC > AT, AT > TA, and GC > TA, with AT > GC being the predominant mutation (Bastlová and Podlutsky 1996). DNA adduct levels ranged from 0.3 to 15.8 per 10^8 nucleotides (depending on the specific adduct and detection system), which is a similar range to that found in various tissues in smokers (reviewed by Phillips 2002). The NTP agrees with the conclusion of Boffetta *et al.* (2009), which states "limited by their small size, and lack of control for potential confounders, these studies [studies of DNA adducts] provide evidence for a genotoxic effect in humans, probably mediated by the metabolite, styrene-7,8-oxide."

* The draft substance profile only reports the positive genotoxicity studies in experimental animals and does not report that styrene did not cause chromosomal aberrations in any animal system and was generally negative for micronuclei. Positive findings for single strand breaks (comet assay) were only observed after intraperitoneal injection, and inhalation studies were negative in rats (Kligerman *et al.* 1993) and equivocal in mice (Vodicka *et al.* 2001).

  NTP Response: The substance profile is a concise summary of the scientific evidence that supports the listing and is not intended to be a comprehensive review. When evaluating studies in rodents, inconsistencies across studies may be due to differences in the study methodology (such as the route of exposure, length of exposure, species tested, strain tested) or tissue-specific responses. The available *in vivo* studies indicate that styrene causes DNA adducts, single-strand breaks (mice only), sister chromatid exchanges, and polyploidy (only tested in rats). Styrene caused DNA strand breaks in multiple tissues in two strains of mice after intraperitoneal administration. With respect to inhalation exposure, styrene induced single-strand breaks in mice (significant increases in lymphocytes after 7 days of exposure and in endonuclease-III sensitive sites in bone marrow) after 21 days of exposure (Vodicka *et al.* 2001), which is the only study available. The negative findings in rats are limited to one study, which analyzed only one tissue. Sister chromatid exchanges (in all but one study) have been observed consistently in studies in experimental animals and were detected in the liver, alveolar macrophages, lungs, bone marrow, splenocytes, and

lymphocytes of mice and splenocytes and peripheral blood cells of rats. Kligerman *et al.* (1993) found dose-response increases in sister chromatid exchanges in the lung and spleen of mice and peripheral blood lymphocytes of both rats and mice exposed to styrene by inhalation. Most studies in experimental animals were negative for micronuclei formation and chromosomal aberrations.

- The results of cytogenetic studies conducted in humans are inconsistent and contrary to the results reported in *in vivo* studies in experimental animals. The inadequacies of these studies are not discussed in the draft substance profile or background document. The studies do not show clear dose- or temporal-response relationships.

  NTP Response: Human studies follow subjects exposed chronically, while most studies in experimental animals are acute or subchronic studies. Thus, it is difficult to directly compare the results (Cohen *et al.* 2002). The background document text notes the general limitations, such as small numbers of subjects, potential confounding, and assay methodology, of the cytogenetic studies in humans, and the tables provide the necessary information (such as matching subjects or controlling for potential confounders) on the study to evaluate its quality.

  The human epidemiological studies, although limited by small study populations, provide evidence that styrene causes chromosomal aberrations in the lymphocytes of people. A meta-analysis of 22 studies found a positive association (weighted frequency ratio = 2.18, 95% CI = 1.52 to 3.13) between styrene exposure level and chromosomal aberration frequency when exposure levels were dichotomized as greater than or less than a threshold value of 30 ppm for an 8-hour time-weighted average (Bonassi *et al.* 1996). In addition, several studies have reported exposure-response relationships between various measures of styrene exposure (such as styrene air levels, urinary metabolites, or blood levels) and chromosomal aberration frequency (Migliore *et al*. 2006, Camurri *et al.* 1983, Somorovská *et al.* 1999), and other studies found higher risks in the high-exposure group compared with the low-exposure group (Artuso *et al*. 1995, Fleig and Thiess 1978 and Tomanin *et al.* 1992). Sister chromatid exchanges and micronuclei have also been found in lymphocytes of styrene-exposed workers with some studies reporting positive exposure-response relationships (Yager *et al.* 1993, Artuso *et al.* 1995); however, the evidence is weaker than that for chromosomal aberrations. Some studies have found that markers of genetic damage in human lymphocytes are correlated: (1) Laffon *et al.* (2002) found a correlation between single-strand breaks and sister chromatid exchanges, between single-strand breaks and micronuclei, and between sister chromatid exchanges and micronuclei. (2) Brenner *et al.* (1991) also reported a correlation between single-strand breaks and micronuclei.

- Lung tumors in styrene-exposed mice are species-specific and not relevant to humans, and there is no concordance among human and rodent mode-of-action data on the effects of styrene. The comments raised the following points to support this statement.
  - Styrene causes lung tumors in mice via a cytotoxic mechanism.
  - Styrene does not cause lung toxicity in humans or rats.

- Ring oxidation and phenylacetic/phenylaceturic acids pathways play a small role in styrene metabolism in humans, and CYP2F1, the human Cyp2f2 homolog, does not appear to metabolize styrene in humans.
- Metabolism of Cyp2f2 is necessary to cause lung toxicity, and the cytotoxicity of 4-vinylphenol is 10 times more toxic to the mouse lung than styrene and 5 times more toxic than styrene oxide (Carlson *et al.* 2001), indicating that the formation and subsequent metabolism of 4-vinylphenol are responsible for lung cytotoxicity in mice.
- Mouse lungs have a higher fraction of Cyp2f2-containing Clara cells than rat or human lungs; Clara cells are rare in humans.
- Recent research in Cyp2f2 knockout mice supports conclusions that styrene is not a carcinogen. This research found that styrene, administered by gavage for 5 days, caused Clara cell toxicity and induced cellular proliferation in wildtype Cyp2f2 mice but not in Cyp2f2-null mice. Since humans have very small amounts of CYP2F, lung toxicity and tumors are not expected from styrene in humans.

- Another comment stated that the relationship between styrene metabolism in Clara cells and mouse lung tumors that contain alveolar type II cells has yet to be determined. The predominant cell type in the styrene-induced tumors is alveolar type II cells (Cruzan *et al.* 2001).

  NTP Response: The findings of lung tumors in mice are relevant to hazard identification in humans as tissue-site concordance is frequently not observed (EPA 2005). The available epidemiological data have not found evidence that styrene causes lung cancer in humans, although most studies were limited by short follow-up and lacked detailed analyses for lung cancer. However, the human epidemiological studies do provide limited evidence that styrene causes lymphohematopoietic cancers. The induction of lung tumors in mice and lymphohematopoietic cancers in humans has also been observed in studies of exposure to epoxides and other epoxide-forming chemicals including the known human carcinogens 1,3-butadiene and ethylene oxide (NTP 2004). The mechanisms by which styrene causes lymphohematopoietic cancers are not known; however, styrene causes key events such as genetic damage and immunosuppression that are plausible modes-of-action for these types of cancer.

  The mechanisms for induction of lung tumors by styrene exposure in the mouse have not been fully elucidated. Advances in cancer biology suggest that many substances cause cancer by multiple modes of action (Guyton *et al.* 2009). Styrene causes cytotoxicity in the mouse lung, and there is evidence to suggest that cytotoxicity-induced cellular proliferation contributes to lung tumor formation in mice. However, styrene exposure also causes other key events that can lead to tumor formation, such as oxidative stress and genotoxicity; thus, it is reasonable that those modes of action, in addition to cytotoxicity, play a role in styrene-induced cancer, and all these modes of action are not mutually exclusive. The preliminary study in Cyp2f2 knockout mice has not been peer reviewed and thus cannot be considered in the NTP evaluation.

  There is also evidence that styrene may cause lung toxicity in humans. Obstructive airway disease (Chmielewski and Renke 1975, Jedrychowski 1982) and adverse effects to the lower respiratory tract (Lorimer *et al.* 1976, 1978) have been documented in different

groups of workers exposed to styrene. In addition, Ruder *et al.* (2004) reported a statistically significant excess of mortality from pneumoconiosis and other respiratory diseases among highly exposed styrene reinforced plastics workers (SMR = 2.54, 95% CI = 1.31 to 4.44, 12 deaths).

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