



**Comments on the Assessment of the Genotoxicity of Styrene  
Presented in the Draft Substance Profile Prepared for  
Potential Listing of Styrene in the 12<sup>th</sup> RoC**

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# COMMENTS ON THE ASSESSMENT OF THE GENOTOXICITY OF STYRENE PRESENTED IN THE DRAFT SUBSTANCE PROFILE PREPARED FOR POTENTIAL LISTING OF STYRENE IN THE 12<sup>TH</sup> ROC

## EXECUTIVE SUMMARY

Cantox Health Sciences International (Cantox) was requested to provide comments on the assessment of the genotoxicity of styrene as presented in the Draft Substance Profile (DSP) prepared to support the proposed listing of styrene in The National Toxicology Program's (NTP) 12<sup>th</sup> Report on Carcinogens (RoC). In general, both the DSP and the Background Document, identify the key genotoxicity studies, and summarize their results appropriately. What is lacking however, is any meaningful discussion of how these data should be interpreted with respect to the deliberation of whether styrene can "*reasonably be anticipated to be a human carcinogen*". There is an inherent bias to the presentation of "positive" results (*i.e.*, to support a genotoxic effect) without any "weighing" of the results in light of their relevance to assessing the potential human carcinogenicity of styrene.

Specific comments include:

- There is little interpretive analysis of the metabolic data in relation to the results of the *in vitro* genetic toxicity studies available for styrene. Styrene is positive only under conditions where any SO that is formed is not readily detoxified
- The relevance to human risk assessment of *in vitro* studies with human lymphocytes using high concentrations of SO unachievable *in vivo* is questionable
- There is no discussion of the lack of correlation between the results of the *in vitro* and *in vivo* genetic toxicity studies and the results of carcinogenicity bioassays
- The DSP is not balanced in its presentation of the *in vivo* genotoxicity data in animals since it does not present any of the "negative" results (*i.e.*, chromosome aberration and micronuclei)

- The inconsistency of dose- and temporal-response relationships in the *in vivo* genotoxicity data that are considered to be “positive” is not adequately discussed in the DSP or Background Document
- There is no mention in the DSP, and no discussion in the Background Document, of a negative COMET assay (Kilgerman *et al.*, 1993) available for styrene conducted by the inhalation route of exposure, the route of most relevance to humans
- In both the DSP and the Background Document there is no analysis/discussion of the lack of concordance between the findings of DNA adducts studies in animals *versus* the results of carcinogenicity bioassays (*i.e.*, the tissues [liver] with the highest numbers of DNA adducts are not involved in any tumorigenic responses [lungs] to long-term administration of styrene in animals)
- In the DSP and Background Document, there is ongoing bias to “positive” conclusions, since there is no presentation and/or discussion of other points of view, or opinions, regarding the genotoxicity of styrene that are readily available in the peer-reviewed scientific literature (*e.g.*, Scott and Preston, 1994a,b; Nestmann *et al.*, 2005; Henderson and Speit, 2005; Speit and Henderson, 2005; Vodička *et al.*, 2006)
- There is no interpretative analysis of the findings of DNA adducts reported in styrene exposed workers. DNA adducts and single-stranded DNA breaks are the only endpoints well-established to be associated with styrene exposure. These endpoints are indicators of exposure and do not necessarily represent genotoxic risk
- The biological significance of the types of DNA adducts found in the human studies is not adequately discussed, especially in relation to DNA repair
- The results of the cytogenetic studies conducted in humans are inconsistent and tend to show results that are contrary to those reported in the *in vivo* animal studies. These complexities are not adequately addressed in either the DSP or the Background Document
- The inadequacies of the designs (number of subjects, appropriateness of controls, consistency of the data, *etc.*) of many of the DNA adduct and clastogenicity studies are not discussed in either the DSP or the Background Document. Such analyses are available in the scientific literature (Nestmann *et al.*, 2005; Henderson and Speit, 2005)

In conclusion, the genetic toxicity data available on styrene cannot be extrapolated so as to suggest that they indicate a genetic, and therefore, carcinogenic risk, for styrene-exposed human populations.

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# COMMENTS ON THE ASSESSMENT OF THE GENOTOXICITY OF STYRENE PRESENTED IN THE DRAFT SUBSTANCE PROFILE PREPARED FOR POTENTIAL LISTING OF STYRENE IN THE 12<sup>TH</sup> ROC

## 1.0 INTRODUCTION

Cantox Health Sciences International (Cantox) was requested to provide comments on assessment of the genotoxicity of styrene as presented in the Draft Substance Profile (DSP) prepared to support the proposed listing of styrene in The National Toxicology Program's (NTP) 12<sup>th</sup> Report on Carcinogens (RoC). Specifically, Cantox was requested to evaluate the genotoxicity data, both *in vitro* and *in vivo* animal and human, with respect to: concordance with the animal cancer study data, dose-response relationships, strength and consistency of evidence, relationships to the results of epidemiology studies, *etc.*

The DSP for styrene presents a synopsis of the available data, including genotoxicity data, relevant to the assessment of the carcinogenic potential of styrene in humans. A more detailed analysis, with summaries of the individual studies, is presented in the "Background Document" that was completed September 29, 2008. The comments on the DSP, essentially a condensed version of the Background Document, therefore, also pertain to the "Background Document" as well. Where appropriate, comments specific to the Background Document are also presented.

In general, both the DSP and the Background Document, identify the key genotoxicity studies, and summarize their results appropriately. What is lacking however, is any meaningful discussion of how these data should be interpreted with respect to the deliberation of whether styrene can "*reasonably be anticipated to be a human carcinogen*", as per the listing guidelines for inclusion in the RoC. The data are summarized rather matter-of-factly, and presented with the apparent aim to support the listing of styrene in the RoC. As a result of an imbalance in presentation, there is an inherent bias to the presentation of "positive" results (*i.e.*, to support genotoxic effect) without any "weighing" of the results in light of their relevance to assessing the potential human carcinogenicity of styrene.

The comments presented forthwith have been addressed according to the type of data evaluated, *e.g.*, *in vitro*, *in vivo*, and human, followed by a summary and some concluding remarks.

## 2.0 IN VITRO GENOTOXICITY OF STYRENE

The DSP presents/discusses very little of the *in vitro* data available for styrene. The fact that styrene-7,8-oxide (SO) is mutagenic in many *in vitro* systems, in the absence of metabolic activation is mentioned as supporting evidence for its inclusion in a previous RoC (NTP, 2004).

Styrene has been well established to induce chromosome aberrations (CA) and sister chromatid exchanges (SCE) *in vitro* under test conditions that enhance metabolism of styrene to SO and reduce the detoxification of SO (e.g., addition of rat S9 mix and of cyclohexane oxide, an inhibitor of epoxide hydrolase (EH)) (Scott and Preston, 1994a; Bonassi *et al.*, 1996; Cohen *et al.*, 2002). Mixed results have been reported in *in vitro* bacterial mutation assays (IARC, 1994a). The requirement for exogenous metabolism of styrene by the CYP family of enzymes for expression of any mutagenic/genotoxic activity in bacterial systems is noteworthy since the DSP, while indicating that styrene is mutagenic in the presence of metabolic activation, fails to adequately discuss these results in relation to the human metabolism of styrene, namely that there is limited systemic exposure to styrene-7,8-oxide due to its rapid detoxification by epoxide hydrolase (Scott and Preston, 1994a). In fact, based on pharmacokinetic modeling conducted by Sarangapani *et al.* (2002), exposures of lung tissue to SO in humans from inhaled styrene may be up to 100-fold and 10-fold lower than in mice and rats, respectively.

The DSP, and the supporting Background Document, cite and discuss *in vitro* studies with human lymphocytes that have reported that both styrene and SO cause sister chromatid exchange, chromosomal aberration, including micronuclei. Lacking in this discussion is the use of styrene and/or SO concentrations *in vitro* that are far greater, by up to 100-fold, than concentrations achieved in the blood of rodents following inhalation exposure (Luderer *et al.*, 2006), the non-linearity of dose-response (lack of or equivocal effects at low concentrations), and lack of correlation of the “positive” *in vitro* genotoxicity data with results of animal carcinogenicity studies or with epidemiology studies (*i.e.*, styrene has not been shown, even in highly exposed animals, to clearly be associated with the development of malignancies of the hematopoietic system, the cells of which have been the subject of many of the assays reporting positive results).

While styrene shows genotoxic activity in some *in vitro* studies under conditions where SO is not readily detoxified, there is no compelling evidence to indicate that this activity, even if expressed at low rates *in vivo*, is associated with carcinogenic risk in humans. This is indirectly acknowledged in the DSP and in the Background Document, since genotoxicity is not conclusively stated to be the mechanism behind the findings of the cancer bioassays and of alleged “limited” evidence of cancers in humans.

Another aspect of the *in vitro* data discussed in the Background Document worthy of comment is the finding of DNA adducts in human peripheral lymphocytes exposed to SO. Again, this

reactive intermediate, while formed in the liver and peripheral tissues to a lesser extent, does not achieve high concentrations in the blood of humans due to its rapid detoxification. In addition, the concentrations of SO achieved in the lungs are lower than in rodents due to limited capacity for metabolism of styrene in this tissue in humans. As with the *in vitro* SCE and chromosome aberration data, the DNA adduct data are derived from studies that utilized concentrations of SO that could not be achieved in the human body, and did not include systems present that detoxify SO prior to interacting with DNA (*i.e.*, epoxide hydrolase and glutathione).

In summary, the *in vitro* data identify styrene as genotoxic only under certain conditions, and they are of limited relevance to the assessment of the potential genetic risk posed by styrene to exposed human populations

### **3.0 IN VIVO (ANIMAL) GENOTOXICITY OF STYRENE**

The DSP presents very little of the available *in vivo* genotoxicity data available for styrene, or for SO, in experimental animals. These data are covered in the Background Document. This is a key oversight. As presented in the Background Document, the *in vivo* genetic toxicity studies conducted in experimental animals with styrene are at most equivocal. As detailed in Table 5-9 of the Background Document (summary of studies reviewed by IARC, 1994a,b, 2002; Scott and Preston, 1994a), styrene has only consistently been shown to induce SCE (in various cell types in mice and in rat splenocytes and lymphocytes) and single-stranded DNA breaks in various tissues in mice exposed at high doses by i.p. injection (*e.g.*, Walles and Orsen, 1983; Vaghef and Hellman, 1998; Vodička *et al.*, 2001). The DSP contains a statement to this effect. Nothing is mentioned of the fact that styrene does not cause chromosomal aberrations in any animal system. Similarly, responses in the *in vivo* micronucleus assays are generally negative (Luderer *et al.*, 2006). Even within the one mouse micronucleus study reporting “positive” results, the results are not internally consistent for styrene. For example, Vodička *et al.* (2001) reported that while exposure of male NMRI mice to styrene at 350 ppm for 7 days increased the incidence of micronuclei in the bone marrow, similar exposures for 21 days failed to do so. Moreover, in a repetition of this study that utilized the same doses, no evidence of clastogenicity (micronuclei or chromosome aberrations) in male NMRI mice was noted after exposure for 1, 3, 7, 14, or 21 days (Engelhardt *et al.*, 2003). These data show the critical role of mammalian metabolism, detoxification, and DNA repair in the lack of expression of genotoxicity of styrene *in vivo* for many genetic endpoints.

SO has not been shown to induce micronuclei *in vivo*, has shown equivocal results for SCE in mice, and has not tested uniformly positive for chromosomal aberrations in mice (Scott and Preston, 1994a; IARC, 1994b). These data further bear out the complexity of the assessment of the *in vivo* genotoxicity of styrene. That SO, the putative “genotoxic” metabolite of styrene, fails to show significant activity in many classical *in vivo* genetic toxicity assays, underscores the

ambiguity of the data and the difficulty in their interpretation with respect to the potential for styrene and SO to be carcinogenic in humans, particularly by a genotoxic mechanism.

In contrast to the available chromosomal aberration and micronucleus assays, the results of *in vivo* studies of DNA damage and repair are mentioned in the DSP. The DSP states that: "Most animal studies have also demonstrated single-stranded DNA breaks from styrene-7,8-oxide or styrene exposure (Walles and Orsen, 1983; Vaghef and Hellman, 1998; Vodička *et al.*, 2001)." However, no discussion follows. The results referred to relate to the COMET and DNA unwinding; hydroxyapatite separation assays. These are notably sensitive systems associated with potentially high incidences of "false positives", since the systems, while sensitive, may not have high specificity (*i.e.*, identifying non-genotoxic or non-carcinogenic chemicals as genotoxic) (McGregor and Anderson, 1999). For styrene, positive results in the COMET assay were reported, but only for the i.p. route of exposure and only at high, near toxic doses (~100 to ~1,000 mg/kg body weight) (Walles and Orsen, 1983; Vaghef and Hellman, 1998). Bolus dosing by the i.p. route can also saturate detoxification mechanisms, including depletion of glutathione stores (Vaghef and Hellman, 1998). Moreover, SO is detoxified at a faster rate in humans compared to rodents, and, in mice metabolism of styrene to SO is substantially greater than in humans due to the presence of cyp2f2 content and the higher numbers of Clara cells.

One COMET assay conducted by the inhalation route of exposure (Kligerman *et al.*, 1993), the route of exposure of most relevance to humans showed no genotoxic effect of styrene in female Fischer rat peripheral blood lymphocytes following exposure at 125 to 600 ppm for 6 hours/day for 2 weeks. Vodička *et al.* (2001) reported equivocal results for a COMET assay for styrene in which mice were exposed by inhalation for up to 21 days at 175 to 350 ppm for 6 hours/day.

Overall, the results for styrene in COMET assays conducted by relevant routes of exposure present no substantive evidence of a clear genotoxic effect *in vivo*.

The DSP also notes the finding of various DNA adducts, particularly N7-guanine, O<sup>6</sup>-guanine, N1-adenine) in the liver and lung of mice and rats exposed to styrene. The levels of these adducts at tumorigenic doses are generally in the range of 1-3 adducts/10<sup>8</sup> nucleotides. In comparison, DNA adduct levels on the order of 1,000/10<sup>8</sup> nucleotides have been reported for genotoxic carcinogens (Otteneder and Lutz, 2002). While the presence of DNA adducts is an indicator of exposure to SO as a metabolite of styrene, it does not demonstrate genetic damage likely to lead to the development of tumors. The lack of a clear connection between DNA adduct formation and tumor induction is exemplified by the study of Boogaard *et al.* (2000). Boogaard *et al.* (2000) examined DNA adduct formation in the liver, lungs, and isolated lung cells of rats and mice exposed to styrene by inhalation at concentrations of 160 ppm (700 mg/m<sup>3</sup>) for 6 hours. DNA adduct levels in the lung tissue of mice, the target tissue in the 2-year carcinogenicity study (Cruzan *et al.*, 2001), were found to be lower than in liver (Boogaard *et al.*, 2000), a non-target tissue. In addition, DNA adduct levels in the lung, liver and lymphocytes of mice were about the same as in the rat, yet there was no indication of an oncogenic effect in the

latter species (Cruzan *et al.*, 1998). Concentrations of SO in the blood of rats exposed to the non-tumorigenic dose of 1,000 ppm were about 5-fold greater than in mice exposed to a tumorigenic dose of 160 ppm. These data show a lack of correlation of SO exposure, DNA adduct formation, and carcinogenic effect. Otteneder *et al.* (2002) concluded that the susceptibility of the mouse to the development of lung tumors may be based on factors other than, or in addition, to DNA adducts, including: other reactive intermediates, oxidative stress, indirect DNA damage, stimulation of cell proliferation, or cell-specific toxicity. Cantoreggi and Lutz (1993) further concluded from the results of inhalation studies in rats and mice that the covalent binding index (amount of DNA adduct/DNA nucleotides) was so low that significant tumor induction was unlikely to be due to DNA adduct formation alone. Finally, as stated in the NTP Background Document “Philips and Farmer (1994) reported that very low levels of DNA adducts were formed in the forestomach [the target tissue for styrene-7,8-oxide–induced tumors; IARC, 1994b] and liver when tritiated styrene-7,8-oxide was administered by gavage to rats and by i.p. injection to mice (Cantoreggi and Lutz 1992; Lutz *et al.*, 1993). These findings further demonstrate the lack of concordance between DNA adducts and tumor formation.

Beyond the lack of correlation of the DNA adduct data in animals to the results of the carcinogenicity studies in mice and rats, the inhalation exposures used in these studies (*i.e.*, 40 to 1,000 ppm for various time periods) are not indicative of exposures to the general population. Moreover, occupational exposures may exceed 20 ppm, yet evidence of carcinogenicity in humans is lacking even at these levels of exposure.

In addition to the inconsistencies in the results of the DNA adduct studies in animals, the types of DNA adducts are of significant importance in determining potential genetic risk. For example, O<sup>6</sup> SO-guanine adducts have been considered to be potentially mutagenic, while N7 SO-guanine adducts are not since they are unstable, rapidly depurinate, or are readily repaired (Bastlová and Podlutzky, 1996). Kanuri *et al.* (2001) have reported that SO-induced N6 adenine adducts may not adversely affect cell replication or result in point mutations. Similarly, Hennard *et al.* (2001) reported that the R β and S-β N6-adenine SO adducts were non-mutagenic in site-specific mutagenesis experiments and did not block polymerase bypass.

The presence of DNA adducts in the tissues of experimental animals is an indicator of exposure to SO, but does not qualitatively or quantitatively prove that styrene is genotoxic *per se* (capable of causing DNA damage that could lead to the induction of tumors). Rather, given the comparative carcinogenicity data available in mice *versus* rats, there is reason to conclude that genotoxicity is unlikely to play a role in this finding.

Many of the inconsistencies and complexities in the interpretation of the available *in vivo* data discussed above have been previously published in the scientific literature (Nestmann *et al.*, 2005; Speit and Henderson, 2005). These publications have received very little attention in the NTP Background Document and are completely absent from the DSP. This observation highlights the unbalanced treatment of the styrene genotoxicity data in each of the NTP

documents. As discussed above, Speit and Henderson (2005) noted that styrene is weakly positive in indicator tests (*i.e.*, DNA strand breaks and SCE) *in vivo*, but shows no convincing effect on the incidence of chromosome aberration or on micronucleus formation. In light of the metabolism of styrene and rapid detoxification of SO, Speit and Henderson (2005) further concluded that “a direct extrapolation of the results from animal genotoxicity studies to man would not predict genotoxic effects in workers exposed to styrene”. These important discussions and attendant conclusions with respect to the animal genotoxicity data are necessary to present the data in an unbiased balanced perspective. At the very least, given the conclusions of Nestmann *et al.* (2005) and Speit and Henderson (2005), and of others who have reported “positive” genotoxic effects of styrene *in vivo* in animals and humans (Bonassi *et al.*, 1996; Cohen *et al.*, 2002; Vodička *et al.*, 2006), the genetic toxicity data from *in vivo* studies in animals do not support or lead to the conclusion that styrene “can reasonably be anticipated to be a human carcinogen”.

In summary, the DSP document does not adequately present the complexity and conflicting nature of the *in vivo* genetic toxicity data available from animal studies. In this respect, the Background Document presents very limited critical analysis of these studies and oversimplifies their results as can be seen in Section 5.4.6 and in Table 5-18. The lack of coherence of the *in vivo* data (*i.e.*, negative results for micronuclei formation and chromosome aberrations, positive findings at high doses or using the i.p. route of exposure, and the lack of quantitative concordance of the results of the DNA adduct studies with the carcinogenicity study conducted in mice) makes difficult any interpretation within the context of the potential human carcinogenicity of styrene. The lack of critical discussion of the *in vivo* animal genetic toxicity data is further highlighted by the absence of citation and analysis of literature reviews that have presented the complexity and conflicting nature of these data (*i.e.*, Nestmann *et al.*, 2005; Speit and Henderson, 2005). The DSP and the Background Document correctly acknowledge that the possible mechanism(s) by which styrene could be associated with tumor development is not known. As such, styrene cannot be considered a “genotoxic carcinogen”.

## 4.0 HUMAN STUDIES

A number of studies of occupationally exposed populations, most notably workers in the re-inforced plastic industries, were reviewed in the Background Document (NTP, 2008). The individual studies are not mentioned or critiqued in any way in the DSP. Only the Background Document (NTP, 2008), and a few other review articles (Bonassi *et al.*, 1996; Cohen *et al.*, 2002) are cited in the DSP. Critical examination of the human studies is entirely lacking in the DSP. Critical analyses of the human studies, and of the genotoxicity data overall, are presented in the Background Document to a very limited extent.

## 4.1 DNA Adducts

The results of the worker studies that have investigated DNA adducts in occupationally exposed populations are summarized in one paragraph in the DSP. While the paragraph in question is technically accurate, stating that in these populations N2-guanine, O6-guanine, and  $\beta$ N1-adenine adducts have been detected, no further discussion is presented. This leaves the reader with the impression that since DNA adducts were found, they may in some way be related to its alleged carcinogenic activity, particularly since the inherent purpose of the DSP is to present data that support the listing of styrene in the 12<sup>th</sup> RoC. A broader evaluation is presented in the Background Document, but this too does not adequately capture the fact that the simple finding of DNA adducts in humans merely indicates exposure to SO and that SO can bind to DNA. In any case, the minimal critical analysis of the DNA adduct studies presented in the Background Document should also appear in the DSP.

The overarching comment pertaining to the study of DNA adducts, and of reports of DNA damage in workers, is the lack of correlation to human cancer epidemiology data. Although the DSP and the Background Document present data that attempt to link styrene exposure to lymphohematopoietic system cancers, such data by all accounts, including one of the key studies in question (Delzell *et al.*, 2006) are weak at best and do not support a causal association between these types of cancer and styrene exposure. As a result, there is no scientific basis from which to infer that the finding of DNA adducts in workers supports a purported association of styrene with lymphohematopoietic neoplasms.

The shortcomings and limitations of the human DNA adducts studies are not presented in the DSP or in the Background Document. Specific limitations relate to the inclusion of only a small number of subjects and controls, inappropriate nature of the controls (lack of matching), lack of adjustment for potential confounders, and the serial nature of the studies reported. It is unclear how much subject and temporal overlap was present in the studies conducted by the same groups of researchers (*e.g.*, the series of studies conducted by Vodička and associates).

Overall, the rates of SO DNA adduct formation noted in the worker studies are relatively low. In general, individual SO-specific DNA adducts have been reported to be present in the lymphocytes of occupationally exposed individuals at mean frequencies in the range of  $8.0/10^{10}$  to  $1.6/10^7$  nucleotides (Horvath *et al.*, 1994; Vodička *et al.*, 1999, 2003; Koskinen *et al.*, 2001). Maximum SO-specific adduct frequencies ranged up to  $1/10^6$  nucleotides. Quantitatively, potential mutagenic risk associated with such low DNA adduct loads is difficult to extrapolate since the toxicological significance of DNA adduct loads in the range of  $1/10^8$  to  $1/10^7$  normal nucleotides has been reported to be questionable, especially in light of the fact that for certain DNA adducts, the background rates can be in the range of  $1/10^6$  to  $1/10^5$  normal nucleotides (Nestmann *et al.*, 1996).

Much of the human DNA adduct data is rather difficult to interpret. For example, in the Vodička *et al.* (1993, 1994, 1995, 1999) serial studies of the presence of O<sup>6</sup>-guanine SO-DNA adducts in hand lamination workers in Bohemia, at the first sampling time (Vodička *et al.*, 1993) the levels of adducts in styrene exposed individuals were about 5 to 7-fold greater than those of controls, yet failed to achieve statistical significance due to the unusually high adduct levels in one control. However, during repeated sampling over the next few years, the levels of O<sup>6</sup>-guanine SO-DNA adducts remained stable (*i.e.*, did not increase in relation to the initial sampling time), yet were then reported to be significantly greater than the controls. In any case, the finding that DNA adducts did not increase with time of styrene exposure is indicative of an equilibrium existing between rates of DNA adduct formation and adduct removal/repair. The relationship of this dynamic process to genotoxic risk in humans has yet to be adequately explored.

In summary, the human DNA adduct data demonstrate exposure to SO as a metabolite of styrene and show it to be capable of interacting with DNA. However, mere presence of DNA adducts in white blood cells cannot be inferred to support the hypothesis that styrene is associated with development of lymphohematopoietic system cancers in humans. This situation echoes that seen in animals where, for styrene and SO, the low levels of SO-adducts in the forestomach (target tissue in rats and mice) and in the lung (target tissue in mice) are insufficient to account for its carcinogenic activity by a genotoxic mechanism (Phillips and Farmer 1994; Otteneider *et al.*, 2002). Secondly, the human DNA adduct studies were generally exploratory in nature and did not include sufficiently large numbers of subjects, or adequate controls. Finally, as noted above, the levels of DNA adducts reported are low. The finding of DNA adducts in humans occupationally exposed to styrene does not as itself support the consideration of styrene as “reasonably be anticipated to be a human carcinogen”.

## 4.2 Cytogenetic Effects

A number of studies have evaluated the presence of DNA damage, including SCE, chromosome aberration, micronucleus formation, and single-stranded DNA breaks in styrene exposed workers. The DSP states that “The most consistent cytogenetic effects in styrene-exposed workers are single-stranded DNA breaks and induction of chromosome aberrations (Bonassi *et al.*, 1996; Cohen *et al.*, 2002; NTP, 2008). This statement fails to present a balanced view of the data. A review of the studies considered in the Background Document reveals that statistical significance of effects was often marginal, dose- and/or temporal response relationships often were missing, correlations with the excretion of mandelic acid were generally poor, and concomitant exposures to other chemicals could not be excluded. In addition to these issues, the conflicting findings (*i.e.*, both positive and negative), particularly with respect to the endpoints of SCE, HPRT mutations, and micronuclei formation, preclude the establishment of a definitive causal relationship between occupational exposure to styrene and cytogenetic effects in peripheral blood lymphocytes. It is also worth noting that the alleged cytogenetic findings in the human studies tended to be contrary to the results from the animal

studies (*i.e.*, increased frequency of SCE in animals, with equivocal or negative findings in humans; equivocal findings of chromosomal aberrations/micronuclei in humans, no evidence in experimental animals). This lack of internal consistency in the data is shown in some of the tables in the background report, but no discussion of this fact is presented. .

The lack of balance inherent in the DSP and Background Document is further highlighted by the absence of literature citations that have critically analyzed the cytogenetic data from human studies, in particular, Nestmann *et al.* (2005) and Henderson and Speit (2005). The review of Henderson and Speit (2005) concluded that single-stranded DNA breaks to be the only genetic endpoint (beyond DNA adduct formation) that could clearly be associated with styrene exposure. Similarly, Vodička *et al.* (2006), in a review of their studies of human populations exposed to styrene concluded that “This review presents styrene as a relatively weak genotoxicant and mutagen..” and that “It seems at present that the majority of employees exposed to styrene around or below MAC {maximum acceptable concentration} values are not in the {range of} excessive genotoxic or carcinogenic risk, and the current MAC seems to be set up properly for styrene”. These conclusions are not reflected in the language of the Background Document. Likewise, the relatively low potential, if it exists at all, for styrene to cause genotoxic effects in human populations is not presented in either the Background Document or the DSP.

With respect to the reports of single-strand DNA breaks in exposed workers, they are an indicator of exposure and of potential DNA damage, but not of mutagenesis *per se*. As stated by Henderson and Speit (2005), there is a level of DNA damage (single strand breaks) that is unlikely to be of biological significance due to the rapid rate at which such lesions are repaired. That repair is rapid is supported by the findings that biomarkers of exposure (urinary excretion of styrene metabolites) in the studies of exposed workers have not correlated well with the amount of DNA single strand breaks reported (*e.g.*, Maki-Paakkanen *et al.*, 1991; Brenner *et al.*, 1991; Shamy *et al.*, 2002; Walles *et al.*, 1993). Moreover, it is known that the incidence rates of DNA strand breaks as assessed by the methodologies used in the occupational studies are responsive to exercise, time of day, and vitamin C intake (Møller *et al.*, 2000).

The discussion of the chromosome aberration studies, of which there are more than 40, is incomplete and inadequate in both the DSP and the Background Document. While the summary presented in the Background Document (Section 5.4.6 and Table 5-18) indicates both “positive” and “negative” results for chromosome aberrations, no reason or discussion of why this is the case is presented. Rather, the reader is left with the impression that styrene exposure in fact causes these lesions since the glyph in Table 5-18 presented for human populations and chromosome aberrations is “(+)”; stated in the legend to indicate “weakly positive results”. In the review of the human chromosome aberration studies conducted to 2004/2005, Henderson and Speit (2005) employed a number of criteria (*i.e.*, adequacy of sampling, details on the types of aberrations scored, use of coded slides, nature and number of controls, statistical methodologies, control of confounding variables, sample size, *etc.*) to identify

those studies considered as “robust”. Of those, 9 were considered “positive” and 7 “negative”. Henderson and Speit (2005) noted that some of the “negative” studies were well-conducted and involved high levels of exposure to styrene. These authors concluded that the results of the chromosome aberration studies in human populations are essentially un-interpretable and inconclusive due to deficiencies in study designs and/or reporting.

In summary, the human studies that have evaluated DNA damage and various cytogenetic endpoints demonstrate that styrene is associated with single strand breaks in DNA. These data cannot be extrapolated to indicate mutagenic or carcinogenic risk. The data with respect to SCE, chromosome aberration, and micronuclei induction are inconsistent or inconclusive and do not show clear dose- or temporal-response relationships and are not in concordance with the genetic toxicity data available from experimental animals. In the DSP and the Background Document, there are insufficient analysis and discussion of the complexities of the *in vivo* human genetic toxicity data. In addition, neither document attempts to “close the loop” and provide some conclusions with respect to the genotoxicity of styrene. This is essential to assess the carcinogenic risk, if any, posed to humans by styrene. At a minimum, conclusions of several available review articles on the subject (Bonassi *et al.*, 1996; Nestmann *et al.*, 2005; Henderson and Speit, 2005, Cohen *et al.*, 2002; Vodička *et al.*, 2006) could be presented to provide a balanced perspective.

## 5.0 CONCLUDING COMMENTS

In general, both the DSP and the Background Document, identify the key genotoxicity studies, and summarize their results appropriately. What is lacking however, is any meaningful discussion of how these data should be interpreted with respect to the deliberation of whether styrene can “*reasonably be anticipated to be a human carcinogen*”, as per the listing guidelines for inclusion in the RoC.

Specific comments include:

- There is little interpretive analysis of the metabolic data in relation to the results of the *in vitro* genetic toxicity studies available for styrene. Styrene is positive only under conditions where any SO that is formed is not readily detoxified
- The relevance to human risk assessment of *in vitro* studies with human lymphocytes using high concentrations of SO unachievable *in vivo* is questionable
- There is no discussion of the lack of correlation between the results of the *in vitro* and *in vivo* genetic toxicity studies and the results of carcinogenicity bioassays

- The DSP is not balanced in its presentation of the *in vivo* genotoxicity data in animals since it does not present any of the “negative” results (*i.e.*, chromosome aberration and micronuclei)
- The inconsistency of dose- and temporal-response relationships in the *in vivo* genotoxicity data that are considered to be “positive” is not adequately discussed in the DSP or Background Document
- There is no mention in the DSP, and no discussion in the Background Document, of a negative COMET assay (Kilgerman *et al.*, 1993) available for styrene conducted by the inhalation route of exposure, the route of most relevance to humans
- In both the DSP and the Background Document there is no analysis/discussion of the lack of concordance between the findings of DNA adducts studies in animals *versus* the results of carcinogenicity bioassays (*i.e.*, the tissues [liver] with the highest numbers of DNA adducts are not involved in any tumorigenic responses [lungs] to long-term administration of styrene in animals)
- In the DSP and Background Document, there is ongoing bias to “positive” conclusions, since there is no presentation and/or discussion of other points of view, or opinions, regarding the genotoxicity of styrene that are readily available in the peer-reviewed scientific literature (*e.g.*, Scott and Preston, 1994a,b; Nestmann *et al.*, 2005; Henderson and Speit, 2005; Speit and Henderson, 2005; Vodička *et al.*, 2006)
- There is no interpretative analysis of the findings of DNA adducts reported in styrene exposed workers. DNA adducts and single-stranded DNA breaks are the only endpoints well-established to be associated with styrene exposure. These endpoints are indicators of exposure and do not necessarily represent genotoxic risk
- The biological significance of the types of DNA adducts found in the human studies is not adequately discussed, especially in relation to DNA repair
- The results of the cytogenetic studies conducted in humans are inconsistent and tend to show results that are contrary to those reported in the *in vivo* animal studies. These complexities are not adequately addressed in either the DSP or the Background Document
- The inadequacies of the designs (number of subjects, appropriateness of controls, consistency of the data, *etc.*) of many of the DNA adduct and clastogenicity studies are not discussed in either the DSP or the Background Document. Such analyses are available in the scientific literature (Nestmann *et al.*, 2005; Henderson and Speit, 2005)

In conclusion, the genetic toxicity data available on styrene cannot be extrapolated so as to suggest that they indicate a genetic, and therefore, carcinogenic risk, for styrene-exposed human populations. As discussed in our comments, the data remain ambiguous and inconsistent. In a recent review of the genotoxicity of styrene in relation to carcinogenic risk, Vodička *et al.* (2006) concludes: "the majority of employees exposed to styrene around or below current MAC values are not in the excessive genotoxic or carcinogenic risk..". Such a conclusion is clearly inconsistent with the consideration of styrene as "reasonably anticipated to be a human carcinogen".

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