Peer Review Workshop for EPA's Draft Toxicological Review of Carbon Tetrachloride

Post-Meeting Comments

Submitted to:

National Center for Environmental Assessment
Office of Research and Development
U.S. Environmental Protection Agency
Washington, DC 20460

Submitted by:

Eastern Research Group, Inc.
110 Hartwell Avenue
Lexington, MA 02421

November 3, 2008

Contents

Introduction 1
Janusz Z. Byczkowski, DABT, D.Sc., Ph.D. 3
Gary Ginsberg, Ph.D. 21
Dale Hattis, Ph.D. 35
Lisa M. Kamendulis, Ph.D. 77
Lawrence H. Lash, Ph.D. 91
Madhusudan G. Soni, Ph.D., FACN 107
Appendix A. Reviewer List 117
Appendix B. Charge to Reviewers 121
Appendix C. Observer List 129
Appendix D. Workshop Agenda 133

Introduction

The Integrated Risk Information System (IRIS) is a U.S. Environmental Protection Agency (EPA) database containing Agency consensus scientific positions on potential adverse human health effects that may result from chronic (or lifetime) exposure, or in select cases less-than-lifetime exposures, to chemicals in the environment. IRIS currently provides health effects information on over 500 chemical substances. IRIS contains chemical-specific summaries of qualitative and quantitative health information in support of two steps of the risk assessment process: hazard identification and dose-response evaluation. IRIS information includes a reference dose (RfD) for non-cancer health effects resulting from oral exposure, a reference concentration (RfC) for non-cancer health effects resulting from inhalation exposure, and an assessment of carcinogenicity for both oral and inhalation exposures. Combined with specific situational exposure assessment information, the health hazard information in IRIS may be used as a source in evaluating potential public health risks from environmental contaminants.

The IRIS program within EPA's National Center for Environmental Assessment (NCEA) developed a *Toxicological Review of Carbon Tetrachloride*, which updates an assessment that was posted to the IRIS database in 1987. Carbon tetrachloride was nominated for reassessment by EPA's Office of Air and Radiation to support potential regulatory actions under the Clean Air Act. Eastern Research Group, Inc. (ERG), an EPA contractor, organized an independent peer review of EPA's *Toxicological Review of Carbon Tetrachloride*. The review document contains a chronic oral RfD, a chronic inhalation RfC, and a quantitative cancer assessment. ERG identified six nationally recognized experts (Appendix A) to conduct this review:

- Janusz Byczkowski, Consultant
- Gary Ginsberg, Connecticut Department of Public Health
- Dale Hattis, Clark University and George Perkins Marsh Institute
- Lisa Kamendulis, Department of Pharmacology and Toxicology, Indiana University
- Lawrence Lash (Chair), Department of Pharmacology, Wayne State University School of Medicine
- Madhusudan Soni, Soni & Associates

ERG provided the reviewers with a charge (Appendix B), which asked for their comments on the various aspects of the document. In the first stage of the review, the experts worked individually to prepare written pre-meeting comments, which were provided to all reviewers and EPA prior to a one-day peer review workshop. In the second stage, ERG convened the one-day workshop, on October 14, 2008, at a venue in Arlington, Virginia. Five of the six reviewers participated in the workshop; Dr. Kamendulis was not able to attend.

The meeting was open to the public and attended by eight observers and two ERG employees (Appendix C). Appendix D provides the workshop agenda. The meeting format included an opportunity for public comment, but no observers provided comments. After the meeting, reviewers revised their pre-meeting comments to reflect their views as they had evolved based on the workshop discussions. The reviewers' final post-meeting comments are provided in this report.[1] These comments reflect the individual opinions of the reviewers.

[1] Since Dr. Kamendulis was unable to attend the workshop, her post-meeting comments are a reflection of her views after reading the other reviewers' pre-meeting comments.

Janusz Z. Byczkowski, DABT, D.Sc., Ph.D.

Consultant

From the Academy of Medicine in Gdansk, Poland, Dr. Janusz Byczkowski earned his M.Sc. in Toxicology in 1970, his Ph.D. in Pharmacology in 1975, and his D.Sc. in Biochemical Pharmacology in 1979. He also received his Diplomate in General Toxicology of the American Board of Toxicology in 1998. Dr. Byczkowski's major areas of expertise include toxicology, pharmacology, human health risk assessment, quantitative dose-response assessment, computational toxicology, benchmark dose (BMD) modeling, physiologically-based pharmacokinetic (PBPK) modeling, and exposure modeling.

Dr. Byczkowski has 40 years experience in research and scientific consulting. For over 10 years he has served as a Consultant and Reviewer supporting the U.S. Environmental Protection Agency (EPA) and other agencies, including 8 years as Risk Assessment Coordinator for Ohio EPA. He performed, reviewed, and corrected countless site-specific human health and ecological risk assessments and remedial investigations and feasibility studies (RI/FS). He also wrote many technical documents and risk assessment guidelines. Working as a research scientist, Dr. Byczkowski performed and/or directed about a hundred scientific projects in the field of pharmacology and toxicology, and developed and wrote ACSL codes for several PBPK/toxicodynamic models. He also wrote many peer-reviewed publications and several invited reviews, including handbook chapters on physiologically and biologically based mathematical modeling. As a consultant, Dr. Byczkowski contributed chapters to several U.S. EPA reviews and white papers. As a risk assessor, he derived chronic and sub-chronic Provisional Toxicity Values for cancer and non-cancer end points of several chemicals and contributed chapters to many draft risk assessment issue papers.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

   The difference between academic dissertation and Toxicological Review support document is its utility. The intent of Toxicological Review is is to provide scientific support and rationale for the hazard and dose-response assessments in IRIS, quantitatively expressed as appropriate toxicity reference values. This Toxicological Review has been prepared from a huge amount of data and qualitative information, with the scientific rigor adequate for academic dissertation. It is logical and relatively clear, but it is not concise. The text contains a lot of narrative descriptions of experiments and their results, often redundant, which perhaps, in many instances could be left synthesized in the appropriate tabular format. Otherwise, the scientific evidence for both cancer and noncancer hazards has been presented accurately and objectively, but for the risk assessor/manager, sometimes with only minimal academic experience and often very busy, it may be difficult to distill the evidence necessary for site-specific risk assessment.

2. **Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.**

   This Toxicological Review addresses all relevant studies on effects of carbon tetrachloride, published in the publicly available literature.

3. **Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.**

   It seems that all important research has been already referred to and discussed adequately in this Toxicological Review.

4. **Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?**

   The sources of uncertainty in Sections 5 and 6 were appropriately identified and described. However, in the opening paragraph of Section 5.3, this document states:

   "*As presented earlier in this section (5.1.2 and 5.1.3 for the RfD; 5.2.2 and 5.2.3 for the RfC), the uncertainty factor approach (U.S. EPA, 2002, 1994b) was used to derive the RfD and RfC for carbon tetrachloride. [...] Because information specific to carbon tetrachloride was unavailable to fully inform many of these extrapolations, default factors were generally applied.*"

   but later:

   "*A UF of 3 was used to account for toxicodynamic difference between animals and humans...*"

   "*...a 10-fold UF was used to account for uncertainty in extrapolating from laboratory animals to humans in the derivation of the RfD associated with this 10-fold UF...*"

   "...a default UF of 10 was used to account for uncertainty associated with human variation in the derivation of the RfD and RfC..."

"*...subchronic toxicity studies were used, and a UF of 3 was applied to extrapolate those data obtained from a study of subchronic exposure to chronic exposure...*"

So, even though the uncertainty factors were appropriately selected and adequately discussed, from the narrative description in section 5 and elsewhere it does not seem that indeed "*default factors were generally applied*". Each of the typically used in IRIS five default factors covers single order of magnitude (i.e., 10^1; U.S. EPA 2002), including:

A = 10 for animal to human;

D = 10 for incomplete to complete data;

H = 10 for average human to sensitive human;

L = 10 for LOAEL to NOAEL;

S = 10 for subchronic to chronic.

Moreover, the clarity of text could be improved by listing in the separate table the applied uncertainty factors, using typical IRIS symbols: UF_A; UF_D; UF_H; UF_L; UF_S and providing final numerical values in each category, analogous to the transparent graphical presentation in Figs. 5-1 to 5-3 and 5-6 to 5-8.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   The Bruckner et al. (1986) study (the same as the one selected in the 1991 revision of IRIS CCl_4 toxicity reference values documentation) has been adequately described in Sections 4.2.1.1, 5.1.1 and 5.1.5; presented in the tables 4-13, 4-15, 5-1 and 5-2; quantitatively summarized and extensively discussed in Section 5.1.2; and partly interpreted in 6.2.1. While the selection of this study is by all means justified and criteria/rationale have been adequately described, the discussion could be consolidated to make reasoning for this selection even more transparent.

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

   In section 5.1.2 (the last paragraph on p. 180) this document states:

"*…use of elevated serum SDH activity as the critical effect for derivation of the RfD is supported by results of a study examining the use of serum liver enzymes as predictors of hepatotoxicity (Travlos et al., 1996). The relationship between the activity of serum liver enzymes (ALT, SDH, ALP, and TBA [total bile acids]) and liver histopathology was examined for 50 chemicals and three chemical mixtures...*"

The increase of SDH activity in plasma has been well documented and used as a marker of toxic liver injury following the treatment with CCl_4, and other hepatotoxicants (e.g., acetaminophen, cadmium chloride, leukotriene, ethyl and allyl alcohols, etc.). Since its relative activity may rise an order of magnitude higher than that of aminotransferases (e.g., in rats challenged with Cd, plasma SDH rose 30- to 300-fold, depending on the dose, while aspartate aminotransferase only 3- to 40-fold, respectively; Toxicol. Appl. Pharmacol. 74:308-313,1984), it may be considered to be a very sensitive biomarker and its selection as a critical effect is scientifically justified. Even though by itself, the increased SDH activity is not an "adverse effect", it is a sensitive indicator of hepatocellular injury. The selection of increased serum SDH activity as a critical effect has been adequately explained in this Toxicological Review.

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

The BMD modeling has many advantages over the alternative "NOAEL or LOAEL" method: 1) it is not limited to doses tested experimentally; 2) is less dependent on dose spacing; 3) it takes into account the shape of the dose-response curve; 4) it gives comparable results across chemicals and endpoints; and 5) provides flexibility in determining biologically significant responses. However, it also requires a good scientific/professional judgment to take advantage of this flexibility. Benchmark response (BMR) is usually defined as a predetermined change in the response rate of an adverse effect, but in this document, basing on professional judgment, an indicator of adverse effect rather than the adverse effect itself has been predetermined.

In section 5.1.2 (the last paragraph on p. 177), this document states:

"*…All of the models for continuous data in U.S. EPA's BMD software […] were fit to the 10-week SDH data. An increase in SDH activity two times the control mean, representing an increase in serum enzyme level considered to be biologically significant, was used as the benchmark response (BMR). Several expert organizations, particularly those concerned with early signs of drug-induced hepatotoxicity, have identified an increase in liver enzymes compared with concurrent controls of two to fivefold as an indicator of concern for hepatic injury…*"

and then (the second paragraph on p. 181):

"*…The $BMDL_{2X}$ of 5.46 mg/kg-day estimated from the increase in serum SDH activity in male rats in the Bruckner et al. (1986) study was used as the POD for derivation of the RfD. Use of the modeled BMDL provides an inherent advantage over use of a NOAEL or LOAEL by making greater use of all of the data. The BMDS was able to achieve adequate fit to the SDH data, providing a better estimate of the dose-response relationship for this endpoint than for other endpoints monitored, which were less sensitive and/or had data less suited to dose-response analysis…*"

Furthermore, the $BMDL_{2x}$ was adjusted to reflect the time weighted average dose (p. 182):

"… $BMDL_{2X\text{-}ADJ} = BMDL_{2X} \times 5$ *days/7 days* …"

and as indicated on p. 184:

"… *a BMR represented by an increase in SDH activity two times the control mean was selected under an assumption that it represents a minimal biologically significant change*…"

So, in light of the above quoted authors' explanation and a quite substantial body of literature regarding this subject, it seems that the BMR selected for use in deriving the POD is scientifically justified. The selection of SDH activity two times above the control mean as a biologically significant response, corresponds to less than 10% of the maximum stimulatory response and is consistent with the default $BMR_{0.1}$ (used for dichotomous data, because the 10% response is also at or near the limit of sensitivity in most bioassays). However, in cases where too low BMR for continuous data were selected, fitting the different models resulted in different BMDL estimates. Thus, the derivation of $BMDL_{2x}$ is reasonable as a justifiable compromise.

4. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

   - **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

   - **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

   A composite uncertainty factor (UF) of 1000 was applied in derivation of RfD for CCl_4 and the UF components are explained in Sections 5.1.3. (p. 183 to 185) and 5.3. (p. 219 to 213), including justification for subchronic to chronic and database uncertainty factors, each of 3 ($\sim 10^{0.5}$).

   While selection of uncertainty factors is always judgmental and by definition – bears uncertainty, it seems that in this Toxicological Review, the uncertainty factors were appropriately selected and adequately discussed, however, please see the General Comment # 4 above, for suggestions on improvement of the clarity of presentation.

   $UF_A = 10^1$ for animal to human extrapolation is the typical, default factor, sufficiently described and explained on p. 183.

$UF_H = 10^1$ for average human to sensitive human variability is reasonable and it has been objectively described.

$UF_S = 10^{0.5}$ for subchronic to chronic uncertainty is an exception from default, but it seems justified and has been described sufficiently.

$UF_D = 10^{0.5}$ for incomplete to complete data is also an exception, but it seems reasonably selected and has been objectively described.

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   In the existing, 1991 revision of IRIS CCl_4 toxicity reference values documentation, the RfC was not developed because the adequate chronic study was not available. Seven years later, the JBRC (1998) unpublished study were performed and partially published by Nagano et al. (2007 a, b), providing the opportunity for quantification of CCl_4 chronic inhalation toxicity. The complete JBRC (1998) report was not available for this reviewer, but from description by the authors of this Toxicological Review, presented in Section 5.2.1., and the partial publications by Nagano et al., it seems that JBRC (1998) was a high quality chronic study with the exposure duration very appropriate for derivation of RfC.

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   Fatty changes in the liver is a *bona fide* adverse effect of CCl_4 intoxication in both experimental animals and human subjects. There is a large body of literature dealing with this subject and the explanation provided by authors in this Toxicological Review, Section 4.6.2., reasonably describes and discusses this issue.

   In addition to the possible mode of action, presented in the Section 4.6.3., the disruption of balance between supply of free fatty acids to the liver and transport of triglycerides from the liver seems to be the major mechanism in generating fatty liver by CCl_4 (similarly to ethionine or phosphorus). Once the enzymes of the triglyceride cycle in the liver become saturated (or inhibited), the triglycerides accumulate in this organ. Presumably, the rate-limiting step is the synthesis of VLDL for export of triglycerides from the liver. Peroxidative decomposition of cytoplasmic membrane structural lipids and, possibly, interference with apoprotein, inhibits synthesis of VLDL and impacts triglyceride secretory mechanism. The interference with triglyceride cycle and synthesis of VLDL has been extensively studied in liver lesions caused by CCl_4 and is described in textbooks of toxicology (see: Hodgson, E. and Levi, P.E. "Introduction to Biochemical Toxicology", Chapter 20 Hepatotoxicity. Appleton & Lange, Norwalk, 1995).

So, the selection of fatty changes in the liver as the critical effect seems to be scientifically justified and adequate to the potential adverse health effects of exposure to CCl_4.

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

In the section 4.6.2. (the last line on p. 139 and the first paragraph on p. 140), this document states:

"*...There is evidence that liver effects produced by carbon tetrachloride are proportional to the product of concentration and time (C × T) (Plummer et al., 1990)...*"

and then (the second paragraph on p. 143):

"*...the F344 rat is known for its high incidence of spontaneous, age-related CPN (Hard and Seely, 2005; Chandra and Frith, 1993/94). Chandra and Firth (1993/94) reported a background incidence of CPN of 88.8% in male and 74.5% in female F344 rats based on an examination of 491 controls from several 2-year carcinogenicity/chronic toxicity bioassays. CPN can be seen as early as 3 months and severity of the lesion increases with age. The presence of CPN can confound kidney lesion diagnosis (Hard and Seely, 2005)...*"

and finally, in the section 4.6.3 (the last paragraph on p. 144 and the first paragraph on p. 145):

"*...Subchronic gavage studies report that the liver is the sole target organ (see Section 4.6.1), probably related to a first-pass effect. The literature for carbon tetrachloride also suggests that the rat liver is a more sensitive target organ compared to the kidney following exposures of subchronic duration (e.g., Nagano et al., 2007a; JBRC, 1998; Bruckner et al., 1986; Adams et al., 1952). Additionally, there are no adequate chronic studies of carbon tetrachloride (other than Nagano et al., 2007b; JBRC, 1998) to confirm whether the kidney may be a more sensitive target organ than the liver following chronic exposure (see Section 4.6.2)...*"

Traditionally, hepatotoxicity is believed to be an early adverse effect of exposures to CCl_4 (see: Comment # B2, above, and *Hodgson, E. and Levi, P.E. "Introduction to Biochemical Toxicology", Chapter 20 Hepatotoxicity. Appleton & Lange, Norwalk, 1995*) and there is a large body of literature supporting this tradition. Also, from the quoted above explanations by authors of this Toxicological Review it is clear that hepatotoxicity can be directly linked with exposures to CCl_4 (qualitatively and quantitatively). In contrast, proteinuria, which potentially could be considered as an indirect biomarker of CCl_4 nephrotoxicity, has a high background incidence, does not fulfill all of the modified Hill criteria and cannot be linked quantitatively with exposures to CCl_4. Moreover, the intensity of proteinuria in rats increases with age of the animals (even those unexposed). Thus, the authors of this Toxicological Review appropriately conclude (the last line of the second paragraph on P. 143) that:

"*...The above uncertainties raise questions as to the relevance of the finding of proteinuria in 5-ppm rats to human health assessment...*"

This conclusion is logically justified and the decision to choose hepatotoxicity over proteinuria as the critical effect seems to be scientifically sound.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   For remarks on BMD modeling *versus* alternative approach, see the Comment # A.3., above.

   In the Section 5.2.2.2. (the second paragraph on p. 199), this document states:

   "*...Internal doses associated with a benchmark response (BMR) of 10% extra risk were calculated. A BMR of 10% extra risk of fatty changes in the liver was selected because the POD associated with this BMR fell near the low end of the range of experimental data points (see plots in Appendix D). As noted U.S. EPA (2000), "[t]he major aim of benchmark dose modeling is to model the dose-response data for an adverse effect in the observable range (i.e., across the range of doses for which toxicity studies have reasonable power to detect effects) and then select a 'benchmark dose' at the low end of the observable range to use as a 'point of departure'."...*"

   The default $BMR_{0.1}$ was selected for dichotomous data in derivation of inhalation $BMDL_{0.1}$, because the 10% response was at or near the limit of sensitivity, as it is usually the case in most bioassays. So, in light of the above quoted authors' explanation (including the quotation from U.S. EPA (2000) guidelines), it seems that the BMR selected for use in deriving the POD is scientifically justified. This explanation is clear and convincing. The authors of Toxicological Review also selected the best available models to fit the data, applying appropriate statistical criteria (as described in the third paragraph on p. 199).

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?**

   The description of PBPK modeling applied to extrapolate animal to human CCl_4 dosimetry (Appendix C) is a potential source of confusion because of the overwhelming amount of detailed information, often redundant (e.g., repetitive discussion of different values of V_{maxC} in different models), sometimes inconsistent with the previous Section 3.5 (e.g., different values of QCC and QPC ascribed to the same model by Paustenbach et al.) and not always transparent relevance to POD extrapolations (e.g., discussion of Thrall et al. study, including data from personal communication, on p. C-2). As pointed out in General Comment #1, above, such a detailed discussion could be appropriate for academic dissertation, but in IRIS Toxicological Review it may be perceived as a lack of focus. The overall presentation also suffers from the lack of explicit source codes of PBPK model(s) used in POD extrapolations for derivation of RfC (both, CSL and CMD files).

   There are inconsistencies in reporting PBPK model parameterization. For example, human cardiac output (QC) and alveolar ventilation (QP) reported by Paustenbach et al. (1988) as 256 L blood/hr and 254 L air/hr, respectively, listed in Table 3-6 on p. 19 (n.b., there is an error in the reference to Paustenbach et al. "*(1998)*" in footnote [C]) would translate for a 70 kg default subject into QCC and QPC of 10.6 L blood/hr

per $kg^{0.75}$ and 10.5 L air/hr per $kg^{0.75}$ or about 13 L/hr per $kg^{0.7}$ respectively. However, in the Table C-2 on p. C-5, the same parameters QCC and QPC are listed as 15 L/hour-kg BW with reference to the same publication by Paustenbach et al. (1988), whereas 15 L/h/kg x 70 kg = 1050 L/hr (over 4 times more than in the Paustenbach model).

It is this Reviewer belief, that in general, the uncertainty factors relevant to animal and human variability should be applied to internal dosimetrics in POD extrapolations using PBPK model for derivation of reference values, especially for a short acting chemicals which clear from the organism faster than accumulate. Otherwise, paradoxical results can be expected (for example, see: *Byczkowski, J.Z.: At What Dose Chloral Hydrate Can Be Really Hazardous to Human Health? Society for Risk Analysis Annual Meeting, San Antonio, TX, 2007, Risk 007: Agents of Analysis M5.74, p. 52 (2007)*. For an example of how to apply uncertainty factors to internal dosimetrics in derivation RfC, see: *Byczkowski, J.Z.: TN & A, Inc. 1999 Draft Risk Assessment Issue Paper for: Deriving a Provisional Subchronic Inhalation RfC for Toluene (CASRN 108-88-3) Using Physiologically-Based Pharmacokinetic Modeling*).

Even though, for a short acting/fast metabolized chemical, the difference between application of UFs to internal vs. external dosimetrics may disappear when integrated variable is selected for POD (e.g., the total amount metabolized, or the area under the curve of concentration over time), or when a cumulative effect is considered as an end point (e.g., carcinogenesis), this may happen only for linear pharmacokinetics. Apparently, CCl_4 displays nonlinear pharmacokinetics, perhaps, mainly due to a suicidal inhibition involved in its metabolic clearance. Its toxicodynamics is even further complicated by lipid peroxidation, limits of free radical quenching and antioxidant protection (for quantitative considerations, see: *Byczkowski,J.Z., Channel,S.R., Pravecek,T.L., and Miller,C.R. (1996) Mathematical model for chemically induced lipid peroxidation in precision-cut liver slices: Computer simulation and experimental calibration. Computer Methods Progr. Biomed. 50, 73-84*). Therefore, facing nonlinear pharmacokinetics and toxicodynamics, it would be more appropriate to apply the uncertainty factors to internal dosimetrics, rather than to the predicted human external exposure concentration of CCl_4.

In the derivation of RfC (and then, a cancer slope factor, SF) in this Toxicological Review, two rates were selected as internal dosimetry, both are time-averaged values: MCA (from arterial blood concentration of CCl_4) and MRAMKL (from AUC of rate of CCl_4 metabolism over time). Since the animal exposure dosage was also adjusted from 6/24 hrs and 5/7 days to the average continuous exposure of 24 hr/day - 7 days/week, the dynamics of the PBPK model prediction has been lost. It seems thus questionable, whether in such an application mode the PBPK model was still capable of capturing a dosimetry that would be mechanistically relevant to the biological effects of short acting free radicals, metabolically generated from CCl_4.

6. Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

- **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

A composite uncertainty factor (UF) of 100 was applied in derivation of RfC for CCl_4 and the UF components are adequately explained in Sections 5.2.3. (p. 202 to 204) and 5.3. (p. 219 to 213). It seems that in this Review, the uncertainty factors were appropriately selected and adequately discussed, however, please see the General Comment # 4 above, for suggestions on improvement of the clarity of presentation and the Comment B.5 for suggestion to apply uncertainty factors to the internal dosimetric, rather than to the predicted human external exposure concentration of CCl_4.

$UF_H = 10^1$ for average human to sensitive human variability it is reasonable and has been objectively described and explained on pp. 202 - 203 .

$UF_A = 10^{0.5}$ for animal to human extrapolation is an exception from default, but it seems justified and has been described sufficiently.

$UF_D = 10^{0.5}$ for incomplete to complete data is also an exception, but it seems reasonably selected and has been objectively described.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 Guidelines for Carcinogen Risk Assessment (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

In Section 6.2.3 (under "Relevance to humans" on p. 255), this document states:

"*...There is no evidence in humans for hepatic cancer associated with carbon tetrachloride exposure. The experimental animal literature, however, shows carbon tetrachloride to consistently induce liver tumors across species and routes of exposure. Further, there are similarities between experimental animals and humans in terms of carbon tetrachloride metabolism, antioxidant systems, and evidence for the liver as a sensitive target organ. Together, this evidence supports a conclusion that experimental evidence for liver cancer is relevant to humans.*

Pheochromocytomas, on the other hand, were observed in only one species (the mouse). [...]

The relevance of mouse pheochromocytomas to humans is similarly unknown, although parallels between this tumor in the mouse and human led investigators to concluded that the mouse might be an appropriate model for human adrenal medullary tumors (Tischler et al., 1996). [...] Overall, this evidence supports a conclusion that experimental evidence for pheochromocytomas is potentially relevant to humans..."

Given that the hepatotoxicity by CCl_4 is demonstrable in humans and that it has been consistently demonstrated in many species of experimental animals, it is logical to postulate by analogy, that hepatocarcinogenicity also could be mechanistically relevant to humans. However, there is no such a parallelism with mouse pheochromocytomas. Thus, the conclusion that "*…experimental evidence for pheochromocytomas is potentially relevant to humans…"* bears great degree of uncertainty. It has been adequately discussed in the document, but perhaps, the uncertainty regarding pheochromocytoma in humans should be better emphasized in this section (6.2.3. Relevance to humans).

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

While the genotoxicity analysis provided in this Toxicological Review is impressive and the conclusions are perhaps sound, the overall interpretation misses one important mechanistic aspect of the CCl_4 mode of action. As already suggested a dozen years ago by McGregor and Lang (1996; *Carbon tetrachloride: Genetic effects and other modes of action. Mutation Res. 366: 181-195*) - the lack of clear evidence of DNA damage by this compound may indicate that the genetic damage in mammalian systems could result from "*interactions with proteins, rather than DNA and it could be induced secondarily to the toxicity of* CCl_4". The same authors also concluded that "*there would appear to be a number of possible routes toward* CCl_4 *carcinogenesis that do not involve direct interaction with DNA*". Indeed, CCl_4 induces c-fos and c-jun and in a time-dependent manner increases levels of AP-1. Moreover, in Kupffer cells, the primary radical as well as secondary products of CCl_4 metabolism and lipid peroxidation increase the DNA binding of NF-kappaB, which may induce the expression of cytokines, e.g., TNF-alpha.

It appears to this Reviewer that the discussion of MOAs involved in hepatocarcinogenicity of CCl_4 considers only the two extreme alternatives in a somewhat simplistic manner, e.g., either cytotoxicity/regeneration, or genetic damage, and avoids discussing the crucial epigenetic mechanisms which are most probably involved in both cancer and noncancer end point effects caused by environmentally-relevant, low concentrations of this pro-oxidant chemical.

Typically, a massive cytotoxicity, with the associated apoptosis (and at the extreme – necrosis) which can eventually stimulate regeneration, are demonstrable at high concentrations of pro-oxidant chemicals, mostly when tissue levels of free radical scavengers and natural antioxidants became depleted. Actually, the increased proliferation rate, which is a *sine qua non* attribute of carcinogenicity (e.g., it may be conveniently traced by the increase in ornithine decarboxylase and S-adenosylmethionine decarboxylase activities), appears much earlier and at significantly lower concentrations of the pro-oxidant chemical than those needed for a noticeable cytotoxicity (see the included pictogram, Figure 1, reproduced from *Byczkowski,J.Z., & Kulkarni,A.P.: Oxidative stress and pro-oxidant biological effects of vanadium. In: Vanadium in the Environment. Part 2: Health Effects, Chapter 12 (Nriagu,J.O. Ed), pp. 235 - 264, J.Wiley & Sons, New York, 1997*). Both enzymes, ornithine decarboxylase and S-adenosylmethionine decarboxylase, are required for spermidine synthesis, which is generally considered to be one component of the pleiotropic rapid growth response in any tissue. Treatment of rats with CCl_4 *in vivo* dramatically

increases concentrations of N1-acetylspermidine and putrescine in the liver, and moreover, it was demonstrated in cultured rat hepatocytes that the increase in spermidine or spermine levels are essential for hepatocyte proliferation. However, as a biomarker - both enzymes display relatively narrow dose-response characteristics. In the tissue treated with pro-oxidant chemicals, at concentrations high enough to produce noticeable apoptosis or necrosis, the ornithine decarboxylase and S-adenosylmethionine decarboxylase activities fell below the untreated controls.

In the dose-response to CCl_4 (and several other pro-oxidant chemicals too) it appears that with increasing exposure dose - the increased proliferation rate, with its potential for carcinogenesis, precedes necrosis, and even before the natural antioxidant levels get depleted and lipid peroxidation takes over - the proliferation drops to a very low rate. The biomarkers of cellular proliferation relate to the dose of pro-oxidant nonlinearly. At high concentrations of CCl_4 and its metabolites which deplete natural antioxidants/scavengers, initiating massive lipid peroxidation and making the unprotected DNA vulnerable to free radical attack and thus genotoxicity - usually a massive necrosis occurs instead of the cellular proliferation (see the Figure 1, reproduced below, for a combined model of dose-response to a typical pro-oxidant chemical; please note the nonlinearities and modeling discontinuities, represented by the bars).

It seems, thus, that the genotoxicity which can be observed at very high concentrations of CCl_4 in oxidatively unprotected genetic models, is not directly relevant to the potential low-dose proliferative responses to CCl_4, perhaps mediated epigenetically at the level of cellular signal transduction (for example *via* protein tyrosine phosphorylation) at the environmentally relevant doses (which are at least a couple orders of magnitude lower than the necrotizing levels). It is likely that the epigenetic mechanisms (e.g., oncogene derepression or activation) rather than genotoxicity or necrosis/regeneration may be responsible for carcinogenicity observed in CCl_4 –treated animals.

Therefore, as already concluded in the Toxicological Review, the genotoxicity at low exposures is not a very plausible mode of CCl_4 action. The quoted above modeling provides additional hint that genotoxicity as a MOA for CCl_4 hepatocarcinogenicity would require a prior depletion of natural antioxidant protection, but in the tissue models when such a depletion of antioxidants has been achieved, necrosis rather than the cellular proliferation was observed.

So, while the MOAs implied already in this Toxicological Review for liver carcinogenicity, promoted by CCl_4, have been transparently and objectively described in the document, their relevance to humans remain highly uncertain and perhaps, additionally, the epigenetic MOA should be considered (which would be also consistent with the non-cancer effects).

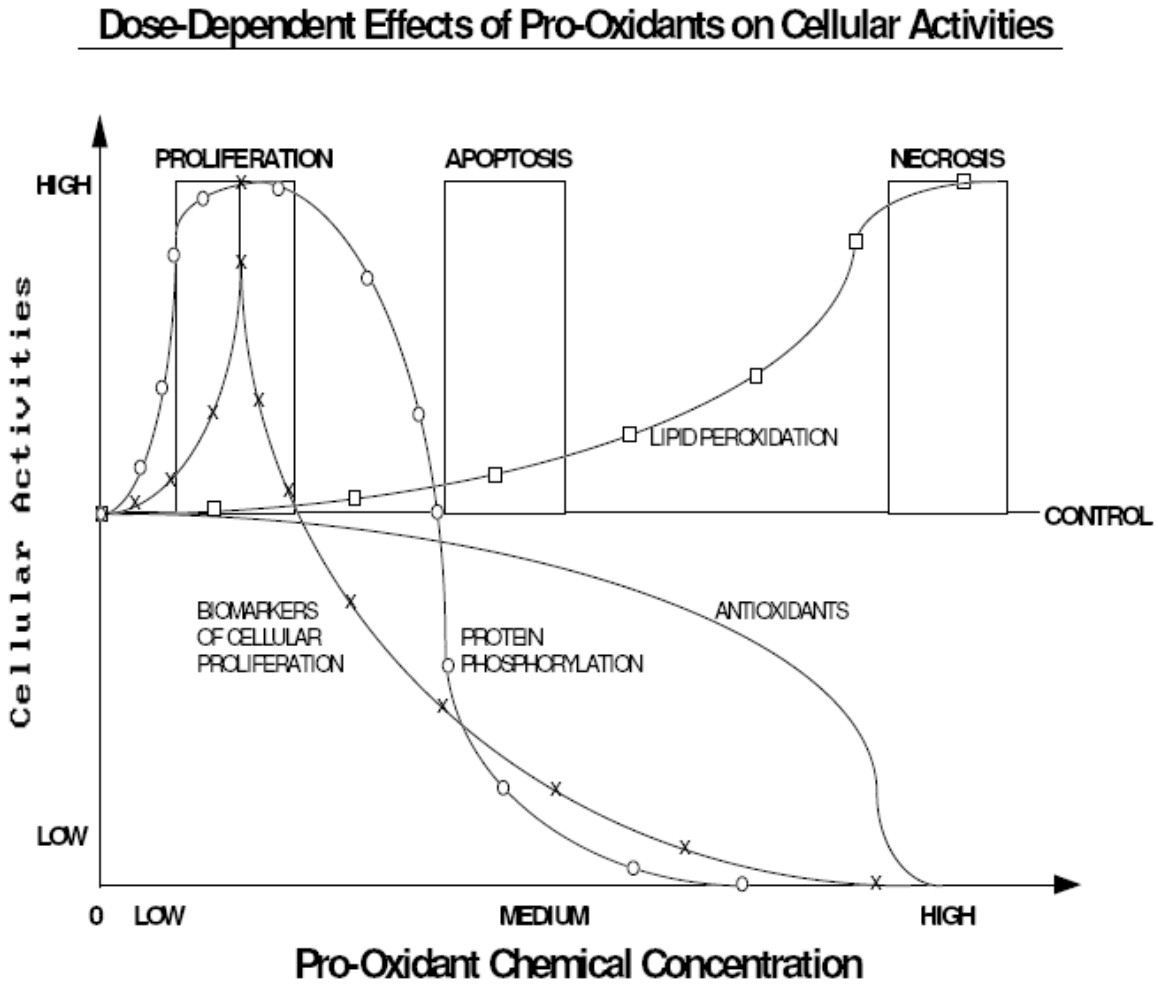


Figure 1 reproduced from Byczkowski & Kulkarni (1997). Effects of different concentrations of pro-oxidant chemicals on cellular function and activities: □—□ lipid peroxidation; —— vitamin E-type antioxidant level; ○—○ protein tyrosine phosphorylation; X—X ornithine decarboxylase and S-adenosylmethionine decarboxylase activities. The curves are computer-generated results of simulation of: lipid peroxidation and antioxidant depletion (BBPD computer program developed by Byczkowski et al., 1995, based on data by Tappel et al., 1989), protein tyrosine phosphorylation (BBPD computer program developed by Byczkowski and Flemming, 1996, based on data by Vroegop et al., 1995 and experimental results of Heffetz et al., 1990), ornithine and S-adenosylmethinine decarboxylation (BBPD computer program developed by Byczkowski and Flemming, 1996, based on Corcoran et al., 1994).

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   Obviously, the epigenetic model discussed in Comment C.2. above, has been developed with data from experiments using single static doses of prooxidant chemicals and thus, it remains highly uncertain how the biomarkers of cellular proliferation would behave under the intermittent on-off exposures to CCl_4. If the pro-oxidant stressor affects the tissue system for a relatively short time (e.g., 6 hrs) and disappears (e.g., for another 18 hrs and/or 2 days of weekend; see Comment B.5. above), and then the cycle is repeated again, many times (see Figure 2.A. reproduced below), this gives the tissue chance to heal after the oxidative stress and the organ has time to regenerate (see: *Rao,P. S., Mangipudy,R.S. and Mehendale,H. M. (1997) Tissue injury and repair as parallel and opposing responses to CCl4 hepatotoxicity: a novel dose-response. Toxicology 118(2-3): 181-193*). For fast clearing parent compound and its short-lived metabolites, this kind of on-off intermittent exposures, typically applied in animal bioassays, produce a different kinetics than a near steady-state human exposure scenario (e.g., assumed for RfC). The relation with the original experimental data may be even further distorted when an integrated variable is used as a dosimetric term in theoretical modeling of dose-response in humans (see the included below Figure 2, reproduced from *Byczkowski,J.Z. (1999) in: TN & A, Inc. Draft Risk Assessment Issue Paper for: Deriving a Provisional Subchronic Inhalation RfC for Toluene (CASRN 108-88-3) Using Physiologically-Based Pharmacokinetic Modeling*).

   In any event, under assumption that the short-lived free radical metabolites of CCl_4 and the peroxidative products are responsible for hepatocarcinogenicity, it may be unrealistic to expect a linear proliferative response *versus* time-averaged integrated CCl_4 dosimetrics (for discussion of the mechanistic relevance of time-weighted dosimetry, see the Comment B.5., above).

   It seems, therefore, that a nonlinear approach to dose-response, consistent with both epigenetic cancer and noncancer MOA (e.g., represented by RfD/RfC), would be more appropriate and more relevant to potential hepatocarcinogenesis by CCl_4 in humans than the linear extrapolations or even a simplified MOA involving hepatocellular cytotoxicity and regenerative hyperplasia.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

   The issue of mouse pheochromocytomas, which appeared during the treatment with CCl_4, have been adequately described qualitatively and quantitatively, and characterized sufficiently in this Toxicological Review. However, their relevance to human cancer risk is highly uncertain as it is discussed under Comment C.1. (see above).

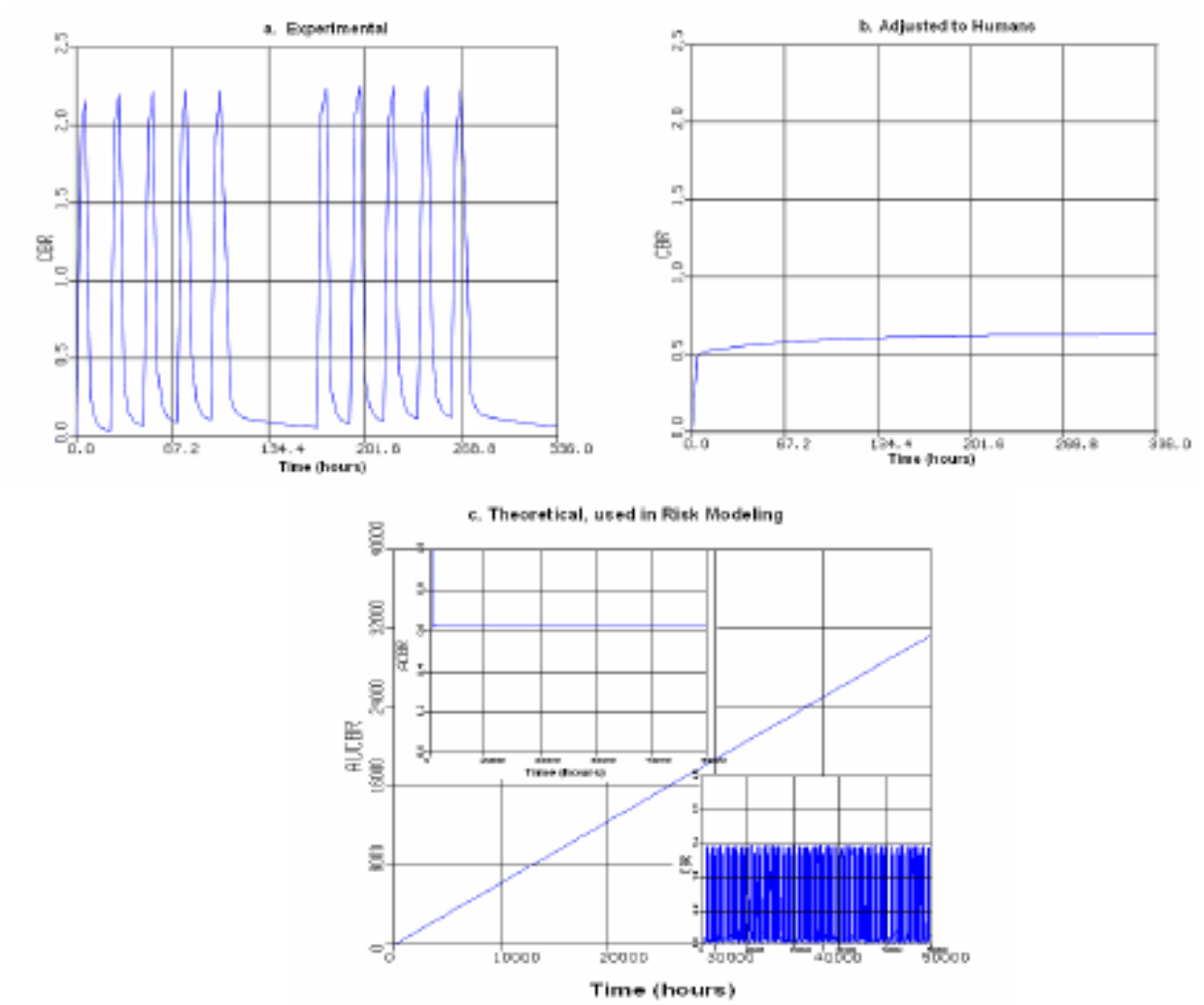


Figure 2 reproduced from Byczkowski (1999). The results of PBPK modeling of internal dosimetry for fast clearing chemical: a. under experimental exposure conditions; b. extrapolated to near steady-state exposure conditions; c. the resultant simulated (predictive) kinetics of integrated and time-weighted variables.

5. **Nonlinear approach: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.**

Since in the case of a free-radical generating, prooxidant chemical, such as CCl_4, the assumption of a threshold (due to limited levels of the natural scavengers of free-radicals and antioxidants; e.g., vitamin E, C, GSH, etc.) and a non-linear dose-response (discussed in the Comment C.2. above) seems perfectly logical, this Reviewer believes that the RfD/RfC could be preferably used to asses cancer risk to humans from exposures to CCl_4. While the linear approach to estimate CCl_4 cancer risk is certainly health-

protective, it can result in exaggerated risk estimates in comparison to the alternative approach, e.g., using the epigenetic MOA which has been argued in Comment C.2 (see above). This alternative approach to cancer risk has been insufficiently explored in this Toxicological Review.

6. **Linear extrapolation: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?**

   For reasons already discussed in Comment C.2. (see above), this Reviewer believes that the linear extrapolation of cancer data to low dose exposures to CCl_4 is not a preferable approach. It would be very difficult to defend such an approach based on scientific reasoning and currently available data. Also, the data for mouse pheochromocytomas with an uncertain relevance to humans should not be used in derivation of cancer risk value.

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

   Critical comments regarding the PBPK model use in derivation of RfC (see comment B.5. above) hold also for application of the same model for route-to-route extrapolation of dosimetry in cancer risk estimates. As emphasized in Comment C.3. (see also Figure 2, above), the use of time-weighted CCl_4 dosimetry has a questionable relevance to mechanisms of carcinogenesis.

8. EPA's 2005 Guidelines for Carcinogen Risk Assessment provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?

The recommendation to apply a linear low-dose extrapolation approach for assessment of CCl_4 cancer risk along with its justification is not convincing to this Reviewer, who suggests to use an alternative nonlinear approach, as discussed in Comments C.2. and C.6 (see above). Moreover, applying to human risk assessment the linear extrapolation with the final oral cancer slope factor of 7E-2 $(mg/kg/d)^{-1}$ implies that the *de minimis* cancer risk (1E-6) for CCl_4 in drinking water would require enforcing as low concentration as 0.5 ug of CCl_4/L of water (0.5 p.p.b). This concentration is well below practical quantifiable limits for CCl_4 and even below the method detection limit of EPA Method 624 for purgables (according to 40CFR part 136).

References:

Byczkowski,J.Z., Channel,S.R., Pravecek,T.L., and Miller,C.R. (1996) Mathematical model for chemically induced lipid peroxidation in precision-cut liver slices: Computer simulation and experimental calibration. Computer Methods Progr. Biomed. 50, 73-84

Byczkowski,J.Z., & Kulkarni,A.P.: Oxidative stress and pro-oxidant biological effects of vanadium. In: Vanadium in the Environment. Part 2: Health Effects, Chapter 12 (Nriagu,J.O. Ed), pp. 235 - 264, J.Wiley & Sons, New York, 1997.

Byczkowski,J.Z. (1999) in: TN & A, Inc. Draft Risk Assessment Issue Paper for: Deriving a Provisional Subchronic Inhalation RfC for Toluene (CASRN 108-88-3) Using Physiologically-Based Pharmacokinetic Modeling.

Byczkowski, J.Z.: At What Dose Chloral Hydrate Can Be Really Hazardous to Human Health? Society for Risk Analysis Annual Meeting, San Antonio, TX, 2007, Risk 007: Agents of Analysis M5.74, p. 52 (2007).

Hodgson, E. and Levi, P.E. "Introduction to Biochemical Toxicology", Chapter 20 Hepatotoxicity. Appleton & Lange, Norwalk, 1995.

McGregor,D. & Lang,M. (1996) Carbon tetrachloride: Genetic effects and other modes of action. Mutation Res. 366: 181-195.

Rao,P. S., Mangipudy,R.S. & Mehendale,H. M. (1997) Tissue injury and repair as parallel and opposing responses to CCl4 hepatotoxicity: a novel dose-response. Toxicology 118(2-3): 181-193.

Gary Ginsberg, Ph.D.
University of Connecticut Health Center
Yale School of Public Health

Dr. Gary Ginsberg is an Assistant Professor of Community Medicine at the University of Connecticut Health Center and is adjunct faculty at the Yale School of Public Health. He has recently served on the National Academy of Sciences Panels on Human Biomonitoring and on Improving U.S. Environmental Protection Agency (EPA) Risk Assessment Methods. He is a member of the Federal Advisory Committee evaluating EPA's approaches to protecting children's health (the Children's Health Protection Advisory Committee) and he also serves on EPA's Scientific Advisory Board. Dr. Ginsberg received his Ph.D. in toxicology from the University of Connecticut and conducted post-doctoral research at the Coreill Institute for Medical Research. He has published in the areas of carcinogenesis/mutagenesis, physiologically-based pharmacokinetic (PBPK) modeling, early life windows of vulnerability, and genetic polymorphisms.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

   Response: This Toxicological Review is clear and logical following a natural sequence beginning with basic hazard, pharmacokinetic and mechanistic information, with this information then applied to the task of selecting key studies, points of departure (PODs) and extrapolation techniques for developing separate cancer and non-cancer toxicity values for the oral and inhalation dose routes. The manner in which these values are derived is generally explained well, but as described below, certain aspects of the pharmacokinetic modeling are not sufficiently described. In terms of being concise, the document seems redundant with the same issues raised and explained in pretty much the same manner in numerous locations. For example, the understanding of cancer MOA at high dose as opposed to low dose is described repeatedly in Sections 4, 5 and 6. Discussion of the MOA and supporting data can be shortened with reference to the initial text location to decrease redundancy and improve document flow (e.g., As described in Section 4.7.3.4, there is evidence to support non-linear cytotoxic MOA for liver cancer at high doses but the evidence of liver tumors at an otherwise non-toxic dose along with other mechanistic considerations, leaves open the possibility of a low dose linear MOA).

2. **Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.**

   Response: Colby, et al (1994) Toxicology 94: 31-40. Adrenal activation of carbon tetrachloride: role of microsomal P450 isozymes.

   There are numerous initiation-promotion studies employing high dose carbon tetrachloride to induce necrosis, hepatic regeneration and promotion of low dose initiators in two stage carcinogenesis protocols (e.g., Tsuda, et al. Jap J Canc Res 84:230-236,1993). These types of studies and references should be part of the evidence that carbon tetrachloride is a well known promoter at high dose. Further, this literature should be examined to see if data are available for the evaluation of initiating potential of CCl4 in such 2 stage designs.

3. **Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.**

   Response: Improved understanding of cancer mechanism in the liver may be gained by classical initiation-promotion liver studies in which low doses of carbon tetrachloride are given in conjunction with promoters (partial hepatectomy, phorbol esters, etc) to determine whether it has initiating potential in rodent liver. Studies into the mechanism of the adrenal tumors would be very helpful to understand if parent compound or metabolite are the key dose metrics for PK modeling and whether there is a potential non-linearity in the dose response. The paper published by Colby et al. 1994 (see above) may be a useful start in that endeavor. Additional epidemiology of exposed workers is worthwhile to follow up on the suggestive evidence of lymphocytic cancer and further explore the potential for adrenal, liver and other tumors. The epidemiology studies may be enhanced by phenotyping individuals for CYP2E1 level as this varies based upon life style (e.g., alcohol ingestion), disease status (e.g., diabetes), genetics (polymorphisms), and exposure to other xenobiotics (other solvents, drugs). The epidemiology studies may also be enhanced by genotyping individuals for glutathione transferase polymorphisms and for other factors that may modify anti-oxidant and cellular defense status. As pointed out in the document, the data gaps in the areas of reproductive and developmental toxicity can be the subject of future testing to decrease the need for a database uncertainty factor. Finally, although not particularly likely, the potential

for carbon tetrachloride to be endocrine disruptive (hormonal mimic or impairment of hormonal systems) should be explored.

4. **Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?**

Response: The characterization of uncertainty in Sections 5 and 6 could be more complete and more descriptive. It is currently very qualitative, with these sections mostly just listing the various uncertainties. Some thought should be given to weighting these uncertainties in terms of how much they affect the confidence in the overall assessment (low, medium or high importance). For example, Table 5-20, Pages, 244-245, could potentially have a column for this ranking in which, for example, the uncertainty regarding human relevance could be ranked as low (text describes both liver and adrenal tumors as likely relevant), but that some other uncertainties (e.g., low dose MOA) could be rated as high. Other key uncertainties not specifically elaborated in text or tables: 1) Dose metric for adrenal tumors: the choice was made to use arterial concentration of parent compound rather than amount metabolized; without identification of an MOA for this endpoint, PK and dose-response modeling based upon parent compound is a reasonable default but is uncertain – this should be acknowledged; 2) Interaction with other chemicals: exposure to CCl4 will occur against a backdrop of other exposures which may induce or inhibit CYP2E1 or detoxification pathways. 3) Disease processes – it would seem to be worth specific mention of diabetes as a condition that could elevate CYP2E1 levels, leading to additional uncertainty over population variability. I would also add genetic polymolrphsims to the sources of variability listed, particularly in GSTs and other anti-oxidant defense systems. 4) Uncertainty regarding effect of time-weight averaging exposure in the Japanese inhalation bioassay: the low dose is said to be below the range of toxicity, which makes sense based upon the TWA exposure concentration (0.9 ppm). However, this was a 5 ppm exposure concentration for the duration of exposure, an exposure level shown in other studies to be able to induce hepatoxicity (Table 4-15). This raises the question of whether hepatoxicity may be occurring and then repaired so the net result is no evidence of toxicity at this dose, yet there may be toxicity occurring on an acute basis during the exposure interval. This may affect thinking about the low dose MOA. I located an unclear attempt at expressing this in the text (2nd para, page 238); this statement needs to be made coherent and further developed in the uncertainty section.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

Response: The choice of Bruckner et al. (1986) may in fact be the best choice for RfD derivation, although consideration should be given to the study showing that CCl4 can attack macromolecules at relatively low acute exposures, as indicated by radiolabeled CCl4 binding to nuclear and especially mitochondrial DNA at the lowest dose tested (3.2 mg/kg, Levy and Brabec, 1984). While this is not direct evidence of hepatotoxicity, it is important to keep in mind that leakage of SDH may not be the most sensitive indicator. This endpoint indicates gross damage to the plasma membrane such that large

macromolecules can escape into the circulation. There are a variety of important upstream events such as lipid peroxidation, GSH depletion and dysregulation of calcium homeostasis that may occur at lower dose. Evidence of DNA binding at a dose below the BMDL for SDH leakage suggests that other CCl4 effects may be detectable at lower doses, although this is not known because careful dose response studies for these effects have not been reported down to low dose. The possibility of low dose biochemical perturbations that are part of the hepatotoxic MOA should be considered as an additional source of database uncertainty in RfD derivation. In support of using Bruckner, et al. as the primary study is that SDH is a well accepted index of hepatotoxicity in which relatively small changes from baseline can be detected. Further, the dose response for SDH leakage in rats from this study (LOAEL of 10 mg/kg/d, NOAEL of 1 mg/kg/d) is generally consistent with histopathological findings (5 mg/kg/d x 10 days, minimal cytoplasmic vacuolation – Smialowicz, et al. 1991) and enzyme leakage and histopathology in mice (LOAEL of 8.6 mg-kg-d, NOAEL of 0.86 mg/kg/d – Condie, et al. 1986).

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

   Response: See answer to Q#1 directly above. Within the limits of the testing done and parameters measured, this may be the most useful and reasonably sensitive endpoint to date. However, there is uncertainty due to data gaps in reproductive testing and the potential for low dose biochemical effects that are part of the hepatoxic MOA. Further, statements on Page 193 point out that liver histopathology was more sensitive than liver enzyme leakage for POD selection in the RfC derivation. This may also be true for oral exposure but there is no long-term oral study with adequate sensitivity and histopathology to test this. This adds to the uncertainty in using Bruckner et al.'s SDH data as the primary outcome for risk assessment.

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   Response: BMD derivation is transparently presented in Appendix B. The only concern is the choice of the 10 week as opposed to the 12 week data. The rationale given was that the exact number of rats are known (5/grp) at 10 weeks but only the range of animals used (7-9/grp) is known for the 12 week data. This does not make the data unusable and BMD analysis can be conducted by assuming 8 animals/group at all doses. The dose response at 12 weeks is slightly steeper (difference between the 10 mg/kg/d dose vs control/low dose is slightly greater) and the greater N at 12 weeks suggest that the BMD modeling may turn out slightly differently. This should be checked, especially since the RfD is being raised 6 fold from the pre-existing value based solely on using BMD instead of LOAEL/NOAEL methodology (same dataset, same UFs). The creation of a 6 fold less health protective value based solely on changed methodology merits full assessment of that methodology to ensure that it is robust and defensible.

4. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

   - **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

   - **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

   Response: The intra- and inter-species UFs are appropriate. However, the lack of a PBPK model for refining the RfD derivation requires further explanation given the use of this technique to extrapolate kinetics across species for inhalation exposure. Are there no oral data with which to calibrate a model? Is it not possible to derive an oral model based upon extrapolation from the inhalation model (this is actually done to derive an oral cancer slope factor from the inhalation model). More explanation is needed.

   The subchronic to chronic extrapolation does not use a full 10 fold UF but rather 3 fold, largely on the basis that inhalation studies failed to show a difference between subchronic and chronic dose response. However, there are 2 reasons why inhalation may not be as sensitive as oral exposure to the buildup of toxicity from CCL4 dosing: 1) first pass delivery from oral but not inhalation exposure; 2) gavage dosing delivering higher acute dose compared to inhalation, which is more evenly spread out than the bolus nature of gavage dosing. These factors combine to cause the peak exposure at the target site to be greater after oral exposure. This may more readily lead to repetitive and cumulative damage from oral exposure. The uncertainty in this cross-dose route extrapolation combined with the uncertainty created by not actually having useful long-term oral studies, makes the subchronic to chronic extrapolation uncertain. Thus, there is justification for the use of the full 10 fold UF for this extrapolation, and the justification provided in this document for only 3fold is rather weak.

   It could be argued that the database uncertainty factor of 3 fold is too low based upon points raised above about possible upstream effects in the form of lipid peroxidation, GSH depletion, macromolecular binding, and derangement in calcium homeostasis. However, low dose mechanistic studies are unavailable and it may not be obvious if such perturbations were found, at what point would they be considered to be adverse or toxic. Therefore, a 3 fold UF can be acceptable under the current circumstances.

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the**

Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.

Response: The JBRC study is clearly the most appropriate dataset for RfC derivation among the candidate datasets. It is tempting to consider the human dataset (Tomensen, et al. 1995) for this purpose because of apparent low dose effects and greater relevance of a human study, but estimates of doses in these worker groups are crude with a fairly wide exposure window (rather than a specific dose) representing each group.

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   Response: This appears to be well justified and it makes sense that lipid accumulation (likely oxidized lipids, which as a waste product can be difficult for hepatocytes to remove) would be a more sensitive indicator than enzyme leakage of CCl4 induced hepatotoxicity from chronic exposure.

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

   Response: this is a questionable call by EPA. The JBRC studies show similar sensitivity of liver and kidney to CCl4-induced toxicity as stated on page 142. The fact that subchronic studies show liver to be more sensitive may merely indicate that the liver is quicker to manifest the toxic insult perhaps because of the opportunity factor (first pass effects) or differences in metabolic activation or detoxification/cellular defenses. Evidence from chronic exposures should be used to drive the RfC to the extent that these data are reliable. The arguments against using the chronic proteinura data are not compelling as relying upon the subchronic study to dictate the dose response for chronic nephrotoxicity may underestimate the potential for the kidney to accumulate damage related to CCl4. The size of the difference between subchronic and chronic effect levels may have something to do with quality and sensitivity of the study as well as the range of exposures tested, in addition to the accumulation of damage. It is logical for proteinuria to be an early signal of renal pathology, with the high frequency in aged animals making interpretation more complex. The description on Pages 142 and 143 should have shown incidence and severity data for this endpoint and related renal toxicity endpoints to better document the relevance (or lack thereof) of proteinuria to CCl4 risk assessment. If nothing else, the proteinuria data add to the uncertainty regarding proper selection of the key endpoint.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify**

and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.

Response: This BMD effort appears to be reasonable and properly justified derivation of POD was reasonably done and conducted well

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?**

   Response: The PBPK modeling should be made more transparent by listing the inhalation concentration in the rodent corresponding to the BMD and BMDL. In that way one could directly compare across species (HEC vs conc in rodent study at BMD). This should appear on Tables 5-6 and 5-7. Additionally, the text should explain the major rodent-human differences that yields greater dosimetry in rodents and the confidence one has that these physiologic and metabolic differences are accurate. For example, the percentage of body fat (0.08 rat, 0.2 human) appears to be a backfit, while another sensitive parameter, metabolic rate, is also a backfit (Table C-2, page C-5). Further, the blood:air partition coefficient was measured as being lower in humans than rodents. The confidence in these data should be described as it is pivotal in creating cross species dosimetry differences. Otherwise, the PBPK modeling appears to be appropriate and reasonably well calibrated. The use of data from competing models as a sensitivity modeling exercise is an added benefit.

6. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

   - **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

   - **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

   Response: These UF choices are generally reasonable, although there may be sufficient uncertainty with regards to proteinuria being the driving end point for risk assessment instead of fatty liver to increase the database UF to 10 fold. In leiu of this, EPA could model the proteinuria data to study the implications of

this apparent lowest LOAEL and either use that determination directly for RfC derivation, or use it to further inform the magnitude of the database UF.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

   Response: I agree with the "likely to be carcinogenic in humans" designation for CCl4. The animal datasets indicate a consistent pattern of liver tumors in a variety of inhalation and oral bioassays, a finding that is very unlikely due to chance. Additionally, the adrenal tumors occurred along a clear dose response pattern in mice by both oral and inhalation exposure. While liver and adrenal tumors have not been found in humans, the human studies are not sufficient for an evaluation of carcinogenic potential. Given that the underlying biochemical and pathologic processes leading to cancer in rodents likely also exists in humans, there is no reason to doubt that CCl4 can increase cancer risk in humans. The suggestion of human lymphatic tumors in more than one study is a potentially important epidemiologic finding that requires follow up. The only potential question about human relevance is the issue of the nature of the response at low, environmentally relevant doses. Animal evidence of liver tumors at an inhalation concentration below that required for hepatotoxicity comes from one dataset and the supporting biochemical/genetic toxicity data for low dose linearity is uncertain. This uncertainty leads to the application of low dose modeling and unit risk calculation as performed in this document, with the assumption of relevance to low environmental exposures in humans. This is appropriate, especially given the likelihood of CCl4 additivity to background damage and promotional processes that have the potential to enhance carcinogenesis, even at low doses.

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

   Response: the emphasis of this document's cancer MOA discussion is on the cytotoxicity/cell proliferation high dose phenomena. While this may be an important component at high dose, there is evidence of a non cytotoxic (or at least not obviously necrotic) mechanism which may involve run down and depletion of cellular defenses, irreversible binding to macromolecules either as the trichloromethyl radical or as reactive lipid hydroperoxides, and genotoxicity. The genetic toxicology database is overall not supportive of mutagenesis or genetic damage as being a primary mechanism. However, Table 4-12 (page 93) points out some important uncertainties in the genotoxicity studies, some of which will be true

for many chemicals (e.g., solvent interference with test result) and some more specific to CCl4(e.g., CYP2E1 down regulation by standard inducing systems). Added to these uncertainties is the fact that in a few studies, CCL4 has showed a genotoxic effect in the absence of S-9 mix, suggesting that there are other types of metabolism inherent to the test system that can dechlorinate CCL4 and lead to radical generation. The formation of radicals (trichloromethyl, lipid hydroperoxides) likely takes place at sub-toxic doses and so merits some consideration as the explanation for the tumor response in the JBRC study at a relatively low, non-toxic levels. In addition, the potential for linearization of the dose-response function at low doses is a reasonably strong possibility due to inter-individual variability in pharmacokinetics, levels of antioxidant defense mechanisms, DNA repair, and background promotional processes.

Overall, the document's inclusion of high dose and low dose MOA possibilities is appropriate although the various MOA discussions in the document tend to emphasize the high dose phenomena. The low dose MOA discussion is brought in secondarily mostly to explain one datapoint rather than as a primary mechanism with sufficient footing to drive low dose extrapolation.

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   Response: While I agree that there is utility in estimating cancer risk according to linear and non-linear projections, the document makes little attempt to bring these very different approaches together into a unified synthesis. What are the implications of the two approaches? Just looking at the contrast between the nonlinear vs linear inhalation approaches (basically the RfC vs the IUR), the RfC derived in Section 5.2 is 0.1 mg/m3 while the IUR is 6E-06/ug/m3, or 0.006/mg/m3. Doing some simple math shows that the RfC represents a cancer risk of: 0.1*0.006 = 6E-04, which is 600 times above de minimis and 6 times about the upper end of the target risk range at superfund sites. The document should provide perspective on the size of this difference and its meaning – i.e., that if one chose the non-linear approach, one would be out of bounds for protecting public health if in fact the low dose linear model is correct. Given the suggestive evidence of low dose carcinogenesis below toxicity thresholds and uncertainties with respect to genotoxicity, this document's recommendation of a linear low dose modeling approach is a prudent way to deal with the uncertainties in a reasonably health protective manner.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

   Response: The pheochromocytoma response in mice occurred in both genders and was clearly dose related. Thus there is no reason to believe this endpoint represents an aritifact. The fact that the tumors are benign does not materially detract from their relevance as indicators of a CCL4-induced carcinogenic process. The evidence I cited above (Colby, 1994) for CCl4 activation in rodent adrenal adds to the evidence for this endpoint. This evidence was not cited in the EPA draft document; additional literature

search and evaluation should be conducted regarding the potential MOA for adrenal tumors when making revisions to this document. This being said, I agree with EPA's characterization of the adrenal tumors as relevant to humans and characterizing the dose response as uncertain. Greater emphasis should be placed on the uncertainties in PBPK modeling and cancer risk estimation for this endpoint given the lack of MOA information and uncertainty with regards to key dose metric for estimating internal dose and risk.

5. ***Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.**

   Response: As stated above, the non-linear approach is reasonable to consider, together with its quantitative implications. The disparity between the non-linear and linear approaches is so large that they cannot easily be used in tandem when making risk judgements and either one has to be chosen over the other, or some hybrid approach is needed. For example, if one doesn't rely upon the low dose linear approach, its clear that the potential for low dose carcinogenesis creates a substantial degree of uncertainty (spread in level of health protectiveness is 600 fold). This might cause the advocate of the non-linear approach to use an additional UF for potential carcinogenicity (e.g., 10x) to get the target dose down in a range that has a better chance of protecting against both cancer and non-cancer endpoints. Such an approach is not novel and has been used for Group C carcinogens in certain regulatory settings (e.g., USEPA, ODW). However, the approach in this document of choosing the low dose linear approach is the most clear, prudent and scientifically defensible approach.

6. ***Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?**

   Response: The cancer modeling approaches were reasonable explorations of the dose response at low dose. However, little importance should be given to the run of the inhalation data in which the top doses were discarded as that is a dose response involving only a low dose and a control. Single dose studies provide minimal data for dose response modeling and so this exercise should just be seen as a screening level cross check. Other techniques to test whether the response at low dose is compatible with the remainder of the dose response or whether there is a dose-response break point might be more helpful in

determining whether the dose response might be different if more sub-toxic doses were available. The switching of tumor endpoint when going from the inhalation unit risk to the oral slope factor appears to not make much sense given that both the oral and inhalation slope factors are based upon the same (inhalation) bioassay. In that bioassay, the liver tumor response occurred at lower dose than the adrenal response. Therefore, the explanation must lie in the PBPK modeling extrapolation from inhalation to oral dosimetry and the shift in key dose metric between adrenal (MCA) and liver (metabolized dose) endpoints. The manner in which these factors modify the oral dose response to make liver tumors the risk driving endpoint should be explained given that it seems to fly in the face of the dose response data presented in Table 4-6. Further, the uncertainties in this approach should be clarified. Given the uncertainties in the MOA and PBPK modeling of the adrenal tumor dose response, it may be more appropriate for the inhalation slope to stick with the same endpoint as the oral slope (female liver tumors). This would only affect the oral slope by a factor of 2 (6E-06/ug-m3 down to 3E-06/ug-m3) and would simplify the analysis. However, a straightforward explanation of the reasons for switching endpoint with dose route and how the modeling led to this result would be important if the draft approach is kept. Along these lines, my observation is that a systemic target site (like adrenal tumors) would logically be the risk driver for inhalation exposure while liver should be the driver for oral exposure. That's because oral exposure leads to first pass metabolism in the liver, creating greater internal dose and opportunity for carcinogenic insult in liver. In contrast, inhalation exposure leads to greater systemic doses of parent compound and more opportunity for extra-hepatic targeting of the tumor response. However, we still need the clarifications requested above on this matter of switching target sites and dose metrics when switching dose route.

The objective of using a BMR of 5% for all tumor sites makes sense (have POD as far removed from hepatotoxic portion of dose response as possible). However, the backing off to the 10% BMR for all tumor endpoints other than female rat liver is not well justified and should be described graphically (e.g., where is the end of the dose response range vs where does the 5 vs 10% BMR lie?). Further, the potential risk implications should be described (would you tend to get higher slope factor with 5 vs 10%BMR?).

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

Response: The EPA assessment is basically correct that oral studies provide inadequate data for dose response assessment given the inadequate designs in some cases, and the high doses used and excessive mortality in other cases (e.g., NCI, 1977). The one possible exception is the adrenal tumor response seen in the oral NCI mouse study in which high doses yielded approximately a 50% response in male mice and 20% response in female mice. This could provide interesting comparison to the inhalation dose response for this endpoint after correction for internal dose differences across dose routes. Since the oral slope factor is relying upon liver tumors and not adrenal tumors, this comparison may turn out to be moot but still a very interesting point of reference and confirmation of the predictability of the inhalation to oral modeling extrapolation.

Regarding the inhalation to oral extrapolation, this is not well explained, which is unfortunate because this is a pivotal step in the construction of the oral slope factor. In the absence of useable oral cancer data, there is logic to conducting PBPK modeling dose route extrapolation to derive the equivalent oral human

dose associated with the tumor dose response in animal studies. However, the current modeling exercise fails to provide any oral data for calibration or validation of the model, so it appears to be based solely upon first principles and assumptions. The key parameter is rate of uptake of CCl4 from the g.i. tract (RGIL), a parameter that apparently is backfit (from where, how?) but could potentially be based upon pharmacokinetic studies with CCl4 or other similar chlorinated solvents that were gavaged. The HED for a particular blood level will also depend upon assumptions of whether human oral exposure is relatively constant or bolus in nature. None of these assumptions are described. Given all this, it is difficult to ascribe a particular level of confidence to the oral dosimetry modeling.

8. **EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?**

   Response: As stated in response to earlier questions, the recommendation in this draft document to use low dose linear modeling is reasonable. This has been well thought out and the alternative (non-linear extrapolation) has, to a degree, been brought up for consideration.

Dale Hattis, Ph.D.

Clark University

Dr. Dale Hattis is a Research Professor with the George Perkins Marsh Institute at Clark University. For the past three decades he has been engaged in the development and application of methodology to assess the health, ecological, and economic impacts of regulatory actions. His work has focused on approaches to incorporate interindividual variability data and quantitative mechanistic information into risk assessments for both cancer and non-cancer endpoints. Recent research has explored age-related differences in sensitivity to carcinogenesis and other effects, a taxonomy of different non-mutagenic modes of action for carcinogenesis with likely differential implications for age-related sensitivity, physiologically-based pharmacokinetic (PBPK) modeling of acrylamide dose in rats and humans, and mechanism-based dose response modeling of carcinogenic effects from ionizing radiation. Current efforts are using PBPK modeling to better assess dose response relationships for human birth weight changes and developmental delays associated with exposure to the insecticide chlorpyrifos during pregnancy. Dr. Hattis is a leader in efforts to replace the current system of uncertainty factors for non-cancer effects with distributions based on empirical observations. He is a member of the Clean Air Science Advisory Committee panel reviewing U.S. Environmental Protection Agency (EPA) efforts to reassess the National Ambient Air Quality Criteria for nitrogen oxides and sulfur oxides, and for several years he served as a member of the Food Quality Protection Act Science Review Board. Until recently Dr. Hattis was a member of the Environmental Health Committee of the EPA Science Advisory Board. For 2007, he was the Chair of the Dose Response Specialty Group of the Society for Risk Analysis. He also served as a member of the National Research Council Committee on Estimating the Health-Risk-Reduction Benefits of Proposed Air Pollution Regulations. Dr. Hattis has been a councilor and is a Fellow of the Society for Risk Analysis, and serves on the editorial board of its journal, Risk Analysis. He holds a Ph.D. in Genetics from Stanford University and a B.A. in biochemistry from the University of California at Berkeley.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

The Toxicological Review is clear. Concise?—that would perhaps be an exaggeration. However the authors have addressed a very large literature in a reasonable way. I have two main problems with how they have utilized the available evidence—(1) the use of the rate of metabolism per unit liver tissue dose metric for PBPK modeling with no additional pharmacokinetic correction between species, and (2) the selection of a doubling of a particular enzyme level as the benchmark response, to be identified as the functional replacement for a NOAEL. Briefly, my concerns are:

- The implicit conclusion that there will be equal toxic and carcinogenic effects across species for an equal rate of production of reactive metabolites per unit liver tissue would be correct if the rates of destruction of the reactive metabolites across species are the same. This is possible, but no evidence is advanced to support this assumption. All of the reactive metabolites are capable of reacting with macromolecular cellular constituents without enzyme catalysis. An alternative inference might be drawn from the consideration that free radicals and the highly reactive oxidants are produced by normal utilization of oxygen in the body. The dangerous side effects (including DNA reactions) from the presence of these reactive moieties are partly limited by the presence of numerous antioxidant defense enzymes and cofactors (e.g. superoxide dismutase, catalase, and glutathione). It is not unreasonable to assume that the activities of these defensive processes in different species might be tuned to the rate of oxygen utilization in different species, which is known to follow the body weight$^{3/4}$ rule, similar to the usual pattern for elimination of drugs and the direct DNA reactive agent, ethylene oxide. This pattern leads to systematically slower elimination of the reactive agents in people relative to rodent test species by factors of several fold (with a central tendency proportional to $BW^{-1/4}$) Therefore I think that unless both the production and the loss of the reactive metabolites can be included in pharmacokinetic models based on reasonable empirical data, EPA should assume that the reactive metabolite internal dose will follow the same $BW^{-1/4}$ rule as is routinely used for parent chemicals that have not been subject to PBPK modeling. This would increase the estimates of the human equivalent doses and cancer potency by several fold, and decrease the RFC similarly.

- The identification of a doubling of SDH as the benchmark response (p. 177) is commendably based on expert opinions that this is a reasonable "indicator of concern" by expert groups, and a "toxicologically significant response" by the author of the primary study selected for BMD modeling. However it is puzzling that this response level is then assumed to correspond to the equivalent of a NOAEL for the application of uncertainty factors to derive the RfD. If this is the lower confidence limit on the dose that produces a "toxicologically significant response" why shouldn't it rather be treated as the equivalent of a LOAEL—or, at the very least, a point intermediate between a NOAEL and a LOAEL, perhaps requiring somewhat less than the full traditional 10-fold LOAEL/NOAEL uncertainty factor?

  In the absence of the expert opinion my impression is that the approach would be to identify a shift in the mean of 1 standard deviation (based on observations in the control group) as the BMR. I don't entirely approve of this formula because in my view it leads to much too large BMR's for inhibition of fetal growth responses. However the reasoning behind some sort of a standard deviation-based formula is reasonable in the absence of other information--that the biological system will control more important parameters more tightly—so the standard deviation of the normal population is an indication of the biological importance of keeping a particular parameter within narrow limits. In

this case the standard deviation for the control group is $0.4*5^{0.5} = 0.9$ so 1 standard deviation above the mean would be about 4.4 IU/ml rather than the doubling to 7 IU/ml that was used for the BMR. In my opinion alternative calculations of the BMD should be made on this basis. A doubling of the group mean enzyme level, as used for the present calculations, represents a movement for the average animal of about 3.5/0.9 = 3.9 standard deviations. This seems to me to be too much to be considered the equivalent of a NOAEL. If the levels are normally distributed, it would mean that the enzyme level of the average member of the exposed group has been raised to a level expected in only about 1/100,000 of the unexposed group.

2. Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.

I found a few in the literature (mostly very recent) that seem relevant for inclusion in the final analysis. The two papers below use initiation-promotion systems that seem to shed light on carbon tetrachloride's mode of action and dose response relationships. I have not been able as of this time to obtain the papers for a thorough reading. The abstracts of the papers are:

The inhalation exposure of carbon tetrachloride promote rat liver carcinogenesis in a medium-term liver bioassay.

Tsujimura K, Ichinose F, Hara T, Yamasaki K, Otsuka M, Fukushima S.
Toxicol Lett. 2008 Feb 15;176(3):207-14. Epub 2007 Dec 3.
Hita laboratory, Chemicals Evaluation and Research Institute, 3-822 Ishii-machi, Hita City, Oita 877-0061, Japan. tsujimura-kazunari@2ceri.jp

The potential of carbon tetrachloride (CCl4) to induce pre-neoplastic lesions in rat liver using a medium-term liver assay (Ito method) for the prediction of carcinogenicity was examined by nose-only inhalation exposure of male rats (15/group) to CCl4 vapor at concentrations of 0, 1, 5, 25, 125 ppm for 6h/day, 6 day/week, for a period of 6 weeks. The numbers and area of glutathione S-transferase placental (GST-P) positive foci were then determined. Additionally, other histopathological observations on the livers were recorded and serum chemical parameters and CCl4 concentrations in blood were measured. The areas and numbers of GST-P positive foci significantly increased in the CCl4-exposed rats at 25 and 125 ppm; but not at concentrations of 1 and 5 ppm. CCl4 blood concentration 24h after initiation of exposure in the 125 ppm group remained at about 5% of the 6h maximum concentration. These data from CCl4-exposed rats clearly show that inhalation exposure can be used in the rat medium-term liver assay, the method is available for the screening of volatile chemicals and is therefore a useful tool in cancer risk assessment. This is the first report of the use of inhalation exposure in this medium-term predictive assay.

Interactions in the tumor-promoting activity of carbon tetrachloride, trichloroacetate, and dichloroacetate in the liver of male B6C3F1 mice[*1]
Richard J. Bull, , Lyle B. Sasser and Xingye C. Lei
Toxicology, Volume 199, Issues 2-3, 1 July 2004, Pages 169-183

Abstract

Interactions between carcinogens in mixtures found in the environment have been a concern for several decades. In the present study, male B6C3F1 mice were used to study the responses to mixtures of dichloroacetate (DCA), trichloroacetate (TCA), and carbon tetrachloride (CT). TCA produces liver tumors in mice with the phenotypic characteristics common to peroxisome proliferators. DCA increases the growth of liver tumors with a phenotype that is distinct in several respects from those produced by TCA. These chemicals are effective as carcinogens at doses that do not produce cytotoxicity. Thus, they

encourage clonal expansion of initiated cells through subtle, selective mechanisms. CT is well known for its ability to promote the growth of liver tumors through cytotoxicity that produces a generalized growth stimulus in the liver that is reflected in a reparative hyperplasia. Thus, CT is relatively non-specific in its promotion of initiated cells within the liver. The objective of this study was to determine how the differing modes of action of these chemicals might interact when given as mixed exposures. The hypothesis was that the effects of two selective promoters would not be more than additive. On the other hand, CT would be selective only to cells not sensitive to its effects as a cytotoxin. Thus, it was hypothesized that neither DCA nor TCA would add significantly to the effects produced by CT. Mice were initiated by vinyl carbamate (VC), and then promoted by DCA, TCA, CT, or the pair-wised combinations of the three compounds. The effect of each treatment or treatment combination on tumor number per animal and mean tumor volume was assessed in each animal. Dose-related increases in mean tumor volume were observed with 20 and 50 mg/kg CT, but each produced equal numbers of tumors at 36 weeks. As the dose of CT was increased to ≥100 mg/kg substantial increases in the number of tumors per animal were observed, but the mean tumor size decreased. This finding suggests that initiation occurs as doses of CT increase to ≥100 mg/kg, perhaps as a result of the inflammatory response that is known to occur with high doses of CT. When administered alone in the drinking water at 0.1, 0.5 and 2 g/l, DCA increased both tumor number and tumor size in a dose-related manner. With TCA treatment at 2 g/l in drinking water a maximum tumor number was reached by 24 weeks and was maintained until 36 weeks of treatment. DCA treatment did not produce a plateau in tumor number within the experimental period, but the numbers observed at the end of the experimental period were similar to TCA and doses of 50 mg/kg CT. The tumor numbers observed at the end of the experiment are consistent with the assumption that the administered dose of the tumor initiator, vinyl carbamate, was the major determinant of tumor number and that treatments with CT, DCA, and TCA primarily affected tumor size. The results with mixtures of these compounds were consistent with the basic hypotheses that the responses to tumor promoters with differing mechanisms are limited to additivity at low effective doses. More complex, mutually inhibitory activity was more often observed between the three compounds. At 24 weeks, DCA produced a decrease in tumor numbers promoted by TCA, but the numbers were not different from TCA alone at 36 weeks. The reason for this result became apparent at 36 weeks of treatment where a dose-related decrease in the size of tumors promoted by TCA resulted from DCA co-administration. On the other hand, the low dose of TCA (0.1 g/l) decreased the number of tumors produced by a high dose of DCA (2 g/l), but higher doses of TCA (2 g/l) produced the same number as observed with DCA alone. DCA inhibited the growth rate of CT-induced tumors (CT dose=50 mg/kg). TCA substantially increased the numbers of tumors observed at early time points when combined with CT, but this was not observed at 36 weeks. The lack of an effect at 36 weeks was attributable to the fact that more than 90% of the livers consisted of tumors and the earlier effect was masked by coalescence of tumors. Thus, the ability of TCA to significantly increase tumor numbers in CT-treated mice was probably real and contrary to our original hypothesis that CT was non-specific in its effects on initiated cells. It is probable that the interaction between CT and TCA is explained through stimulation of the growth of cells with differing phenotypes. These data suggest that the outcome of interactions between the mechanisms of tumor promotion vary based on the characteristics of the initiated cells. The interactions may result in additive or inhibitory effects, but no significant evidence of synergy was observed.

3. Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.

In the light of my responses under question #1, one type of research that would clearly be helpful would be measurements in comparable rat and human liver metabolism systems of the rates of destruction of the reactive metabolites of carbon tetrachloride or steady state concentrations of those metabolites as indexed by rates of formation of metabolite-specific adducts. If metabolite elimination rates are in fact slower in people than in rodents, then steady state concentrations of metabolites should be greater in human than in the rodent systems for a given rate of metabolite formation. To be fully credible, such comparisons

should be done with fresh liver systems (e.g. slices, isolated hepatocytes) that preserve as much of the in vivo concentrations of enzymes and cofactors as possible.

Improved relatively short term research on dose response relationships would also be helpful using a liver cancer initiation-promotion system such as that described in the Tsujimura et al. (2008) paper listed above. In that kind of system I would also like to see testing where various amounts/durations of carbon tetrachloride are administered either before known promoters of liver tumors or after known initiators. This might well greatly improve our information on dose response for different kinds of cancer-enhancing activities for this compound.

In parallel with these studies, I think it would also be helpful to eludicate dose response relationships for more sensitive tests of liver effects, including cell replication lipid peroxidation and s-adenosylmethionine depletion.

4. **Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?**

The implicit assumption of passive destruction of the reactive metabolites at identical rates in humans vs rodents should be articulated, together with the mechanistic reasoning and prior experience with other chemicals that could lead to different assumptions as to the appropriate causal dose metric (gross metabolism rate vs AUC of the active metabolites) and interspecies projection rules for causally-relevant delivered dose.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

The choice of the Bruckner et al. (1986) 12-week oral gavage study is not clearly incorrect. The discussion in the document articulates reasonable bases for considering it a bit better than competing alternative subchronic studies. More problematic is the apparent need to base the RfD determination on a single study, and even a single data set within that study, rather than some integrative calculation across different data sets.

Within the Bruckner et al study, there is good reason to focus on the data for SDH, as this appears to be the most sensitive of the enzyme activities to the influence of carbon tetrachloride at the lowest doses. However I question the exclusion of the data for the 12 week time point.

The excuse for this exclusion is that the precise number of animals is not given for the groups studied at different doses for this time point, preventing calculation of a unique standard deviation for input into the BMDS software. However the number of animals is specified within a reasonably narrow range of 7 to 9 animals. The implications of this 7 to 9 animal range for the uncertainty in the standard deviations at each

group are shown in Table 1 on the next page. For this table, columns 3-6 show different estimates of the standard deviation of the SDH measurements depending on whether the 12 week dose group data come from groups of 7, 8, or 9 rats respectively (based on the simple formula $SD = SE * N^{0.5}$, where SD is the standard deviation, SE is the standard error, and N is the number of animals in the group). Column 7 shows the average of the three estimates, which in all cases is very close to what would be expected if the groups had 8 rats. Column 8 then shows the standard deviation of the uncertainty distribution arising from an equal weighting of the 7, 8, and 9 rat calculations. Finally, column 9 shows the coefficient of variation (CV) for this uncertainty—the standard deviation from column 8 divided by the group mean from column 7. For all groups it can be seen that the standard deviation is a bit more than 6% of the mean.

The 10-week exposure data, by contrast, allow a precise calculation of standard deviations for each group because they are reportedly based on precisely 5 animals per group. Unfortunately, just because one can do an unambiguous calculation of the standard deviation, that does not mean that there is not some statistical uncertainty in the resulting estimates. For comparison with the 6.3% CV found for the 7-9 rat/group uncertainty in Table 1, I did some Monte Carlo simulations of the uncertainty in calculated standard deviations from groups of 5 randomly drawn animals from a population having the mean and reported standard deviation for the SDH levels in the control group for the 10-week animals (mean of 3.5 and standard deviation of 0.4*50.5 = 0.894 IU/ml). The results for 3 runs of 5,000 trials each are shown in Table 2. It can be seen that the inherent uncertainty of the precisely calculated standard deviation for the 5 animal groups has an inherent sampling error that is much larger (CV of more than 36% of the mean) than the added uncertainty due to the 7-9 animal range of animals per group for the 12 week exposures.

But there is more. When we go from the 5-animal groups modeled in Table 2 to 7-9 animals per group, we of course decrease the statistical sampling error uncertainty. For comparison with Table 2, Table 3 shows the sampling error uncertainty in the estimate of the standard deviation for groups of 8. It can be seen that increasing the number of animals per group from 5 to 8 leads to an appreciable decrease in the uncertainty in the estimated standard deviation—from a CV of over 36% to a CV of just under 27%.

Now what happens when we add back the uncertainty in whether there are 7, 8, or 9 animals (as in Table 1) to the decreased uncertainty from statistical sampling errors modeled in Table 3? Table 4 gives these results. It can be seen that all things considered, despite the uncertainty in numbers of animals per group, the uncertainty in the standard deviations that can be estimated for the 12-week exposure groups is less than the uncertainty in the estimates of standard deviation for the 10-week exposures that were selected for BMD analysis (a CV of 27.5% for the 12-week exposure groups vs a CV of 36.7% for the 10-week exposure groups, assuming the same mean and standard deviation). Therefore at the very least the 12-week results should be preferred; but it would be even better to do the ultimate BMD calculations for both periods of exposure, and then combine the results in some reasonable way (e.g. calculation of an arithmetic or geometric mean for the resulting Points of Departure, perhaps weighted by the inverse of the variance of the estimated points of departure calculated from the two sets of data).

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

Within the Bruckner et al study, there is good reason to focus on the data for SDH, as this appears to be the most sensitive of the enzyme activities to the influence of carbon tetrachloride at the lowest doses.

Table 1
Uncertainty in the Standard Deviation for Different Dose Groups Arising from Uncertainty in Whether Each Dose Group Had 7, 8, or 9 Rats

Group (mg/kg-day)	Mean SDH (IU/ml) 12 weeks	Std error	Standard Deviations for 7-9 rats: 7 rats	8 rats	9 rats	Mean of SD for 7-9 rat cases	SD of Uncertainty among 7-9 rat cases	CV of SD for 7-9 rat cases
0	3.2	0.4	1.058	1.131	1.200	1.130	0.071	0.063
1	1.9	0.1	0.265	0.283	0.300	0.282	0.018	0.063
10	8.7	2	5.292	5.657	6.000	5.649	0.354	0.063
33	145.7	57.9	153.2	163.8	173.7	163.6	10.3	0.063

Table 2
Uncertainty in the Standard Deviation of SDH Levels Calculated for the Control Group for the 10 Week Animals (5 Animals Per Group), Based on 3 Monte Carlo Simulation Runs of 5000 Trials Each

	Mean of SD	SD of SD	CV of SD
1st Run	0.831	0.307	0.369
2nd Run	0.840	0.308	0.367
3rd Run	0.837	0.305	0.364
Average of 3 runs	0.836	0.307	0.367

Table 3
Uncertainty in the Standard Deviation of SDH Levels Calculated for the Control Group for the 10 Week Animals if There Had Been 8 Animals Per Group—the Average for the 12 Week Exposed Animals, Based on 3 Monte Carlo Simulation Runs of 5000 Trials Each

	Mean of SD	SD of SD	CV of SD
1st Run	0.867	0.232	0.268
2nd Run	0.865	0.231	0.268
3rd Run	0.864	0.233	0.270
Average of 3 runs	0.865	0.232	0.269

Table 4
Uncertainty in the Standard Deviation of SDH Levels Calculated for the Control Group for the 10 Week Animals if There Had Been 7-9 Animals Per Group—the Average for the 12 Week Exposed Animals, Based on 3 Monte Carlo Simulation Runs of 5000 Trials Each, Incorporating Both the Sampling Error Uncertainty and the Uncertainty in the Number of Animals Per Group (7-9, equally weighted)

	Mean of SD	SD of SD	CV of SD
1st Run	0.861	0.236	0.274
2nd Run	0.865	0.238	0.275
3rd Run	0.865	0.237	0.275
Average of 3 runs	0.863	0.237	0.275

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD.**

   BMD modeling is the best approach for analyzing the data.

   Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.

   As I mentioned in my responses to the general charge questions, I think that the 2x SDH increase is to large a change to be considered the functional equivalent of a NOEL and treated as the POD for application of uncertainty factors. For convenience I repeat my rationale for this, and my discussion of an alternative approach:

   The identification of a doubling of SDH as the benchmark response (p. 177) is commendably based on expert opinions that this is a reasonable "indicator of concern" by expert groups, and a "toxicologically significant response" by the author of the primary study selected for BMD modeling. However it is puzzling that this response level is then assumed to correspond to the equivalent of a NOAEL for the application of uncertainty factors to derive the RfD. If this is the lower confidence limit on the dose that produces a "toxicologically significant response" why shouldn't it rather be treated as the equivalent of a LOAEL—or, at the very least, a point intermediate between a NOAEL and a LOAEL, perhaps requiring somewhat less than the full traditional 10-fold LOAEL/NOAEL uncertainty factor?

   In the absence of the expert opinion my impression is that the approach would be to identify a shift in the mean of 1 standard deviation (based on observations in the control group) as the BMR. I don't entirely approve of this formula because in my view it leads to much too large BMR's for inhibition of fetal growth responses. However the reasoning behind some sort of a standard deviation-based formula is reasonable in the absence of other information--that the biological system will control more important parameters more tightly—so the standard deviation of the normal population is an indication of the biological importance of keeping a particular parameter within narrow limits. In this case the standard deviation for the control group is 0.4*50.5 = 0.9 so 1 standard deviation above the mean would be about 4.4 IU/ml rather than the doubling to 7 IU/ml that was used for the BMR. In my opinion alternative calculations of the BMD should be made on this basis. A doubling of the group mean enzyme level, as used for the present calculations, represents a movement for the average animal of about 3.5/0.9 = 3.9 standard deviations. This seems to me to be too much to be considered the equivalent of a NOAEL. If the levels are normally distributed, it would mean that the enzyme level of the average member of the exposed group has been raised to a level expected in only about 1/100,000 of the unexposed group.

4. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

I agree with this choice.

- **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

I agree with this choice.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

I agree with this choice.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document?

Yes

Please comment on whether the application of these uncertainty factors has been scientifically justified?

I believe they have been adequately justified.

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   I believe this is the best choice. A close second, in my view would be the human occupational study. That study could be interpreted better by estimating the mean exposures within the three groups of workers by fitting the exposures to a lognormal distribution, but the discussion in the document provided inadequate information to do this. What would be needed is the numbers of workers in each of the exposure groups

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   I believe this is a reasonable choice.

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

   I agree with this choice. However it seems to me that the document would benefit from showing analyses of implications for the RfC had proteinuria been used as the basis for calculating an RfC. At the expert panel meeting some commenters had the impression that the proteinuria response was actually more sensitive in picking up toxicologically significant effects from carbon tetrachloride. Alternative RfC calculations using this endpoint would clarify this and hence make the consequence of the choice of liver effects as the primary focus for the RfC more transparent.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   I think these choices are reasonable.

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?**

   As indicated in my comments for the general questions, I have a major disagreement with the choice of dose metric and its interspecies projection. For convenience, I repeat those comments here:

   The implicit conclusion that there will be equal toxic and carcinogenic effects across species for an equal rate of production of reactive metabolites per unit liver tissue would be correct if the rates of destruction of the reactive metabolites across species are the same. This is possible, but no evidence is advanced to support this assumption. All of the reactive metabolites are capable of reacting with macromolecular cellular constituents without enzyme catalysis. An alternative inference might be drawn from the consideration that free radicals and the highly reactive oxidants are produced by normal utilization of oxygen in the body. The dangerous side effects (including DNA reactions) from the presence of these reactive moieties are partly limited by the presence of numerous antioxidant defense enzymes and cofactors (e.g. superoxide dismutase, catalase, and glutathione). It is not unreasonable to assume that the activities of these defensive processes in different species might be tuned to the rate of oxygen utilization in different species, which is known to follow the body weight$^{3/4}$ rule, similar to the usual pattern for elimination of drugs and the direct DNA reactive agent, ethylene oxide. This pattern leads to systematically slower elimination of the reactive agents in people relative to rodent test species by factors of several fold (with a central tendency proportional to BW$^{-1/4}$) Therefore I think that unless both the

production and the loss of the reactive metabolites can be included in pharmacokinetic models based on reasonable empirical data, EPA should assume that the reactive metabolite internal dose will follow the same $BW^{-1/4}$ rule as is routinely used for parent chemicals that have not been subject to PBPK modeling. This would increase the estimates of the human equivalent doses and cancer potency by several fold, and decrease the RFC similarly.

6. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

   I agree with this choice.

   - **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

   As indicated above, I believe a $BW^{-0.25}$ correction should be added here to account for likely slower elimination of the active metabolites in people relative to rats. This would lower the RfC by a factor of about $(70/0.25)^{0.25} = 4$ fold.

   - **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

   I agree with this choice.

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

   Yes, with the exception of the failure in the document to correct for the likely difference in detoxification rate of the active metabolites in people vs rodents.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

   I agree with the conclusion and thing the justification is well made.

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

   I generally agree with EPA's analysis and conclusion. I would emphasize, however, that reactive metabolites are expected to be formed at low and high doses. Where an upward turning ("nonlinear") mode of action is present as well as a linear mode of action, the linear processes will dominate the dose response relationship at low doses, albeit at cancer incidence rates that will not be directly detectable with ordinary sizes of animal groups as the dose gets relatively low.

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   As per the previous comment, where linear and upward-turning nonlinear modes of action are present in the same system the low-dose dose response will tend toward linearity. In my view, therefore, I think EPA should do the best it can to estimate the low dose slope, and not present the "non-linear" threshold-implying calculations. I append to these comments as Appendix A an extended excerpt from a white paper that a colleague and I did for EPA a little more than a year ago discussing the relevant issues.

   In addition, one of the likely components of the carbon tetrachloride of action that is mentioned in the document is the depletion of S-adenosyl methionine. Therefore the following discussion of the dose response relationship for dichloroacetate seems relevant, as it too is thought to act via this process. The discussion is from a paper in press in *Critical Reviews in Toxicology* (Hattis, D, Rahmioglu, N, Verma, P., Hartman, K., Kozlak, M, and Goble, R. "A Preliminary Operational Classification System for Non-Mutagenic Modes of Action for Carcinogenesis." Critical Reviews in Toxicology, 2008, in press.)

   Among environmental chemicals, one of the stronger candidates for a hypomethylation mode of action is dichloroacetate (DCA). Dichloroacetate exposures lead to decreased methylation of the promoter regions of the proto-oncogenes c-*jun* and c-*myc* and increased expression of the corresponding m-RNAs (Tao et al 2000). These effects are preventable by dosing with methionine, a precursor of S-adenosylmethionine, the methyl donor in the pathway leading to transfer of methyl groups to DNA. Methionine dosing also prevents the induction of liver cancer by dichloroacetate in mice (Pereira et al. 2004).

   In this context it is of interest that an extensive 2-year carcinogenesis dose response study is available for DCA (Carter et al. 2003). This study yielded dose response information for both fully developed liver cancers (Figure 14) and premalignant liver foci of various histological types (Figure 15). Contrary to the

usual expectation for a mode of action of this type, the data do not indicate appreciable nonlinearity over the fairly wide dose range studied. They fail to reject even a simple linear stochastic interpretation of dose-response (P = 0.46 for a two-parameter model containing only a term for background and a term for the linear slope). [The "linear-stochastic" dose response function represented by the line in Figure 14 is directly derived from the expectation for a Poisson process in which individual cancer transformation events occur independently within individual animals:

$$\text{Fraction of animals with at least 1 tumor} =$$
$$1 - \text{fraction of animals with zero tumors} = 1 - e^{-m}$$
$$\text{(where m is average number of tumor transformations per animal)}$$

Rearranging, the number of tumor transformations per animal (plotted on the y axis for Figure 15) is

$$m = -\ln(1 - \text{fraction of animals with tumors})$$

where "ln" indicates natural (base e) logarithm.]

Because of the expectation for nonlinear dose response for this case, another option for dose response modeling would be a traditional probit-type analysis, which assumes a lognormal distribution of thresholds for individual animals (for more detailed description, see Hattis 2008; Hattis and Burmaster 1994). Figure 16 shows the same data from Figure 14 analyzed in this way. One benefit of this form of analysis is that it yields an estimate of the interindividual variability among the animals in the form of the geometric standard deviation of the assumed lognormal distribution of individual thresholds (3.1 in this case, the antilog of the log probit slope in the plot shown). The model fit by maximum likelihood in Figure 16 also does not depart significantly from the data (p = 0.42 for a 3-parameter model, where estimates were made of the background incidence of tumors, the probit slope, and the intercept).

Figure 14

Dose Response Relationship for Fully Developed Carcinomas in the Liver--Data of Carter et al. (2003) and Linear Stochastic Model Fit

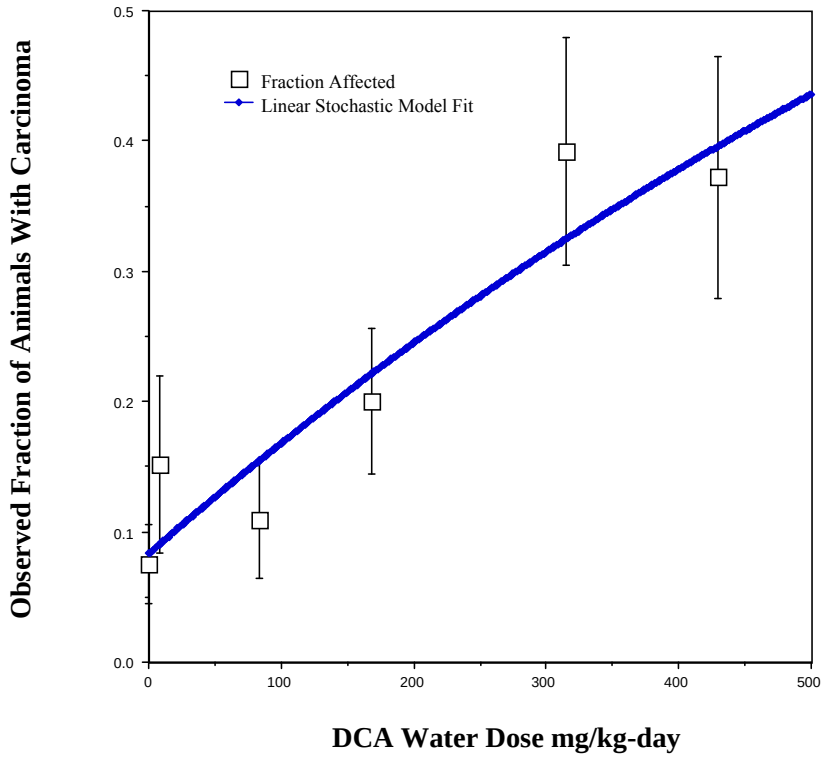
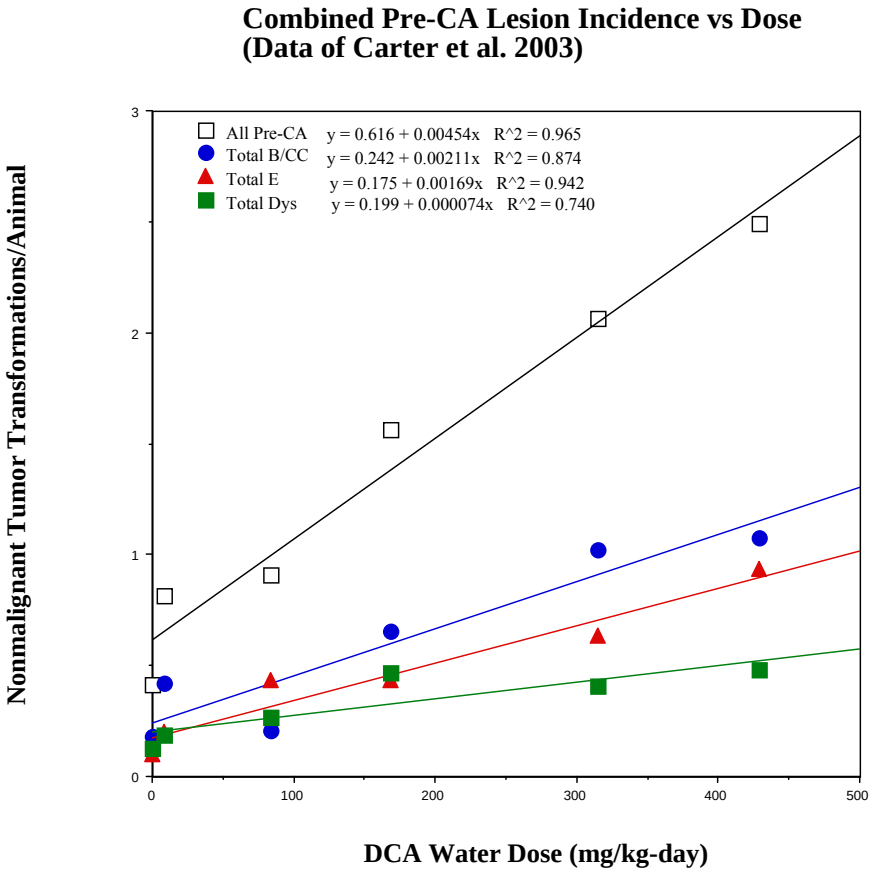


Figure 15



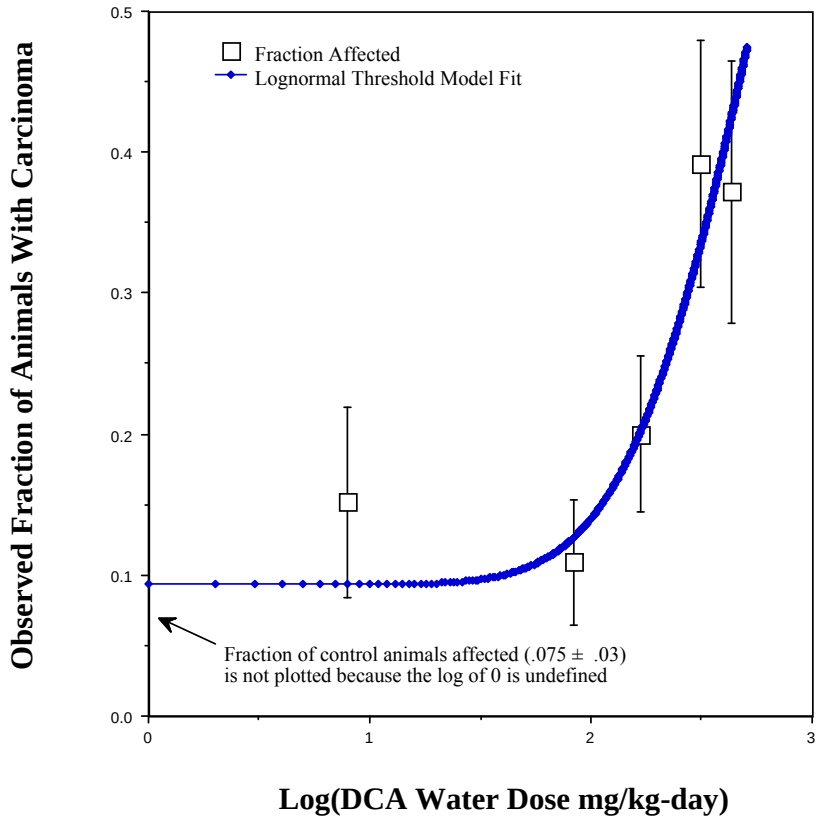
B/CC = Basophilic and/or clear cell

E = Eosinophilic

Dys = Dysplastic

Figure 16

Dose Response Relationship for Fully Developed Carcinomas in the Liver--Data of Carter et al. (2003) and Lognormal Threshold Distribution Model Fit



This case, with its rich detail of precursor lesions and mode of action hypotheses via SAM depletion, reduction of promoter methylation, and enhanced expression of proto-oncogene, seems to be a prime candidate for the development of a quantitative biologically-based model for carcinogenesis via a putative alteration in epigenetic control of gene expression.

References cited in this response:

Carter, J. H., Carter, H. W., Deddens, J. A., Hurst, B. M., George, M. H. and DeAngelo, A. B. (2003). Carcinogenesis in the male B6C3F mouse given the drinking water chemical dichloroacetic acid. *Environmental Health Perspectives* 111:53-64.

Hattis, D. (2008). Distributional analyses for children's inhalation risk assessments. *Journal of Toxicology and Environmental Health* 71(3):218-226.

Hattis, D. and Burmaster, D. E. "Assessment of Variability and Uncertainty Distributions for Practical Risk Analyses" Risk Analysis, Vol. 14, pp. 713-730, 1994.

Pereira, M. A., Wang, W., Kramer, P. M. and Tao, L. (2004). Prevention by methionine of dichloroacetic acid-induced liver cancer and DNA hypomethylation in mice. *Toxicological Sciences* 77:243-248.

Tao, L., Yang, S., Xie, M., Kramer, P. M. and Pereira, M. A. (2000). Effect of trichloroethylene and its metabolites, dichloroacetic acid and trichloroacetic acid, on the methylation and expression of c-Jun and c-Myc protooncogenes in mouse liver: Prevention by methionine. *Toxicol Sci* 54(2):399-407.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

I believe EPA's approach is entirely appropriate in this case. However I believe it would also be informative to include an alternative linear-low dose model estimate based on the liver tumors only. Further, it appears from the liver tumor results that a large fraction of the animals with pheochromocytomas must also have had liver tumors. If this is the case, then I think this is worth noting in the discussion.

Some of the other panelists expressed skepticism about the potency of carbon tetrachloride as calculated from the pheochromocytoma data set using a linear projection from the point of departure. As suggested at the review meeting, I think it would be useful to put the result in perspective by showing where it fits among the slope factors calculated for other small-molecular weight chlorinated hydrocarbons (e.g. vinyl chloride, methylene chloride, etc). As indicated in the previous paragraph, I also think it would be useful to calculate and report the slope factor that would be indicated just for the liver tumors by themselves.

5. ***Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the**

utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.

As indicated above and in Appendix A, I don't believe a low-dose nonlinear assumption is compatible with the expected linear production of DNA reactive metabolites at low doses. It could perhaps be compatible with an assumption that production of such metabolites only occurs with the manifestation of toxicity, but in this case one would also have to postulate that there is no such production even for liver cells that are on their way to necrotic death due to other causes, including normal background turnover in these cells. In general I think some normal turnover and replacement should be considered likely. I believe there is some low rate of normal background turnover of liver cells.

6. ***Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?**

   I generally agree with the EPA analyses and choices in this respect. I have some reservations about the exclusive use of a probit model for the pheochromocytoma Bayesian analysis because it implies an individual-threshold type dose response for which there is no specific justification.

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

   I agree that EPA has sound reasons for concluding that the available carcinogenesis studies by the oral route are considerably less than ideal because of the relatively short less-than-lifetime periods of dosing and observation, the use of only a single dose group vs. a control, and the excessively high incidence of tumors in at least some studies. It is not impossible to use these data, but they are far from ideal.

   I generally agree with the use of PBPK modeling to do route to route projections of doses and risks, and I think it is reasonable and preferable in this case. However there is some contradiction in the document because PBPK modeling was not used (and I believe it was said it could not be used) earlier in the

document in connection with the interspecies projection of the RfD. A 10-fold uncertainty factor was substituted instead. Somehow, the judgments in these two sections of the same document should be reconciled.

8. **EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?**

   Yes, I believe it has been well described. As reinforcement, see my discussion in Appendix A and my response to 3, 5, and 6 above.

Appendix A

Excerpt from
Uncertainties in Risk Assessment for Carcinogenesis: A Road Map Toward Practical Improvements

Dale Hattis and Robert L. Goble

December 21, 2007

The Resurgent Controversy Over the Expectation for Low Dose Linearity in Responses to Genetically-Acting Carcinogens

In pursuing our road-map to uncertainties in cancer risk assessment, there is really no way to avoid discussing the resurgent controversy over an issue that had been regarded as essentially settled for at least a couple of decades. Continuing through the current EPA cancer guidelines (U. S. EPA 2005), the standard assumption is that if an agent acts by primary reactions with DNA, then it should be expected that, at the limit of low doses, a linear no-threshold dose response relationship should prevail. To understand the origins of this and alternative concepts that continue to be advanced in the current literature, it is helpful to review some of the history of the intellectual discussion as it manifested itself before the older1986 version of the EPA cancer risk assessment guidelines (U.S. EPA 1986). After that, we will assess the merits of some of the more recent challenges to the low dose linearity theory and suggest ways forward to fairly represent this central uncertainty in projection of low dose cancer risks for genetically active agents.

The roots of the historical controversy can be traced to a basic difference between different sets of disciplines in mental models of biological systems, and the ways that chemicals and other perturbing influences can cause effects. The disciplines of physiology, traditional toxicology and pharmacology tend to foster a view of biological systems as complex interacting webs of processes. These systems are seen as exquisitely designed so that perturbation of any one parameter automatically gives rise to countervailing adaptations that, if the perturbation is not too large, will keep the system functioning within normal limits without serious or long lasting harm. This mental model leads directly to a general expectation that there should be thresholds in dose response; for any toxicant that acts by overwhelming some set of homeostatic processes there should be a dose below which the system can handle the perturbation without a meaningful adverse effect.

A different vision of some fundamental life processes arose from the ex-physicists who created the discipline of molecular biology in the decades after the end of World War II (e.g., Stent, 1963). This is the notion that there is a basic fragility in some functions that are central to life. When both somatic and germ cells divide, an enormous amount of information must be faithfully copied and distributed between the progeny cells. Mistakes can occur in this copying, and a change at even a single place in the DNA can give rise to important adverse (or, very rarely, beneficial) effects if by chance the mistake happens in just the wrong place in the DNA of the wrong cell. This leads to an intuition that even a single molecule of a DNA reactive chemical has a small but finite chance of doing lasting damage if it happens to react with the wrong place on DNA and if the DNA lesion is not repaired by the next time the DNA is copied.

In the 1970s and early 1980s it was recognized that basic bimolecular reaction kinetics require a fundamental linearity between the concentration of DNA reactants and relevant sites on DNA. However it was also recognized that there were many opportunities for at least high-dose nonlinearities both before and after DNA reaction in the sequence of events from intake of a DNA reactive agent (or a metabolic precursor) into the body to the ultimate manifestation of tumors (Hattis 1990).

In the 1970s some looked to pharmacokinetics as a potential source of threshold dose response relationships that might intervene between toxicant intake and the delivery of DNA reactive molecules to the nucleus of relevant cells. Figure 1 is an illustration similar to one that was published in Science (attributed to researchers at Dow Chemical) that attempted to make this pharmacokinetic-based threshold idea plausible. In the diagram, liquid (representing a continuous dosage of a toxicant) flows into a tank with two triangular holes. The level of liquid rises in the tank until some begins to flow out of the lower of the two holes (representing a high-affinity metabolic pathway producing a “safe” metabolite). A further rise occurs until the amount of liquid flowing out of the tank equals the amount flowing in. If the inflow is small enough that it can be completely balanced by flow out of the lower hole, then the liquid

will not rise to the level of the higher hole (representing the lower affinity enzyme producing the dangerous metabolite). Thus the analogy predicts a threshold of inflow into the tank, below which all of the metabolism is via the "safe" high affinity pathway.

Unfortunately, this representation of the competition between higher and lower affinity metabolic pathways is not compatible with conventional Michaelis-Menten enzyme kinetics (Hattis, 1990; Slikker et al. 2004). Using the basic Michaelis/Menten equation, the rate of the activating reaction (producing the dangerous metabolite, D) is:

$$\frac{dD}{dt} = \frac{V_{max}[C]}{K_m + [C]} \qquad (1)$$

where [C] is the concentration of substrate (the form of the toxicant that is absorbed from the environment), Vmax is the maximum rate of the reaction that produces the dangerous metabolite, and Km (the Michaelis constant) is the substrate concentration at which the reaction proceeds at half of its maximum velocity (Vmax). Similarly the rate of the competitive detoxifying reaction (producing the safe metabolite, S) is:

$$\frac{dS}{dt} = \frac{V_{max}'[C]}{K_m' + [C]} \qquad (2)$$

The [C]'s in the denominator of both equations can be neglected at low doses when they become small relative to the K_m's. At low doses we can therefore find the ratio of the substrate [C] that goes by the dangerous and safe metabolic pathways by simply dividing the two equations:

$$\frac{\text{rate of D production}}{\text{rate of S production}} = \frac{V_{max}[C]/K_m}{V_{max}'[C]/K_m'} \qquad (3)$$

and because the numerator [C]'s now cancel, it can be seen that we are left with a ratio of four constants. This means that below the dose region where there is appreciable saturation of the enzymes producing either the safe or the dangerous metabolite, the fraction of the substrate taken by each pathway approaches a constant, independent of dose. There are no dose rate effects in this low dose region (where [C] is much less than both Km's), there can be no thresholds, and indeed the system must operate linearly at the limit of low dosage, albeit with a different distribution of metabolism between "safe" and "dangerous" pathways than would be observed at higher doses. At the limit of high dose, the ratio of production of the dangerous to the safe metabolites is governed only by ratio of the two Vmax values;

whereas at lower doses the Km's become progressively more involved. If the higher affinity (lower Km) pathway produces the dangerous metabolite, then the fraction of material metabolized by the dangerous pathway will be greater than at the highest saturating doses, resulting in a convex-upward dose response relationship for DNA damage (e.g. the pattern seen for vinyl chloride). On the other hand, if the safe pathway has the lower Km then the portion of the chemical processed by the safe pathway will be greater at lower doses than is seen at higher doses. In the abstract of a paper (Gehring and Blau, 1978) describing a process model for carcinogenesis from electrophilic agents, Perry Gehring, (then a leader of the toxicology group at Dow Chemical) acknowledges that there should be an expectation for some "albeit negligible" carcinogenic risk from genetically acting chemicals at low doses.

It is well to emphasize that the basic Michaelis-Menten equation applied above is not simply an empirical formula. It is well grounded in fundamental mechanistic considerations of receptor association and dissociation kinetics with reasonably wide applicability (Hoel, 1985), The maximal velocity, Vmax, arises because there are a limited number of enzyme molecules available to catalyze the reaction, and each enzyme molecule is necessarily constrained to operate at a finite maximal rate (which varies according to substrate) in converting substrate into its product. The fact that the reaction proceeds linearly at low doses (with a rate constant of Vmax/Km) arises from the fact that the reaction is limited by the rate of diffusion of the substrate molecules into the active site of the enzyme—a rate that must be linear with substrate concentration at the limit of low doses. In the light of this Figure 2 offers a more accurate molecular-scale vision of the competition between enzyme-mediated activating and detoxifying processes. Each small substrate molecule has a "random walk" through a cellular compartment as it rebounds from collisions with other molecules. At the limit of low dosage, when there are few or no other similar substrate molecules around, the substrate molecule must have a finite chance of encountering the active site of each type of enzyme (or, similarly, a transport molecule taking it to a different compartment). Therefore each type of enzyme or macromolecular transporter must have finite opportunity to process the substrate molecule at the limit of low dosage.

The basic Michaelis-Menten enzyme equation form applies with equal force to active transport processes (in which specialized molecules utilize energy to pump specific molecules or ions into our out of cells), and to DNA repair processes. Thus the fundamental expectation for low dose linearity and high dose saturation applies similarly to these other components of the causal chain between external exposure and the generation of somatic mutations that are components of carcinogenesis. At the limit of low substrate concentration the Michaelis Menten enzyme/transport reaction rates are limited by the rate of diffusion of substrate molecules into the active sites of the enzymes/transport molecules; and those diffusion processes, given a specific temperature, are linear functions of substrate concentrations. At the limit of high concentration (where the substrate concentration is very much larger than Km), the reaction must approach a finite maximal rate (which, of course, varies according to the substrate) because there are a limited number of enzyme molecules and each one must have a limited capacity to process substrate.

With this as background, we can now examine the bases for some more recent claims that thresholds should be expected at low doses for genetically acting agents. A convenient starting point for this examination is a special issue of Mutation Research published in 2000 by participants at a conference sponsored by the European Centre for Ecotoxicology and Toxicology Of Chemicals (ECETOC), essentially a trade association research arm of European chemical companies.* Without going through

---

* The ECETOC web site (www.ecetoc.org) lists the Mutation Research special issue as one of their publications [ECETOC Publication No. 28 Dose-response and threshold-mediated mechanisms in mutagenesis - Mutation Research Special Issue (Published January 2000, volume 464], and describes the organization as follows:

> "…established in 1978 as a scientific, non-profit making, non-commercial association, financed by 50 of the leading companies with interests in the manufacture and use of chemicals. A stand-alone organization, it was

the threshold claims from each of the papers in this collection individually (Kirshch-Volders et al. 2000; Schulte-Hermann et al. 2000; Muller and Kasper 2000; Moustacchi 2000; Parry et al. 2000; Swenberg et al. 2000; Lowell 2000; Madle et al. 2000; Henderson et al. 2000; Crebelli 2000; Kirkland and Muller 2000; Speit et al. 2000; Parry 2000), three main types of arguments stand out:

- Multiple targets. Some specific modes of genotoxicity are reported to depend on multiple interactions between chemicals and target macromolecules (rather than the single-DNA-reactant-molecule DNA adduct formation mechanism discussed above). If the number of target interactions required to produce an effect is large, the resulting low dose dose response relationship can be expected to be highly upward-turning, and well approximated by a threshold.

- Multiple barriers. A molecule of a chemically reactive agent much pass multiple transport, potential detoxification, alternative targets for reaction other than DNA, and DNA repair hurdles in order to cause a permanent change in DNA sequence or chromosomal damage. The multiplicity of these hurdles makes it unlikely that any single molecule could cause an actual mutation along the pathway to carcinogenesis. If these multiple barriers do not produce an "absolute threshold" they can at least be expected to lead to a "pragmatic" or "practical" threshold below which exposure is of no real biological consequence.

- Inducible detoxification, apoptosis, and/or DNA repair processes. One result of exposure to a toxicant may be the induction of the levels of a variety of cellular and genomic defense processes. If this induction is effective enough, and occurs at low enough doses, it is possible that the prevention "good" that results from avoidance or repair of mutagenic damage from background processes may even be great enough to exceed the direct mutagenic harm done by the toxicant itself. This gives rise to "hormetic" dose response relationships in which the net mutagenesis and carcinogenesis is even reduced by some range of exposures to the toxicant compared to background (zero dose) levels.

Modes of Genetic Action Requiring Multiple Interactions with Macromolecular Target Molecules or Structures

The first paper in the Mutation Research special issue (Kirsch-Volders et al. 2000) gives a good theoretical mathematical account of the dependence of the shape of the dose response curve on the number of macromolecular targets that must be "hit" in order to produce an effect. Essentially, if a single hit on DNA, an alpha or beta tubulin structure, or topoisomerase is sufficient to cause an effect (assuming imperfect repair) then the fundamental math calls for a single hit Poisson process:

$$\text{Probability of Effect/Target} = 1 - e^{-m} \qquad (4)$$

where m is the average number of "hits" per target. In cases where the number of hits per target needed to cause an effect is larger than 1 (e.g. according to the authors, where the target is the spindle drawing chromosomes to different progeny cells during mitosis, or the nuclear membrane, by a mechanism that is not detailed in the paper), then the appropriate Poisson term for n hits required per target is substituted:

established to provide a scientific forum through which the extensive specialist expertise in the European chemical industry could be harnessed to research, review, assess and publish studies on the ecotoxicology and toxicology of chemicals."

$$\text{Probability of Effect/Target} = 1 - e^{-m}\frac{m^n}{n!} \qquad (5)$$

(where the notation n!, translated to English as "n factorial" means n X n-1 X …. All the way down to 1.)

The larger the n, the more steeply upward-turning the resulting curve will be—increasingly resembling, but not the same as a curve with a true threshold of zero probability of effect for a finite dose.

Later on, Kirsch-Volders et al (2000). add:

> "To be able to clearly assess a threshold, the spontaneous frequency of the analysed endpoint should be very low, ideally equal to zero; indeed a too high spontaneous background will lead to additive effects and a difficult estimation of small increases at low dose level."

This comment undermines considerably the generality of the earlier application of multi-hit analysis to putative multi-target mutation/chromosomal damage mechanisms at very low doses. Essentially it says that in order for the multi-hit formulas to apply at the limit of low dosage, the genetically active agent must cause genetic changes by a mechanism that is somehow distinct from all the processes that cause the appreciable background of genetic changes from all other endogenous and exogenous agents, as well as the imperfections in functioning of the apparatus of polymerases, spindle proteins etc. that maintain, copy and transmit the genetic material.

The essential low-dose linearity of agents that act in concert with background processes was discussed in some of the foundational papers that derived the methods for inferring low dose cancer risks in the 1970s (e.g. Crump et al. 1976). This general expectation can be illustrated with a simple example of a two-stage mutation process in which there is a background of 1 arbitrary unit and an expectation for 1 additional induced unit per 1 mg/kg continuous dose of a mutagenic agent (Table 2, adapted from Hattis and Smith, 1986). It can be seen that at high doses, the dose response relationship between excess tumors over background vs dose of the inducing agent appears almost perfectly proportional to $(\text{dose})^2$. This is because at high doses, far above the background rate of the tumors, the agent predominantly acts by causing both mutations in the two-step process. As the dose is reduced to regions where it causes mutations that are a small fraction of background rates, the induced mutations predominantly cooperate with mutations that result from the background processes—leading to an increment in tumors over background that approaches linearity with dose of the added inducing mutagen.

One example of a process that may involve multiple targets is the action of spindle poisons such as vinblastine and colcemid (Parry et al. 2000). Older observations by Elhajouji et al (1995, 1997) are often cited as evidence of thresholds for agents that inhibit spindle function, and indeed the paper of Elhajouji et al (1995) does present plots for four different agents (carbendazim, nocodazole, mebendazole and colchicine) showing relatively sharp dose dependent transitions in the production of centromere-positive micronuclei. These plots utilize linear, rather than log-transformed x-axes, in contrast to other analyses we evaluate below. In vivo data are available in a recent report by Choudury et al. (2004). However even in this case, we believe it is worth compiling data on background rates and mechanisms of spindle malfunction to assess the extent of potentially interacting background processes and the uncertainties in quantitative analysis this might produce in specific future cases.

It is also sometimes worth examining the data underlying authors' overviews of their results to see if the indicated form of the dose response relationship has been assessed in as sensitive and even-handed way possible. For example, Touil et al. (2000) present a very detailed set of individual observations of the effects of in vitro treatment of human lymphocytes from 5 people with various doses

of gamma rays. They do a series of statistical comparisons of micronucleus frequencies in the cultures from individual subjects receiving various doses of radiation with comparable untreated cultures from the same individual people. Perhaps influenced by their earlier conclusion favoring thresholds for aneugenic events (Elhajouji et al. (1995, 1997) they observe no statistically significant differences between cultures treated with less than 1 Gy and comparable control cultures, and summarize:

> "For radiation-induced non-disjunction, lower doses (0.1, 0.25, and 0.5 Gy) of gamma-rays did not induced a statistically significant increase in non-disjunction frequencies for all the donors, whereas 1 Gy and above clearly induced a statistically significant increase in the total non-disjunction frequencies for all the donors ($P < 0.05$ at 1 Gy and $P < 0.0001$ at 2 Gy). The aneugenic effect of radiation is less clearly dose dependent at the lower doses, suggesting an apparent threshold below which no change could be demonstrated."

The discussion in the Touil et al. (2000) further supports the expectation of a threshold on the mechanistic multi-target grounds: "If it is assumed that multiple events are required to lead to chromosome loss or malsegregation, the existence of a threshold is probable." They do, however, note that more donors and more doses should be examined before a firm conclusion could be drawn about whether or not a threshold exists for this type of genetic effect.

It occurred to us that a more sensitive way to examine the dose response trends in the existing data of Touil et al. (2000) would be to combine the information in their paper for all five of the individual subjects, and simply plot the means and standard errors for the combined observations at each dose. Figure 3 shows the results of this combined analysis for all micronuclei; Figure 4 shows the combined data for micronuclei with a centromere (indicating a chromosome loss event); and Figure 5 shows data for non-disjunction events projected from observations of nondisjunctions occurring in chromosomes 1 and 17. Additionally, Table 3 shows the results of inverse-variance weighted least squares regressions for each endpoint done with the whole data set, and with only the data points from zero to 0.5 Gy. We would suggest that, viewed in this way, it does not seem warranted to conclude that the data suggest thresholds in the aneugenic responses to radiation. Quite the contrary, the data seem most simply interpretable as linear-no threshold in form. For two of the three endpoints, the data for the 0-0.5 Gy points by themselves show a slope that is statistically significant at $P < .05$. The authors in this case appear to have been mislead by an inappropriate use of a null hypothesis of no difference between the control and each dosed culture separately, rather than letting the data as a whole speak to the issue of dose response form.

Arguments for Absolute Thresholds or "Practical Thresholds" from The Presence of Multiple Transport, Detoxification, and Repair Processes

The "multiple barriers" argument appears to be mostly a rhetorical device since the mathematical implications of mass action at low doses (Figure 2 above) reinforce the expectation of linearity at each step. By reciting the series of opportunities that a molecule of a DNA-reactive agent has to go astray rather than react with DNA and then have the adduct cause a mutation in a gene that matters in a cell that matters for carcinogenesis, a speaker can make it appear very unlikely that such a chain of events could occur (Parry et al. 2000). And indeed, from the standpoint of a single molecule, the probability is necessarily very small. However each physical barrier has some probability of being surmounted by each molecule; each alternate reaction opportunity or detoxification enzyme will divert a finite fraction of the molecules, each DNA repair process will repair the molecules/adducts at a finite rate and therefore a finite fraction of the DNA lesions will persist to the time of the next passage of the polymerase enzyme responsible for copying the DNA. Similarly not all cells with damaged DNA will be removed by apoptosis, and cell cycle checkpoint functions will not perfectly prevent the copying of damaged DNA. If there is a finite rate of DNA lesion generation and a finite rate of DNA repair or removal by apoptosis, then there must be a finite rate of mutation that, at the limit of low dosage where

saturation effects are negligible, must be a linear function of the number of DNA reactive molecules (or their precursors) that enter the system. Moreover once an initial tumor cell is generated there must be a finite probability that it will escape repression by its normal neighbors through gap junction communication and by other immune-based defense processes. The presumption of some of the discussion in the Mutation Research special issue (Herman et al. 2000) seems to be that at low doses some or many of these processes can be assumed to be perfect; but this is just not possible. The dose response relationship for the combined process is a simple multiplicative combination of the component processes. If all of these are linear at low doses, then the combined dose response relationship must also be linear at low doses.

A final refuge of this set of arguments is to distinguish between an "absolute" threshold (a true zero response at a finite dose rate)* and a "pragmatic" or "practical" threshold. Lowell (2000) argues:

> "A 'pragmatic' threshold can be considered as a concentration below which any effect is considered biologically unimportant…. (Lutz, 1998). This term is used in a somewhat similar way to how ECETOC defines a biological threshold except that it implies that there may be effects occurring because of treatment or exposure but these are considered below what might be considered biologically important. An example might be increases that did not exceed the range of responses seen in negative control material in a well-conducted series of experiments. Such a threshold may be defined, in part, with the help of statistical tests. The distinction between the various classifications of thresholds can initiate a philosophical discussion but is not relevant to regulatory risk assessment."

It appears, therefore, that this line of argument reverts to treating an effect that may be present but which cannot be clearly demonstrated as above "the range of responses seen in negative control material" (with some undefined sample size and sensitivity/detection noise) as if it weren't there. It seems to us that risk assessment methods have been created precisely to make fair assessments of the likely magnitude of effects that cannot be directly measured but are still potentially substantial enough that decision-makers and the public may reasonably care about reducing them. Or, put another way, "practical" for a biologist to detect, may not mean the same as of "practical" concern because someone might get hurt. Our view is that terms such as "practical threshold" are inherently confusing in these contexts and do a disservice to transparent public analysis and discussion of likely underlying realities.

There is, of course, merit to the argument that as risks decline to very low levels (often identified as below one in a million on an individual lifetime basis, as assessed by conservative risk estimation procedures), they may fall below a "threshold" of problems that warrant the use of the appreciable regulatory resources needed for mandatory state controls. This, however, is a risk management judgment that must be distinguished from the technical risk analysis. Given an explicit risk management judgment that certain risks are not of interest for regulation, this can be used by analysts to circumscribe their efforts. However the analysts must not be put in a position of camouflaging a risk management judgment as a technical conclusion that there is no risk. Additionally, in some circumstances, even if a risk is assessed to be very small when assessed on an individual risk basis, it may be judged to merit attention if it applies to a large number of people, and if abatement measures are relatively straightforward, low cost, and pose negligible risks of their own. (A 1/million individual risk that applies to the whole U.S. population would be expected to have the potential to affect about 300 people.) Thus we would urge risk

* Lowell (2000) quotes a somewhat different definition of "absolute" threshold attributed to ECETOC: "…a concentration below which a cell would not 'notice' the presence of the chemical. In other words, the chemical is present but does not interact with the cellular target." The precise identification of such a threshold, if it exists is difficult.

managers to consider potential population-wide impacts as well as individual risks and their uncertainties before judging that a particular hazard falls below a "practical threshold" for regulatory concern.

Arguments for Thresholds or Hormetic Dose Response Relationships from Possible Inducible Detoxification or DNA Repair Processes

In contrast to some of the arguments reviewed above, this category of mechanisms does have some potential to produce changes to the low dose linear expectation under some circumstances. Up to this point we have discussed the several processes producing high dose nonlinearities in dose response relationships as if their levels were static—fixed at some baseline level of activity/efficiency in promoting or reducing damage to DNA or subsequent steps in the carcinogenic process. In fact, however, it is not unlikely that the levels of the enzymes that mediate these processes are themselves regulated by feedback mechanisms that respond to influences from the external and internal environment, as do many other components of biological systems (Schulkin, 2003). Mehendale and colleagues have documented what may be a common mechanism for enzyme induction in which binding of a substrate to the active site of an enzyme has the effect of protecting it from some normal degradation processes, leading to higher enzyme levels than would exist if the molecules were not actively processing substrate. This has been observed, for example for CYP2E1 (Chien et al. 1997) for which ethanol binding can apparently prolong the half life by several fold (Roberts et al 1995).* The same group has also explored induction of other mechanisms of protection against liver toxicity from such agents as carbon tetrachloride and acetaminophen (Mehendale et al. 1994; Samant et al. 2006). In some cases repeated moderate "priming doses" of liver toxicants such as carbon tetrachloride can lead to enhanced detoxification and other adaptations that protect against the ordinarily lethal effects of larger challenge doses (Anand et al. 2006; Philip et al. 2006).

In the light of this, it is certainly possible, in theory, that under some circumstances the induction of detoxifying or DNA repair enzymes (e.g. from radiation--Schmerold and Wiestler 1986; Chan et al. 1992) could have the side-effect of preventing or repairing enough "background" damage as to outweigh the the primary damage done by the inducing toxicant over some range of dosage. Whether such possible offsetting effects could extend all the way down to the limit of low dosage depends on the fundamental dose response relationships underlying the induction process(es) and the levels and types of "background" damage of the that are available to be prevented.

Specifying the requirements for this helps illuminate the special nature of the conditions that would be needed to produce a net biological benefit from a particular type of exposure to a genetically active agent:

- "Background damage" (e.g. from the DNA damaging free radicals produced as a byproduct of metabolism, other endogenous DNA reacive agents such as ethylene oxide and possibly formaldehyde, and other exogenous DNA reactive agents) must occur at sufficient rates that offsetting prevention benefits could occur,

* Of course, whether the induction of a particular enzyme is beneficial or adverse depends on the details of the action of the enzyme on pathways of activation vs detoxification of particular toxicants. For example, in the case of the CYP2E1 induction by alchohol, epidemiological studies have found greater carcinogenic risk from vinyl chloride in people with greater alcohol consumption (Tsutsumi et al. 1993; Mastrangelo et al., 2004). CYP2E1 is known to catalyze the reaction by which vinyl chloride is converted to the active metabolite responsible for causing the liver cancers associated with this compound. CYP2E1 induction also enhances the toxicity of thioacetamide (Wang et al. 2000; Ramaiah et al. 2001).

- The usual "baseline" state of expression of the detoxification, DNA repair, apoptosis, or cell cycle check point mediators needs to be sub-optimal. Normally, one would expect that if it were net beneficial to have higher standing levels of a particular enzyme, then that would have been of selective advantage during evolution. Consequently people's normal constitutive detoxification/repair enzyme levels should have been adjusted to at least approximate optimality by natural selection. However, the types and levels of present-day exposures to mutagenic agents could conceivably be different enough from those present during the recent evolution of modern humans that prevailing constitutive levels of defensive enzymes are not perfectly tuned to current exposures. (For example cancer rates in wealthier industrialized countries tend to be very substantially higher than in poorer, less-developed countries. (Harris et al.1985)) In evaluating such possibilities, it is important to bear in mind that both detoxification enzymes and DNA repair enzymes can have adverse biological side-effects themselves. For example the same P450 "detoxification" enzyme that is induced by ethyl alcohol (Daiker et al. 2000; Feierman et al. 2003; Sato 1993) also is involved in the transformation of vinyl chloride to the activated form that reacts with DNA to induce the characteristic liver cancers produced by that compound. Epidemioligic data now exist that high consumers of alcohol are much more susceptible to the carcinogenic effects of vinyl chloride (Mastrangelo et. al 2004). P450s also affect estrogen metabolism including some to genetically active agents. DNA excision repair enzymes, which repair DNA by cutting out small sections of DNA that has been damaged, also do damage themselve by making cuts at some finite rate in sections of DNA that do not contain pre-existing damge (Branum et al. 2001). Thus it is likely to be beneficial, biologically, to induce these enzymes above their baseline levels only when there is sufficient damage in a particular cell that the biological "costs" of the excision repair enzyme itself are outweighed by the need to repair an unusual amount of damage by a relatively rare exposure episode.

- The dose-response relationship(s) for induction of the detoxification and/or repair enzymes has to be steep enough, and the induction long lasting enough, that the prevention benefits are sufficient to offset the primary damage done by the inducing agent.

Human interindividual variability in both background rates of damage and constitutive levels of expression of metabolic, DNA repair and other genomic defense systems in the stem- and progenitor cells that are most likely to be relevant for carcinogenesis further complicates the practical problem of fairly evaluating the likelihood and extent of offsetting protective effects at low doses from any specific agent. Clearly this is an area where improved assessment of cancer risks and uncertainties could benefit from substantial new long term experimental research, and compilation of data bases to allow assessors to better judge the likelihood of different states of the world. We need databases of

- Dose-time-response relationships for enzyme induction (and the time course of reversion to baseline levels of activity) for detoxification and genomic defense processes for common exposures in the human population.

- A better quantitative understanding of the "background" processes that underly the incidence of human cancers of all types (by both molecular pathological pathways and more conventional anatomical site/histological categories). This would be helpful for evaluating both the candidate "hormesis" processes discussed in this subsection and the "interacting background" processes discussed in the first subsection above.

- The background distribution and intensity of external and internal exposures to genetically active agents that are likely to affect the background state of induction of the various genomic defense mechanisms.

- The distribution of human interindividual variability for DNA repair rates for different kinds of DNA damage in different cell types. Some data of this type have been compiled in an earlier analysis of interindividual variability in susceptibility for carcinogenesis (Hattis and Barlow 1996). More recently we have compiled data for interindividual variability in repair and apoptosis responses radiation-induced DNA damage in human lymphocytes from Barber et al. (2000); Chang et al. (2006); Ismail et al. (2007); and Thygarajan et al. (2007). Overall the human internidividual variability in these responses in this cell type is relatively modest (geometric standard deviation of 1.2-1.3) compared to other types of pharmacokinetic and pharmacodynamic parameters we have studied (Hattis et al. 1999; Hattis et al. 2002; Hattis and Lynch 2007). However it should be noted that the importance of genomic defense variability may be amplified for cancer risks because this variability may influence the risks of transitions among multiple stages in the molecular pathological pathways to the ultimate generation of malignant tumors.

References

Anand SS, Philip BK, Palkar PS, Mumtaz MM, Latendresse JR, Mehendale HM. 2006. Adaptive tolerance in mice upon subchronic exposure to chloroform: Increased exhalation and target tissue regeneration. Toxicol Appl Pharmacol. 2006 Jun 15;213(3):267-81.

Barber JBP, West CML Kiltie AE, Roberts SA, Scott D. 2000. Detection of individual differences in radiation-induced apoptosis of peripheral blood lymphocytes in normal individuals, ataxia telangiectasia homozygotes and heterozygotes, and breast cancer patients after radiotherapy. Radiation Research 153:570-578.

Branum ME, Reardon JT, Sancar A. 2001. Repair excision nuclease attacks undamaged DNA. Journal of Biological Chemistry 276:25421-25426.

Chang JL, Chen G, Lampe JW, Ulrich CM 2006. DNA damage and repair measurements in cryopreserved lymphocytes without cell culture—A rproducible assay for intervention studies. Environmental and Molecular Mutagenesis 47:503-508

Chien JC, Thummel KE, Slattery JT. 1997. Pharmacokinetic consequences of induction of CYP2E1 by ligand stabilization. Metabolism and Disposition 25:1165-1175.

Choudhury RC, Palo AK, Padhy A. 2004. Cytogenetic consequences of vinblastine treatment in mouse bone marrow. Chemotherapy. 50(4):171-177.

Crebelli R. 2000. Threshold-mediated mechanisms in mutagenesis: implications in the classification and regulation of chemical mutagens. Mutat. Res. 464:129–135.

Crump K, Hoel D, Langley C, Peto R. 1976. Fundamental carcinogenic processes and their implications to low-dose risk assessment. Cancer Research 36:2973-2979.

Daiker DH, Shipp BK, Schoenfeld HA, Klimpel GR, Witz G, Moslen MT, Ward JB Jr. 2000. Effect of CYP2E1 induction by ethanol on the immunotoxicity and genotoxicity of extended low-level benzene exposure. J Toxicol Environ Health A. 59:181-196.

Elhajouji A, Van Hummelen P, Kirsch-Volders M, 1995. Indications for a threshold of chemically induced aneuploidy in vitro in human lymphocytes. Environ. Mol. Mutagen. 26:292-304.

Elhajouji A, Tibaldi F, Kirsch-Volders M. 1997. Indication for thresholds of chromosome non-disjunction versus chromosome lagging induced by spindle inhibitors in vitro in human lymphocytes. Mutagenesis 12:133-140.

Feierman DE, Melinkov Z, Nanji AA. 2003. Induction of CYP3A by ethanol in multiple in vitro and in vivo models. Alcohol Clin Exp Res. 27: 981-988.

Gehring PJ, Blau GE. Mechanisms of carcinogenesis: dose response. J Environ Pathol Toxicol. 1978 1:163-79.

Gilman P 2006. Response to "IRIS from the inside" (letter to the editor). Risk Analysis 26:1413

Harris, RC Hohenemser C and Kates R 1985. Human and non-human mortality,. Pages 129-56 in Perilous Progress (Kates, R Hohenemser C and Kasperson JX editors) Westview Boulder CO

Hattis, D. 1990. Pharmacokinetic principles for dose rate extrapolation of carcinogenic risk from genetically active agents, Risk Analysis, 10:303-316.

Hattis D, Baird S, Goble R. 2002. A straw man proposal for a quantitative definition of the RfD. in Final Technical Report, U.S. Environmental Protection Agency STAR grant # R825360, "Human variability in parameters potentially related to susceptibility for noncancer risks," Full version available on the web at http://www2.clarku.edu/faculty/dhattis; shortened version Drug and Chemical Toxicology 25:403-436.

Hattis, D, Barlow K. 1996. Human interindividual variability in cancer risks--technical and management challenges. Human and Ecological Risk Assessment 2:194-220.

Hattis D, Banati P, Goble R, Burmaster D. 1999. Human interindividual variability in parameters related to health risks. Risk Analysis 19:705-720,

Hattis D, Lynch MK. 2007. Empirically observed distributions of pharmacokinetic and pharmacodynamic variability in humans—Implications for the derivation of single point component uncertainty factors providing equivalent protection as existing RfDs." In Toxicokinetics in Risk Assessment, J. C. Lipscomb and E. V. Ohanian, eds., Informa Healthcare USA, Inc., 2007, pp. 69-93.

Henderson L, Albertini S, Aardema M. 2000. Thresholds in genotoxicity responses. Mutat. Res. 464:123–128.

Hoel DG. 1985. Incorporation of pharmacokinetics in low-dose risk estimation. Chapter 10 in Toxicological Risk Assessment, Volume I. D. G. Clayson, D. Krewski, and I. Munro, eds.

Ismail IH, Wadhra TI, Hammarstein O. 2007; An optimized method for detecting gamma-H2AX in blood cells reveals a significant interindividual variation in gamma-H2AX response among humans. Nucleic Acid Research, Advance Access published February 12, 2007, doi:10.1093/nar/gkl1169.

Kirkland DJ, Muller L. 2000. Interpretation of the biological relevance of genotoxicity test results: the importance of thresholds. Mutat. Res. 464:137–147.

Kirsch-Volders, M., Aardema, M., and Elhajouji, A. (2000). Concept of thresh-old in mutagenesis and carcinogenesis. Mutat. Res. 464:3–11.

Lovell DP 2000. Dose-response and threshold-mediated mechanisms in mutagenesis: statistical models and study design. Mutat. Res. 464:87–95.

Lutz WK 1998. Dose-response relationships in chemical carcinogenesis: Superposition of different mechanisms of action, resulting in linear-non-linear curves, practical thresholds, J-shapes. Mutation research 405:117-124.

Madle S, von der Hude W, Bronschinski L, Janig GR. 2000. Threshold effects in genetic goxicity: perspective of chemicals regulation in Germany. Mutat. Res. 464:117–121.

Mastrangelo G, Fedeli U, Fadda E, Valentini F, Agnesi R, Magarotto G, Marchi T, Buda A, Pinzani M, Martines D. 2004. Increased risk of hepatocellular carcinoma and liver cirrhosis in vinyl chloride workers: Synergistic effect of occupational exposures with alcohol intake. Environmental Health Perspectives 112:1188-1192.

Mehendale HM, Roth RA, Gandolfi J, Klaunig JE, Lemasters JJ, Curtis LR. 1994. Novel mechanisms in chemically induced hepatotoxicity. FASEB J. 8:1285-1295.

Moustacchi 2000. DNA damage and repair: consequences on dose-responses. Mutat. Res. 464:35–40.

Muller L, Kasper P. 2000. Human biological relevance and the use of threshold-arguments in regulatory genotoxicity assessment; experience with pharmaceuticals. Mutat. Res. 464:19–34.

Parry JM. 2000. Reflections on the implications of thresholds of mutagenic activity for the labeling of chemicals by the European Union. Mutat. Res. 464:155-158.

Parry JM, Jenkins GJS, Haddad F, Bourner R, Parry EM. 2000. In vitro and in vivo extrapolations of genotoxin exposures: consideration of factors which influence dose-response thresholds. Mutat. Res. 464:53–63.

Philip BK, Anand SS, Palkar PS, Mumtaz MM, Latendresse JR, Mehendale HM. 2006. Subchronic chloroform priming protects mice from a subsequently administered lethal dose of chloroform. Toxicol. Appl. Pharmacol. 216:108-121.

Roberts BJ, Song BJ, Soh Y, Park SS, Shoaf SE 1995. Ethanol induces CYP2E1 by protein stabilization. J. Biiological Chemistry 270:29632-29635.

Sato A. 1993. Confounding factors in biological monitoring of exposure to organic solvents. Int Arch Occup Environ Health. 65(1 Suppl): S61-S67.

Samant SP, Dnyanmote AV, Mayurranjan SM, Chilakapati J, Warbritton A, Latendresse JR, Mehendale HM. 2006. Protective effect of type 2 diabetes on acetaminophen-induced hepatotoxicity iin male Swiss-Webster mice. J. Pharmacology and Experimental Therapeutics 162:507-519.

Schulte-Hermann R, Grasl-Kraupp B, Bursch W. 2000. Dose-response and threshold effects in cytotoxicity and apoptosis. Mutat. Res. 464:13–18.

Schulkin J. 2003. Rethinking Homeostasis—Allostatic Regulation in Physiology and Pathophysiology. MIT Press, Cambridge, Massachusetts.

Shackelford RE, Kaufmann WK, Paules RS. 1999. Cell cycle control, checkpoint mechanisms, and genotoxic stress. Environ Health Perspect. 107 Suppl 1:5-24.

Shlyakhter, A. I. (1994). An improved framework for uncertainty analysis: Accounting for unsuspected errors. *Risk Analysis* **14**, 441-447.

Slikker W Jr, Andersen ME, Bogdanffy MS, Bus JS, Cohen SD, Conolly RB, David RM, Doerrer NG, Dorman DC, Gaylor DW, Hattis, D, Rogers JM. Setzer, RW, Swenberg, JA, Wallace K. 20004. Dose-dependent transitions in mechanisms of toxicity." Toxicology and Applied Pharmacology, 201:203-225.

Speit G, Autrup H, Crebelli R, Henderson L, Kirsch-Volders M, Madle S, Parry JM, Sarrif AM, Vrijhof H. 2000. Thresholds in genetic toxicology—concluding remarks. Mutat. Res. 464:149–153.

Stent GS. 1963. Molecular Biology of Bacterial Viruses W.H. Freeman and Company, San Francisco, CA.

Steenland K, Deddens J, Salvan A, Stayner L. 1996. Negative bias in exposure-response trends in occupational studies: modeling the healthy workers survivor effect. Am J Epidemiol. 143(2):202-210.

Swenberg JA, Ham A, Koc H, Morinello E, Ranasinghe A, Tretyakova N, Upton PB, Wu KY. 2000. DNA adducts: Effects of low exposure to ethylene oxide, vinyl chloride and butadiene. Mutat. Res. 464:77–86.

Thyagarajan B, Anderson KIE, Lessard CY, Veltri G, Jacobs DR, Folsom AR, Gross MD 2007. Alkaline unwinding flow cytometry assay to measure nucleotide excision repair. Mutagenesis 22:147-153.

Touil N, Elhajoujli A, Thierens H, Kirsch-Volders M. 2000. Analysis of chromosome loss and chromosome segregation in cytokinesis-blocked human lymphocytes: non-disjunction is the prevalent mistake in chromosome segregation produced by low dose exposure to ionizing radiation. Mutagenesis 15:1-7.

U.S. Environmental Protection Agency. 1986. Guidelines for Carcinogen Risk Assessment (1986). http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=54933 accessed 2/5/07.

U.S. Environmental Protection Agency. 2005. Guidelines for Carcinogen Risk Assessment 2005 http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=116283, accessed 2/5/07.

Table 2
Effect of Background Mutation Rates on the Carcinogenesis Dose-Response Curve at Low Doses, Assuming a Hypothetical Two-Stage Carcinogenic Process

Dose	Rate of 1st Transition (1 extra unit per unit of dose)	Rate of 2nd Transition (1 extra unit per unit of dose)	Relative No. of Tumors (background = 1) (product of two previous columns)	Induced Excess Over Background
1000	1001	1001	1,002,001	1,002,000
100	101	101	10,201	10,200
10	11	11	121	120
1	2	2	4	3
.1	1.1	1.1	1.21	0.21
0.01	1.01	1.01	1.0201	0.0201
0.001	1.001	1.001	1.002001	0.002001

Source: Adapted from Hattis and Smith, 1986.

Table 3
Comparative Results of Inverse-Variance Weighted Least Squares Regressions of the Touill et al (2000) Data for the Means of All Five Subjects—Results Shown for the Full Data Sets (0-2 Gy) and Data Restricted to the 0-0.5 Gy Range

	Mean MN/1000 CB Cells		
	Linear Slope (/Gy)	95% CL	P[a]
Full Data Set	39	35-44	<.0001
0-0.5 Gy Points Only	30	20-38	0.02
	Mean Chromosome Loss (MNCen+)		
	Linear Slope (/Gy)	95% CL	P
Full Data Set	7.2	6.0-8.4	0.0003
0-0.5 Gy Points Only	5.7	-11.7	0.19
	Mean Total Nondisjunction		
	Linear Slope (/Gy)	95% CL	P
Full Data Set	13.7	12.1-15.2	<.0001
0-0.5 Gy Points Only	16.9	11.6-22.1	0.02

[a]These "P" values are the probabilities that one would observe data as steeply sloping upward as was found if the true slope were zero (no relationship between incremental radiation dose and the measured response).

Figure 1
Argument for the Plausibility that Thresholds Might Arise From the Competition Between Metabolic Pathways Producing Safe and Dangerous (DNA Reactive) Metabolites

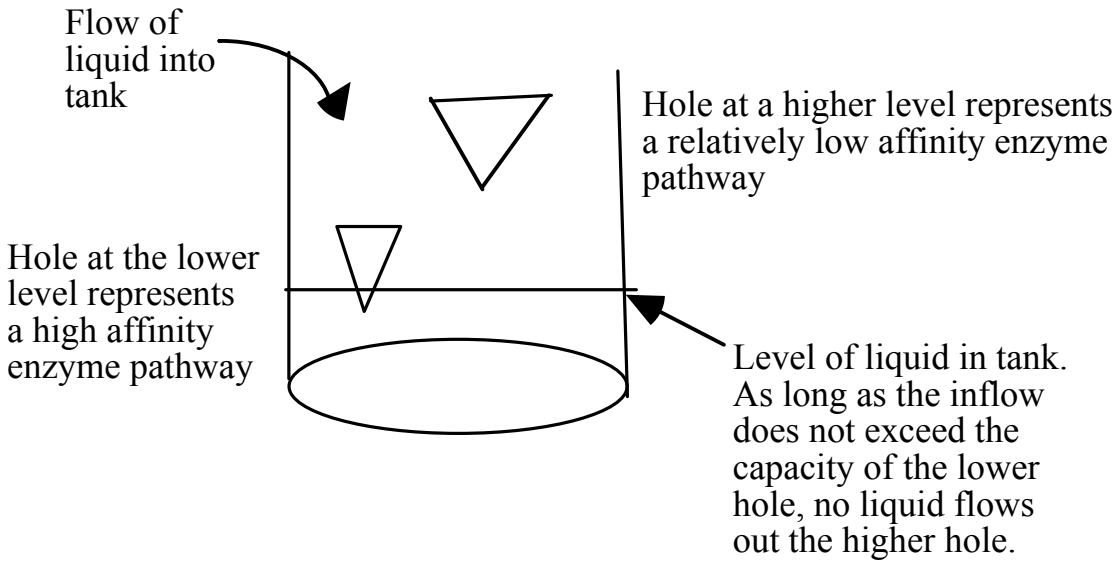


Figure 2
A Molecular Vision of the Low-Dose Competition for Substrate Between Activating and Detoxifying Enzyme Molecules

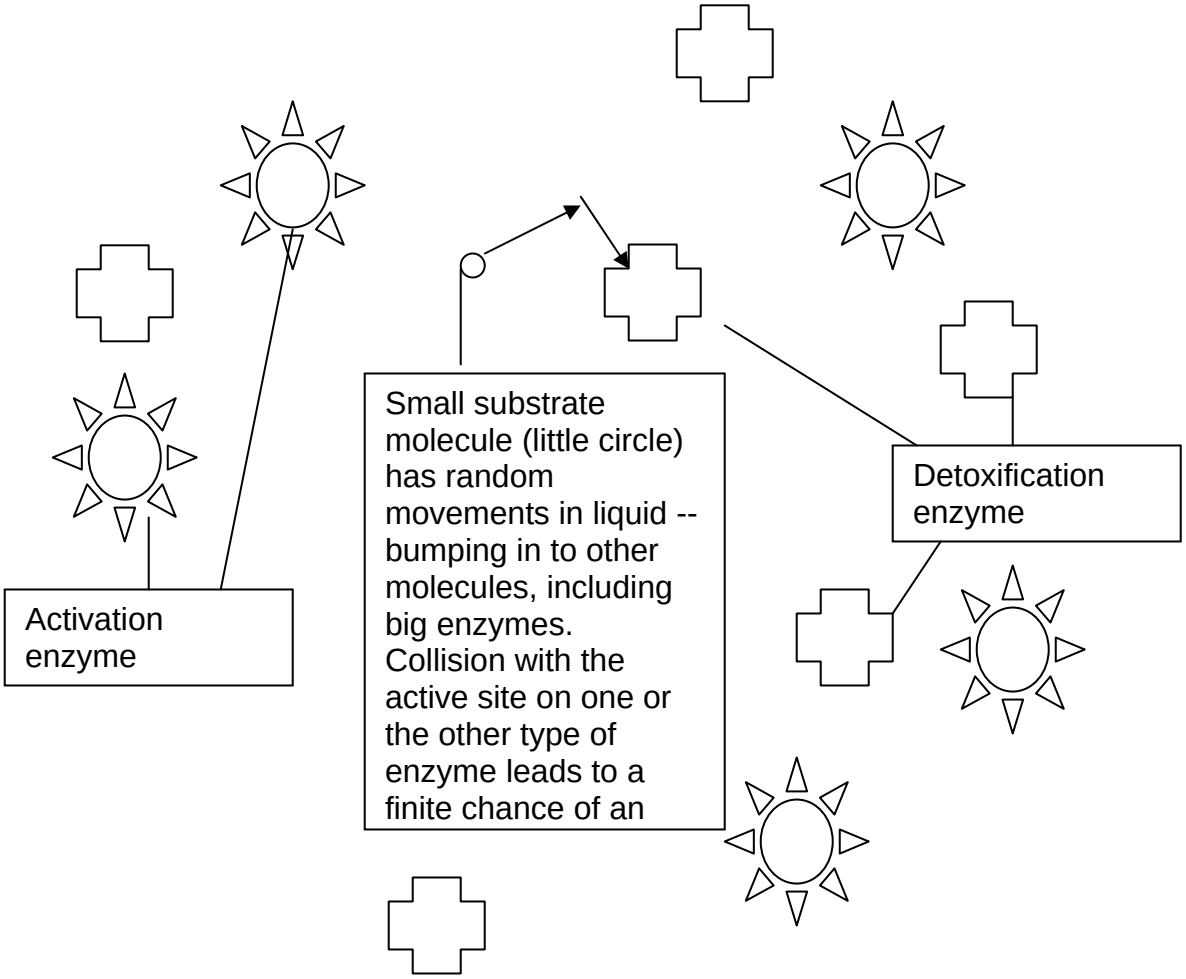


Figure 3

Plot of 5-Subject Averages of Micronuclei/1000 Lymphocytes vs Radiation Dose (Data of Touil et al. 2000)

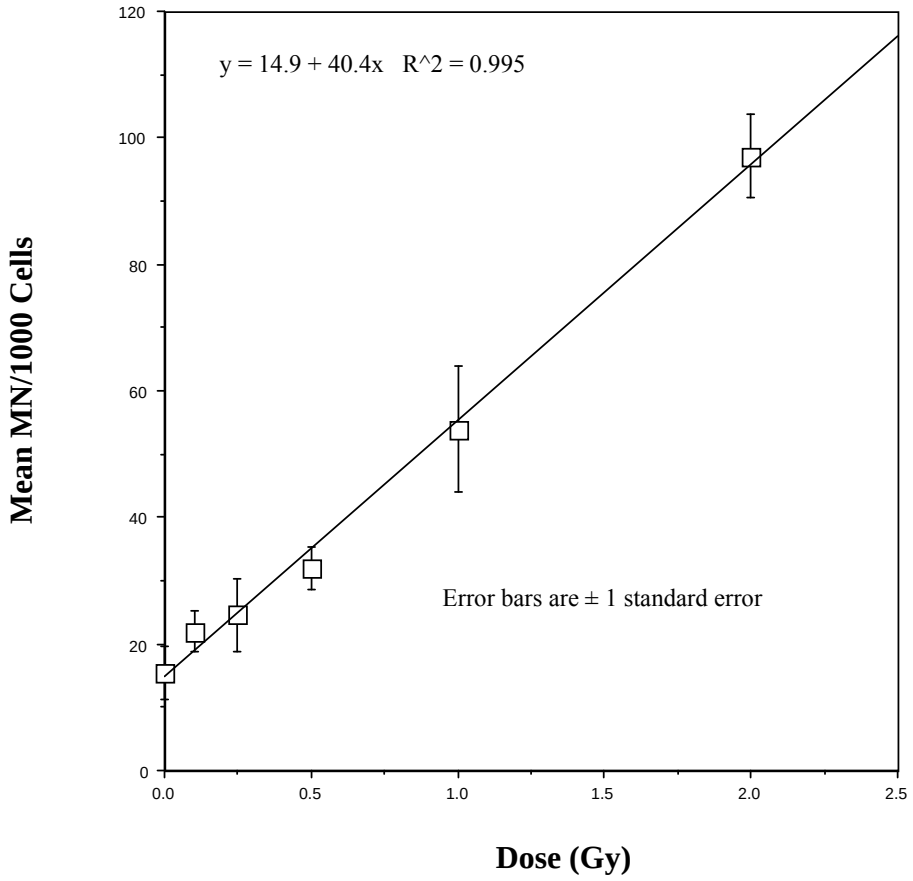


Figure 4

Plot of 5-Subject Averages of Chromosome Loss (MNCen+) /1000 Lymphocytes vs Radiation Dose (Data of Touil et al. 2000)

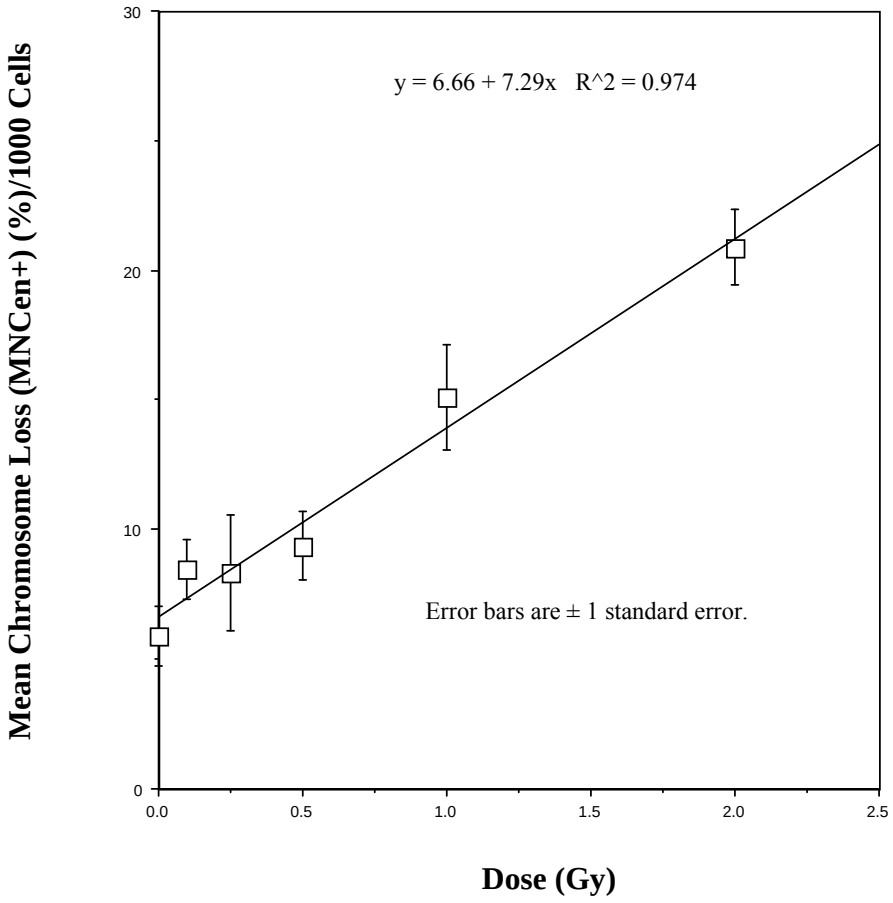
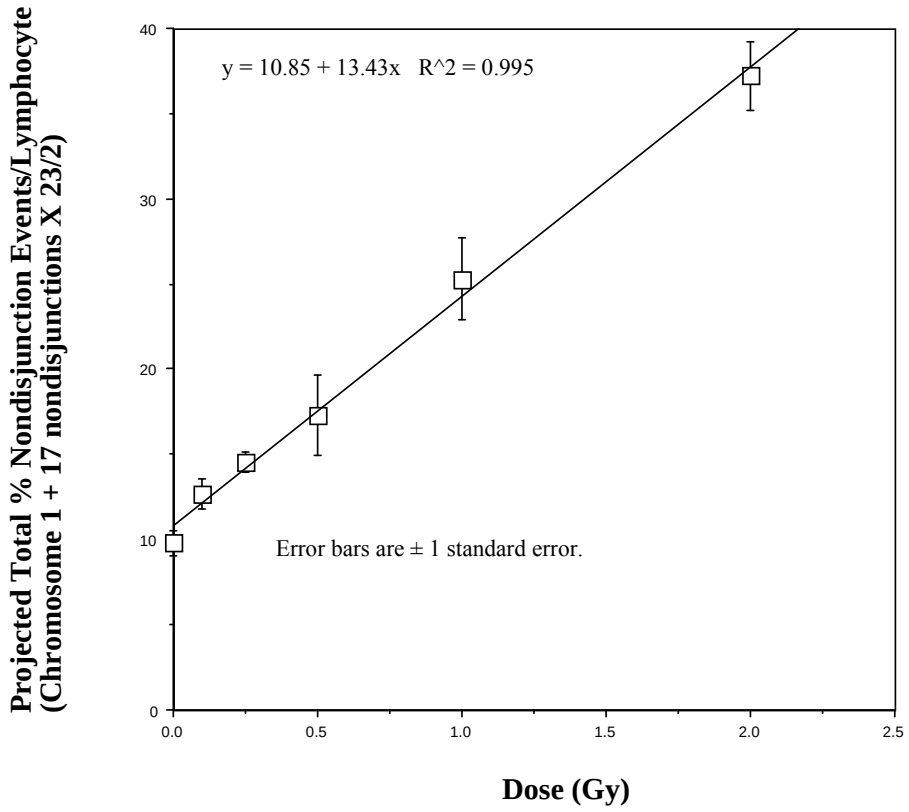


Figure 5

Plot of 5-Subject Averages of Projected Total % Nondisjunction Events/Lymphocyte vs Radiation Dose (Data of Touil et al. 2000)



Lisa M. Kamendulis, Ph.D.

Indiana University School of Medicine

Dr. Lisa M. Kamendulis is an Assistant Professor in the Department of Pharmacology and Toxicology at Indiana University School of Medicine. After completing her undergraduate degree in Biology from the University of Massachusetts at Amherst, Dr. Kamendulis received her Ph.D. Toxicology from the University of New Mexico in 1994, where she studied mechanisms of hepatotoxicity, specifically focusing on the induction of apoptosis. Following her Ph.D. work, Dr. Kamendulis completed post-doctoral studies within the Departments of Pathology and Biochemistry at Indiana University School of Medicine, then acquired additional training as an Assistant Scientist in the laboratory of Dr. James Klaunig at Indiana University. Her current research focuses on examining mechanisms by which environmental agents and therapeutic drugs elicit liver cancer, and uses both *in vivo* and *in vitro* rodent models to examine the role of oxidative stress in modulating growth regulatory signaling networks and gene expression elicited by nongenotoxic carcinogens. She has published over 40 peer-reviewed manuscripts, and book chapters in the areas of Toxicology and chemical carcinogenesis. Dr. Kamendulis has served the science of toxicology both locally and nationally by serving as Councilor and Secretary-Treasurer of the Ohio Valley Society of Toxicology, as a member and chair of the Placement Committee, and as Councilor in the Carcinogenesis Specialty Section of the Society of Toxicology.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

   The toxicological review for carbon tetrachloride is clearly and objectively written. The available data for cancer and noncancer health effects are, in general, accurately described, and presented in a logical and comprehensive manner.

2. **Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.**

   I am not aware of additional studies that should be included in the assessment of cancer and non-cancer health effects of carbon tetrachloride

3. **Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.**

   While I do not view the following as required to derive a scientifically-based conclusions on the available toxicity and carcinogenicity data for carbon tetrachloride, confidence in the database for future assessments of carbon tetrachloride would be increased if 1) multi-generational studies to assess the potential for reproductive and developmental toxicity; 2) enhanced assessment of toxicity at lower dose levels; 3) assessment of genotoxicity and mutagenicity at lower (non-cytolethal) dose levels; 4) characterize carcinogenic activity at lower dose levels (cancer bioassay with lower doses, and/or studies evaluating preneoplastic lesion development at lower dose levels; 5) studies on cancer endpoints in CYP2E1 knockout mice (either cancer bioassay or studies in preneoplastic lesions).

4. **Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?**

   The potential sources of uncertainty have been clearly presented and adequately described. The uncertainties were presented in a transparent manner, and evaluated scientifically. In particular, including Figures 5-1 – 5-3, and 5-6 – 5-8, illustrated the impact of application of the UFs on the derivation of RfD and RfC, which were useful to demonstrate how these UF impact the assessment.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   The Bruckner et al. (1986) study identified serum enzyme changes and liver histopathology as the most sensitive endpoints for carbon tetrachloride after 10 and 12 week exposure to carbon tetrachloride. This

study showed increases in liver enzymes at both the 10 and 12 week timepoints, which were relatively dose-responsive. The other available studies had limitations in experimental design that diminished their utility for deriving an RfD for carbon tetrachloride. The study by Condie et al (1986) found similar changes in liver histopathology, however, the reported results did not include a standard deviation, and thus, these data could be used only to determine an RfD based on NOAEL and LOAEL values (could not do benchmark dose analysis. The rationale for selection of the Bruckner study was objectively provided and adequately described in the Toxicological review. I am unaware of any other data that could be selected as the principal study for deriving an RfD for carbon tetrachloride.

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

   The Bruckner et al. (1986) study ichanges and liver histopathology as the most sensitive endpoints for carbon tetrachloride after 10 and 12 week exposure to carbon tetrachloride. Many experts in hepatotoxicity concur that agents that increase liver enzymes compared with control, even increases as low as 2-fold are indicative of agents that are likely to produce liver injury (EMEA, 2006; Boone et al., 2005; FDA Working Group, 2000). Further, Travlos et al (1996) evaluated a battery of chemicals to characterize various liver enzymes for their relative ability to predict hepatotoxicity. These authors concluded that there was an association between treatment-related increases in alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activities and histopathological changes in the liver. SDH activity had greater positive and negative predictive values than similar changes in ALT. Therefore, increased SDH appears to be the most appropriate critical effect for the derivation of an RfD. The use of SDH as the critical biomarker was thoroughly discussed and documented in the review, and was described in a transparent and objective manner. I am not aware of other endpoints that would be more appropriate to use in the derivation of RfD for carbon tetrachloride.

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   A goal of benchmark dose modeling is to model the dose-response data for an adverse effect in the observable range and then select a 'benchmark dose' at the low end of the observable range to use as a 'point of departure'. This analysis offers an advantage over using NOAEL or LOAEL as this analysis takes in account of all the data from the study, when possible. This type of analysis also eliminates the uncertainty associated with extrapolating from LOAEL/NOAEL values. This is clearly shown in figure 5-1. It is further stated that "although the RfD based on Bruckner et al. (1986) is not the lowest among candidate studies, it is considered the most scientifically rigorous", mainly due to lower UF applied in the RfD derivation. The BMD modeling was appropriately conducted and objectively and transparently described in the Review. I agree that this was the appropriate analysis to determine a POD and am not aware of another approach that would better characterize the available data.

4. Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:

The selection of the UFs applied in the derivation of the RfD were appropriate and scientifically justified. No changes to the value of the UFs applied, or additional UFs are needed. For clarity, the same values should be reported (see p 182 where the RfD was reported as 0.0039 mg/Kg-day versus p 183 where the RfD is reported as 0.004 mg/Kg-day.

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

The default UF of 10 was applied due to a lack of data that would enable intraspecies extrapolation. While known effects exist in a critical step in the toxicity process (metabolism and ontogenic expression of P450 levels), definitive studies characterizing this parameter on hepatotoxicity have not been assessed. Furthermore, many factors (dietary, environmental, etc) can impact metabolism of carbon tetrachloride, therefore, predicting responses across species is difficult and has not been characterized scientifically. Thus the rational for using the default value of 10 is justified.

- **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

I agree with the use of a reduced UF of 3 for the subchronic to chronic extrapolation. Although the chronic oral data is limited, due to low survival, scientifically reliable inferences could be made based on the chronic inhalation study (Nagano et al., 2007). These studies describe that same endpoints associated with liver toxicity occurred in both the subshronic and chronic studies, and occurs shorly after carbon tetrachloride dosing begins. Further, there is consistency in the toxicity endpoints in several studies. Therefore, a full 10-fold UF is not necessary.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

I agree that a UF of 3 should be applied for database weaknesses. The limitations of the database are thoroughly described on p 184-185. It is noted that for developmental toxicity, the dose required is in excess of that needed to produce hepatic toxicity (exceeds the POD), thus since no other toxicities were seen in studies with carbon tetrachloride (short-term, sub-chronic and chronic), it seems apparent that liver toxicity is the most sensitive endpoint, and that if effects are to occur on other organ systems, they would likely arise at doses higher than needed to produce hepatic changes. However, due to an incomplete database, a UF of 3 for database weaknesses is appropriate.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

The application of UFs were scientifically based, and transparently and objectively described in the Review document. The application of all uncertainty factors is scientifically justified.

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   Many animal studies identified liver and kidney as targets of carbon tetrachloride toxicity and could be considered for derivation of an RfC. However, the most comprehensive appears to be the 2-year inhalation bioassay by JBRC (Nagano et al., 2007b; JBRC, 1998). This study used 50 animals/sex/group and examined an extensive set of endpoints of toxicity. Although significant mortality was seen at the high dose group (125 ppm) in this study, histopathological analysis of tissue confirmed the toxic effects in liver, and the findings obtained in this study are consistent with the database for carbon tetrachloride. The selection of this study as the principal study is scientifically justified and adequately documented in the Review. Upon evaluation of the available data, I could not identify another study that could serve as the principal study.

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   The effects observed in the 2-year JBRC study included hepatic and renal changes, benign pheochromocytomas, and increased severity of eosinophilic changes in the nasal cavity of female rats. Of these effects, hepatic changes were considered the most reliable for derivation of an RfC. This is justified because: 1) Renal effects were observed generally at higher concentrations and or lower incidence than liver effects; 2) benign pheochromocytomas were observed only in mice and may represent a strain-specific finding; and 3) eosinophilic changes in the nasal cavity of female rats which alone may not represent an adverse effect - nasal lesions were only of moderate severity in the high-exposure group (which showed severe renal and hepatic effects), and were not accompanied by other adverse effects in the nasal cavity. Thus, liver effects appear to be the most sensitive endpoint for deriving an RfC. Liver enzymes were elevated in the 25ppm group, however, serum enzyme data was not collected for the majority of animals in the high dose group due to low survival. Histopathological analyses was performed on all animals and showed that fatty liver changes, and fibrotic changes were evident at both the 25 and 125 ppm dose groups. The endpoint of fatty liver changes was selected as the critical endpoint for derivation of RfC. The criteria and rationale for this selection is transparently and objectively described in the Toxicological Review. While some fatty liver changes are reversible and do not necessarily result in cellular damage, there is a correlation between the progression of fatty liver changes and development of cellular damage in the liver. Additional discussion and literature citations should be included to firm the association between fatty liver (seen in this study) and assumed cell damage. Fibrotic changes in the liver may be more representative of sustained cellular damage. Therefore, the more biologically relevant endpoint may be liver fibrosis. Since the NOAEL and LOAEL for fatty liver changes and fibrosis were the same, indicating the critical effect as fibrosis would not change the NOAEL and LOAEL values used to derive an RfC for carbon tetrachloride.

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

   As reported in the JBRC study and shown in table 4-2 in the Review document, proteinuria was seen in male and female rats. Proteinuria was found in nearly 100% of the rats in both the control and carbon tetrachloride-exposed rats (all 3 doses). In the carbon tetrachloride-exposed animals, however, rats showed an increase in the severity of proteinuria relative to controls (increased grade 4+ compared to 3+). After two years of exposure to carbon tetrachloride, proteinuria in 5 ppm rats did not appear to progress into clearly adverse renal toxicities, as were seen at and above 25ppm. Therefore, the conclusion was made that the biological significance of proteinuria at 5 ppm is unclear. This conclusion is scientifically based and was transparently and objectively described in the Toxicological review.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   Benchmark dose modeling offers an advantage over using NOAEL or LOAEL as this analysis takes in account of all the data from the study, when possible. This type of analysis also eliminates the uncertainty associated with extrapolating from LOAEL/NOAEL values. It is appropriate to use BMD for determining the POD for the data set. The BMD modeling using fatty liver changes as the critical endpoint was transparently described. However, as noted in comment 2 above, liver fibrosis may be a more biologically relevant endpoint to characterize liver damage.

   If fatty liver remains as the critical effect, then the BMR (using 10% extra risk of fatty liver) for deriving the POD is scientifically justified, since the POD associated with this BMR fell near the low end of the range of experimental data points (plots shown in Appendix D). This was appropriately described in the document. No alternative approaches are suggested for determining the POD.

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?**

   Several well characterized and validated PBPK models exist for carbon tetrachloride via inhalation. Interspecies extrapolation (i.e., rat-to-human) of carbon tetrachloride inhalation dosimetry was accomplished using a human PBPK model described in Paustenbach et al. (1988), Thrall et al. (2000), and Benson and Springer (1999). The application of these models was scientifically appropriate. The modeling was transparently described in the Toxicological Review. Further, the model properly represented the toxicokinetics of the species under consideration, and was applied properly. One point of

consideration is the method used for the calculation of HECs. $VmaxC_R$ values of 0.4 and 0.65 were used, as justified from the available literature. However, the HECs obtained from these values were averaged, and using the lower value might be considered more conservative. If the lower values are used to calculate an RfC, the resulting value would be .118 mg/m^3 versus 0.143 mg/m^3, however, in either case, the number would be truncated and presented as 0.1 mg/m^3. Overall, the model assumptions, parameter values, and selection of dose metrics are clearly presented and scientifically supported. The sensitivity analysis was clearly presented, and appropriately characterized, and the uncertainty in the model estimates were accurately considered.

6. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

The UFs applied for the derivation of an RfC for carbon tetrachloride were appropriate.

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

Similar to the RfD derivation, the default UF of 10 was applied to the RfC derivation due to a lack of data that would enable intraspecies extrapolation. Definitive studies characterizing the many factors that can impact metabolism of carbon tetrachloride have not been assessed. Accurately predicting toxicological responses across species is difficult and has not been characterized scientifically. Thus the rational for using the default value of 10 is justified.

- **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

Using a UF of 3 for interspecies differences is appropriate. Validated PBPK models are available and were appropriately used to derive an RfC for carbon tetrachloride. Due to potential differences in pharmacodynamic parameters that have not been adequately addressed scientifically, a UF of 3 was appropriately applied.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

The weaknesses in the database are described on p 203-204. Due to an incomplete database, a UF of 3 for database weaknesses was appropriately applied.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

The application of UFs for the derivation of an RfC was scientifically based, and transparently and objectively described in the Toxicological Review for carbon tetrachloride. The application of all uncertainty factors is scientifically justified.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

   Carbon tetrachloride was classified as "Likely to Be Carcinogenic to Humans". This descriptor is appropriate when the weight of the evidence is adequate to demonstrate carcinogenic potential to humans but does not reach the weight of evidence for the descriptor "Carcinogenic to Humans". The descriptor "Likely to be carcinogenic to humans" is warranted when supporting data is available which includes, but is not limited to: an agent that has tested positive in animal experiments in more than one species, sex, strain, site, or exposure route, with or without evidence of carcinogenicity in humans; a positive tumor study that is strengthened by other lines of evidence, for example, either plausible (but not definitively causal) association between human exposure and cancer or evidence that the agent or an important metabolite causes events generally known to be associated with tumor formation. Relative to liver tumor formation, both of these situations are applicable to carbon tetrachloride, as such this classification appears appropriate. The scientific justification for the weight of evidence descriptor has been sufficiently, transparently and objectively described in the review. The data available for liver tumors in rats and mice supports this conclusion. For pheochromocytomas, tumors were found only in mice, and predominantly characterized as benign. Thus, this document included the statement (p216) "the finding of pheochromocytomas in the mouse may be a species-specific finding and, as such, may present a less certain human cancer risk than does the finding of liver tumors in experimental animals". I am in agreement with this assessment. Regardless, the document adequately describes the available data in a transparent and objective manner.

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

   The preponderance of data for carbon tetrachloride supports a mode of action (MOA) for liver tumors that includes the following key events: (1) metabolism to reactive intermediates, (2) radical-induced mechanisms leading to hepatocellular toxicity, and (3) sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. These key events are consistent with a hypothesis that liver carcinogenicity occurs at exposures that also induce hepatocellular toxicity and a sustained regenerative and proliferative response, and that exposures that do not cause hepatotoxicity are not expected to result in liver cancer. A number of experimental studies support these key events, which makes this a

biologically plausible MOA. This scientific basis for this MOA and the characterization of uncertainties for this MOA have been adequately addressed and described in the review.

Concerning the potential genotoxicity of carbon tetrachloride at low doses, results of extensive testing for genotoxic and mutagenic potential of carbon tetrachloride are largely negative. There is little direct evidence that carbon tetrachloride induces mutations in mammalian systems; mutagenicity studies in transgenic mice have yielded negative results, as have the vast majority of the mutagenesis studies that have been conducted in bacterial systems. Only under highly cytotoxic conditions was carbon tetrachloride shown to exert genotoxic effects.

At many instances in the review (for example, p 239), it is stated that carbon tetrachloride overall has not been found to be a potent mutagen and that positive genotoxic results are found only at high exposure levels and generally in concert with cytotoxic effects (see Tables 4-8 to 4-11). This finding would support that carbon tetrachloride does not likely induce genotoxic effects through direct binding or damage to DNA. Using a weight of evidence approach, the scientific data shows that carbon tetrachloride is not genotoxic or mutagenic.

The scientific literature supports that doses of carbon tetrachloride that cause toxicity will result in lipid peroxidation, peroxidation products and other radical species; while doses of carbon tetrachloride that are not toxic do not result in lipid peroxidation, peroxidation products and other radical species. The argument being presented is that low (non-toxic) levels of carbon tetrachloride, if tested, would exhibit genotoxicity. Since low doses of carbon tetrachloride that are not toxic will not be associated with radical mediated damage and lipid peroxidation, based on the known biological effects of carbon tetrachloride, it is unlikely that low doses will produce mutation or genotoxicity.

Evidence is accumulating demonstrating a hormetic response (u- or j-shaped curves) for many toxic chemicals. Hormesis is defined as a dose–response curve in which a U, J or inverted U-shaped dose–response is observed; with low dose exposures often resulting in beneficial rather than harmful effects (Calabrese, 2002). In many instances repeated moderate “priming doses” of liver toxicants such as carbon tetrachloride can lead to enhanced detoxification and other adaptations that protect against the ordinarily lethal effects of subsequent larger doses (Anand et al. 2006; Philip et al. 2006). Therefore, low level exposure to carbon tetrachloride would be expected to induce detoxifying and/or DNA repair enzymes and reduce or prevent damage cellular caused by carbon tetrachloride. In light of this biological response, the potential for carbon tetrachloride to exhibit genotoxicity following low level exposures is unlikely.

While extensive description of indirect genotoxicity as a potential MOA has been presented in the Review document, based on the above statements, I do not agree with the conclusions drawn concerning the potential for genotoxicity of carbon tetrachloride at low doses.

Anand SS, Philip BK, Palkar PS, Mumtaz MM, Latendresse JR, Mehendale HM. (2006). Adaptive tolerance in mice upon subchronic exposure to chloroform: Increased exhalation and target tissue regeneration. Toxicol Appl Pharmacol. 2006 Jun 15;213(3):267-81.

Calabrese,E.J. (2002) Hormesis: changing view of the dose–response, a personal account of the history and current status. Mutat. Res., 511, 181–189.

Philip BK, Anand SS, Palkar PS, Mumtaz MM, Latendresse JR, Mehendale HM. (2006). Subchronic chloroform priming protects mice from a subsequently administered lethal dose of chloroform. Toxicol. Appl. Pharmacol. 216:108-121

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   I agree with a nonlinear MOA involving hepatocellular cytotoxicity and regenerative hyperplasia. As stated above, the existing data for carbon tetrachloride supports a MOA for liver tumors that includes the following key events: (1) metabolism to reactive intermediates, (2) radical-induced mechanisms leading to hepatocellular toxicity, and (3) sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. These key events are consistent with a hypothesis that liver carcinogenicity occurs at exposures that also induce hepatocellular toxicity and a sustained regenerative and proliferative response, and that exposures that do not cause hepatotoxicity are not expected to result in liver cancer. All other cancer bioassays that have been conducted show a similar finding – liver toxicity was seen concomitant with liver tumors. This demonstrates concordance of this endpoint among several studies.

   Concerning the cancer MOA at low doses, the Nagano paper reported that a 5ppm dose of carbon tetrachloride did not induce toxicity but did increase the incidence of combined adenoma and carcinomas in the liver of female mice only, although the increase was not significant relative to the study control. When interpreted relative to historical control (from the same laboratory) the increase in the 5ppm carbon tetrachloride group was considered significantly elevated. It is understood that this type of analysis (use of historical control vs study control) can be applied when a positive trend is observed, as was the case in this EPA analysis.

   While the slight increase in tumor response (5ppm, female mice) was observed without evidence of apparent histopatological changes in the liver, the effect at this dose was limited to female mice, and not in male mice or male and female rats. Thus, the biological significance of this finding is questioned. Furthermore, the tumor response at 5ppm in female mice (18%) was considerably lower than the incidence produced by 25 (88%) and 125ppm (98%) carbon tetrachloride in male mice (98%) and or by 125ppm in either male or female rats (80-88%). Epidemiological studies have not identified an association between human exposures to carbon tetrachloride and increase liver cancer incidence. Therefore, I do not agree that the default linear extrapolation approach should be applied due to uncertainty in understanding the cancer MOA at low doses.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

   Pheocromacytomas were observed in mice following carbon tetrachloride exposure (JRBC; Nagano et al., 2007). The tumors produced in the mouse were benign. Guidance provided in the 2005 *Guidelines for Carcinogen Risk Assessment* (U.S. EPA, 2005), states that "benign tumors that are not observed to progress to malignancy are assessed on a case-by-case basis." Since the tumor type seen in the mouse has a human equivalent that is damaging to human health and can lead to fatal sequelae, the EPA

document conducted dose-response modeling for pheochromocytomas and to address the potential cancer risk using linear extrapolation as a default approach.

I do not agree with this approach. This benign tumor was observed only in mice - no increase in the incidence of pheochromocytomas was observed in rats in either NCI (1977) or Nagano et al. (2007b), therefore this may represent a strain-specific finding. These negative results were obtained by two separate routes of administration (oral – NCI, inhalation – Nagano). Further, in epidemiological studies, no increase in pheochromacytomas has been observed in exposed humans. In these human exposures, carbon tetrachloride exposure was confounded by simultaneous exposures to other chemicals, notably co-exposure to ethanol, a chemical that can enhance metabolism to reactive metabolites, and may be expected to exacerbate carbon tetrachloride effects. Based on these factors, a linear extrapolation based on pheochromacytomas in mice is not justified.

5. ***Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.**

   Collectively, an extensive body of literature on adverse effects of carbon tetrachloride supports a mode of action (MOA) for liver tumors that includes the following key events: (1) metabolism to reactive intermediates, (2) radical-induced mechanisms leading to hepatocellular toxicity, and (3) sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. These data are objectively described in the review document.

   The key events are threshold based, which implies that liver tumor response will be nonlinear – occurring at exposures that induce hepatocellular toxicity and a sustained regenerative and proliferative response, while exposures that do not cause hepatotoxicity are not expected to result in liver cancer. Confidence should be placed on an RfD of 0.004 mg/kg-day and RfC of 0.1 mg/m^3 for carbon tetrachloride. These values were scientifically derived, (used BMD approaches, and PBPK modeling when appropriate) and use additional uncertainty factors (1000 for RfD and 100 for RfC) to characterize risk and safeguard human health.

   I am not aware of an alternate nonlinear approach that should be applied to characterize liver cancer risk from carbon tetrachloride.

6. ***Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for**

including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?

While the rationale for the linear approach is transparently presented in the Toxicological Review for carbon tetrachloride, based on the comments provided in response to questions 2-5 above, I do not agree that a linear assessment is justified for carbon tetrachloride.

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

I agree with the conclusion that studies on carbon tetrachloride carcinogenicity by the oral route were insufficient to derive a quantitative estimate of cancer risk. The review applied a human PBPK model to extrapolate inhalation data to the oral route, which assumed continuous infusion of carbon tetrachloride from the gastrointestinal tract to the liver. This approach appears to be justified, and the document appropriately discussed that the degree of uncertainty introduced by these assumptions cannot be quantified. However, this analysis used a low dose linear extrapolation, an approach with which I am not in agreement (see response to questions 2-5 and 8).

8. **EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?**

Section 5.4.3 describes guidelines excerpted from EPA's (2005a) *Guidelines for Carcinogen Risk Assessment*, related to applicability of selecting a nonlinear or linear extrapolation approaches for assessing cancer risk. This section of the document concludes that based on extensive mechanistic data, at high exposure levels, rodent bioassay data reveal a general correspondence between hepatocellular cytotoxicity and regenerative hyperplasia and the induction of liver tumors.

The Toxicological review for carbon tetrachloride describes three areas that suggest that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative mode of action alone. These include 1) increased incidence of liver tumors in the low-dose female mouse in the absence of apparent liver toxicity (Nagano et al., 2007b); 2) increased incidence in pheochromocytomas in mice; and 3) absence of data on low-dose genotoxicity. As commented on in questions 2-5 above, I do not agree with this recommendation. The following paragraphs summarize the rationale for this opinion.

The slight increase in tumor response (5ppm, female mice) was limited to female mice, and not in male mice or male and female rats. The tumor response at 5ppm in female mice (18%) was considerably lower than the incidence produced by 25 (88%) and 125ppm (98%) carbon tetrachloride in male mice (98%) and or by 125ppm in either male or female rats (80-88%). Epidemiological studies have not identified an association between human exposures to carbon tetrachloride and increase liver cancer incidence.

Concerning pheochromacytomas in mice, tumors were classified as benign, were only observed in mice (no increase in rats [NCI (1977); Nagano et al. (2007b)], by two separate routes of administration (oral – NCI, inhalation – Nagano). Epidemiological studies did not reveal increases in pheochromacytomas in exposed humans.

Carbon tetrachloride is largely negative for genotoxicity and mutagenicity; positive genotoxic results are found only at high exposure levels and generally in concert with cytotoxic effects (see Tables 4-8 to 4-11). The argument being presented is that low (non-toxic) levels of carbon tetrachloride, if tested, would exhibit genotoxicity. Since low doses of carbon tetrachloride that are not toxic will not be associated with radical mediated damage and lipid peroxidation, based on the known biological effects of carbon tetrachloride, and on a weight of evidence approach, the scientific data supports that carbon tetrachloride is not genotoxic or mutagenic.

Lawrence H. Lash, Ph.D.

Wayne State University School of Medicine

Dr. Lawrence H. Lash is Professor of Pharmacology at Wayne State University School of Medicine in Detroit, Michigan, where he has been a faculty member since 1988. Prior to that, Dr. Lash earned his B.A. in Biology at Case Western Reserve University in Cleveland, Ohio in 1980, his Ph.D. in Biochemistry at Emory University School of Medicine in 1985, and did a postdoctoral fellowship in Pharmacology and Toxicology at the University of Rochester School of Medicine from 1985 to 1988 with Professor M.W. Anders. At Rochester, he studied the enzymology of cysteine conjugate β-lyase in the bioactivation of the cysteine conjugate of trichloroethylene, *S*-(1,2-dichlorovinyl)-L-cysteine (DCVC), and studied mechanisms of renal cellular injury induced by DCVC. Since coming to Wayne State, Dr. Lash's research program has focused on assessment of factors that determine or regulate renal susceptibility to chemically induced injury, including membrane transport, Phase I and Phase II metabolism, gender and species differences, signaling pathways that modulate response to toxicity, and disease.

Some key findings over the past twenty years have included the following: 1) Discovery and characterization of Na^+-coupled glutathione (GSH) transport across the renal basolateral plasma membrane and identification of the organic anion transporter 3 (Oat3) as one carrier mediating the transport; 2) development of *in vitro* cell models to study nephron segment-specific mechanisms of injury; 3) identification of mitochondria as a major subcellular site of action for nephrotoxic cysteine *S*-conjugates of halogenated solvents, such as trichloroethylene; 4) extension of *in vitro* cell models for primary culture of rat proximal and distal tubules to those from human kidneys; 5) quantitation of metabolism by both GSH conjugation and cytochrome P450 pathways for trichloroethylene and perchloroethylene in liver and kidney from rats, mice, and humans, providing information that can be used to improve pharmacokinetic models that are part of human health risk assessment efforts; 6) demonstration that DCVC induces a range of responses in human proximal tubular cells, including cell death by necrosis (oncosis) and apoptosis, growth arrest, and repair and proliferation, depending on the concentration and time of exposure; 7) showed that human kidney is less susceptible than rat kidney to cytotoxicity induced by DCVC; and 8) showed that compensatory renal hypertrophy and diabetic nephropathy induce oxidative stress in proximal tubules and changes in mitochondrial GSH status. Dr. Lash has published more than 100 original, peer-reviewed research papers and more than 50 reviews and book chapters, and has edited or co-edited four books and a special issue of *Toxicology and Applied Pharmacology* on membrane transport in toxicology.

Dr. Lash has consulted for the U.S. Environmental Protection Agency (EPA) on their human health risk assessments for trichloroethylene, perchloroethylene, trimethylpentane, and barium and the National Academy of Sciences on their report on biomarkers in urinary toxicology. He has served on study sections and review panels for the National Institutes of Health, National Science Foundation, Super Fund review panel of the National Institute of Environmental Health Sciences, and other organizations. He is an Associate Editor for three major peer-reviewed journals in pharmacology and toxicology, *The Journal of Pharmacology and Experimental Therapeutics*, *Toxicology and Applied Pharmacology*, and *Pharmacology and Therapeutics*, and is on other editorial boards.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

The Toxicological Review is well organized, well written, and comprehensive. The goals of the review are clearly presented and the document has objectively and, for the most part, accurately synthesized all the pertinent literature on the evidence for the noncancer and cancer hazards of carbon tetrachloride. The available literature is thoroughly and objectively reviewed, although there are a few cases (see below) where some further comments on the accuracy of the data are needed and a case where a couple of references are not cited. In both these situations, however, the errors or lack of analysis are relatively minor and may not significantly influence the overall evaluation of noncancer and cancer hazards. As discussed below, these factors may modestly influence the specific uncertainty factors that are used.

Page 7, para. 3, last sentence: Values for blood/air partition coefficients are given for humans (2.73 to 4.20 from Fisher et al., 1997 and Gargas et al., 1989) and for rats (4.52 from Gargas et al., 1986). It seems unusual that there should be such a large range of values and, if the lower range of values for humans are correct, then it seems unusual that there would be such a large interspecies difference. Some evaluation of these data are needed.

Page 13, para. 2 and page 22: It is noted that CYP enzyme inactivation is more severe in the rat, being 1 molecule of enzyme lost for every 26 molecules of substrate metabolized, whereas in humans, inactivation is 1 molecule of enzyme lost for every 196 molecules of substrate metabolized. Such a large difference in metabolism-dependent inactivation (7.5-fold less inactivation in humans vs. rats) would be expected to have a large influence on the extent of carbon tetrachloride bioactivation. Hence, this might be expected to significantly increase risk in humans as compared to rats solely based on this one parameter. No comment is made regarding this large discrepancy between species. On page 22, however, Table 3-5 summarizes a comparison of *in vitro* and *in vivo* metabolism among four species (rat, mouse, hamster, and human), and shows human metabolism to have V_{max} values both *in vitro* and *in vivo* that are 27% lower than those in the rat. This difference may, therefore, mitigate some of the effect of omitting consideration of interspecies differences in rates of CYP inactivation.

Page 26, bottom – page 27: The study of Yoon et al. (2007) is discussed regarding the extrahepatic metabolism of carbon tetrachloride. While rat kidney cortex and proximal tubules express reasonable levels of CYP2E1 protein and activity for the oxidative metabolism of another CYP2E1 substrate, trichloroethylene (Cummings et al., 1999, 2000b, 2001), human kidney has been reported by multiple laboratories to not express any detectable CYP2E1 protein (Amet et al., 1997; Cummings and Lash, 2000; Cummings et al., 2000a) and to exhibit little if any oxidative metabolism of trichloroethylene (Cummings and Lash, 2000; Cummings et al., 2000a). Because extrahepatic metabolism is calculated to contribute only a very minor proportion to total metabolism (< 1%), however, acknowledgement of this point will have no significant influence on the conclusions that are reached. For the sake of correctness, however, these interspecies (rodent vs. human) and interorgan (kidney vs. liver) differences in CYP2E1 expression and activity should be properly noted. See the references listed below.

References:

Amet, Y., Berthou, F., Fournier, G., Dréano, Y., Bardou, L., Cledes, J., and Menez, J.-F. (1997) Cytochrome P450 4A and 2E1 expression in human kidney microsomes. Biochem. Pharmacol. **53,** 765-771.

Cummings, B.S., and Lash, L.H. (2000) Metabolism and toxicity of trichloroethylene and S-(1,2-dichlorovinyl)-L-cysteine in freshly isolated human proximal tubular cells. Toxicol. Sci. **53,** 458-466.

Cummings, B.S., Lasker, J.M., and Lash, L.H. (2000a) Expression of glutathione-dependent enzymes and cytochrome P450s in freshly isolated and primary cultures of proximal tubular cells from human kidney. J. Pharmacol. Exp. Ther. **293,** 677-685.

Cummings, B.S., Parker, J.C., and Lash, L.H. (2000b) Role of cytochrome P450 and glutathione S-transferase α in metabolism and cytotoxicity of trichloroethylene in rat kidney. Biochem. Pharmacol. **59,** 531-543.

Cummings, B.S., Parker, J.C., and Lash, L.H. (2001) Cytochrome P-450-dependent metabolism of trichloroethylene in rat kidney. Toxicol. Sci. **60,** 11-19.

Cummings, B.S., Zangar, R.C., Novak, R.F., and Lash, L.H. (1999) Cellular distribution of cytochromes P-450 in the rat kidney. Drug Metab. Dispos. **27,** 542-548.

Page 34, para. 4, lines 8 and 9: Typos: Change “GTT” to “GGT” on line 8 and change “of” to “or” on line 9.

Page 47, para. 1: In the review of the Smyth et al. (1936) study, it is stated that “use of controls was not described, although controls apparently were included in the study.” This statement does not make much sense. On what basis does the EPA conclude that there were controls?

Page 48, para. 1, line 6: Typo: Change “much” to “must.”

*Page 65, para. 1:*In discussing the developmental toxicity studies of Narotsky et al. (1995, 1997a), the document describes the lack of robustness of the study. It then states the authors’ conclusions about the underlying mechanism of the all-or-none fetal resorption response without questioning the strength of this conclusion. Some evaluation is needed.

Page 66, para. 2: In describing the Hamlin et al. (1993) study, which is quite limited in scope, it is unclear what value this study is in elucidating developmental toxicity of carbon tetrachloride.

Page 91-93: In describing the genotoxicity studies, some of the results were unexpected or equivocal. The document concludes this brief section (section 4.4.2.1) with a statement that in vitro carbon tetrachloride results should be interpreted cautiously. While there is nothing wrong with this statement, it does not really identify the problems with the data. Although the document states that there were confounding factors, the reader does not really know what to think of the data or how to use it.

Page 94, para. 1: Genotoxicity studies are described in non-mammalian eukaryotes. The document reports that positive results in studies by Callen et al. (1980) were only observed at the highest test concentration of 34 mM, when there was extensive toxicity. While the document certainly addresses the issue of dose relevance later on, some comment should be made here about the inappropriateness or irrelevance of such a high dose.

Page 95-99: Again, in reviewing several of the genotoxicity studies, both in non-mammalian and mammalian test systems, the document reports results with doses that are very high. While the document does point out that the high degree of toxicity observed at these high doses complicates interpretation of the results, I would like to see a stronger statement about the relevance of these studies.

Page 102, bottom –page 104, top: Mutagenesis studies are reported in transgenic mouse strains. It is unclear what the value is of these mouse strains and how they are potentially useful in elucidating the ability of carbon tetrachloride to cause mutations. A few specific statements about what is unique about these strains are needed.

Page 118, para. 3 (section 4.5): In describing mechanistic studies on the mode of action of carbon tetrachloride, the document states that "representative studies" are summarized. Why were only certain studies summarized? What criteria were used to select which studies to discuss?

Page 126, para. 2, sentence 2: This sentence needs to be rewritten as follows: "Depletion of GSH with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase, which generates the precursor to GSH…, increased…"

Page 126, para. 3, line 3: Correct the sentence to read that "Activation of calcium ATPase triggers the transport of one calcium ion from the cytoplasm to the endoplasmic reticulum…"

Page 132, para. 2, line 5: Correction: NF-κB (where κ = Greek letter kappa).

Page 145, para. 3: The discussion about the kidney as a potential target organ needs to consider species differences, in particular the reported absence of CYP2E1 in human kidney (see discussion above).

Page 146, para. 1: The document states that under the *Guidelines for Carcinogen Risk Assessment* (US EPA, 2005a), carbon tetrachloride is classified as "likely to be carcinogenic to humans." The document should make it clear who is making this conclusion. Is this the finding of an NTP study group?

Page 155, last sentence: The document states that "EPA is choosing to characterize the full range of carcinogenic potential for human exposure to carbon tetrachloride." What does "full range" mean? Is this carcinogenic potential over a complete range of exposure doses? Over a range of doses to which humans can be reasonably exposed? Please define.

Page 165, lines 3-4: The document concludes this section on other possible modes of action and states that, "thus, the possible contribution of a low-dose mutagenic effect in the mode of action or alternative modes of action cannot be excluded." While the second part of the conclusion is fine, I have a concern that the first part, namely about a possible, low-dose mutagenic effect, is misleading. Are there really any data to support this? My suggestion is that the document could state that a "low-dose mutagenic effect cannot be excluded, although there are currently no data to support such a mechanism."

Page 166, top para: In a similar vein as the last comment, the document states that "other (or another) mode of action that are independent of cytotoxicity and regenerative cell proliferation may be operative in this range." Rather than say "may be operative," I would suggest "cannot be excluded."

Page 166, para. 2 (section 4.7.3.5): The section concludes with a statement that the same types of tumors that are found in animals are also found in humans. Are these tumors due to other chemicals? If so, which chemicals? This is important because the document states that liver tumors due to carbon tetrachloride have not been reported in humans.

Page 170, second full para.: There is a statement that "CYP2E1 microsomal protein levels were reduced by 20% (not statistically significant),…" If two parameters are not significantly different from one another by statistical analysis, then there is no difference.

Page 174, para. 2, line 7: The term "hepatonephrotoxicity" is not correct; change to "hepatotoxicity and nephrotoxicity."

Page 177, Table 5-1: All abbreviations should be defined in the tables. While ALT is pretty well known to those who study liver function, SDH is often used by biochemists to define "succinate dehydrogenase" rather than "sorbitol dehydrogenase." What is "OCT"?

Page 212, bottom para.: In the subsection on intrahuman variability, it is stated that there is "an absence of quantitative information on variation in hepatic levels of CYP2E1 or other metabolizing enzymes…" This is not true. CYP2E1 protein levels in human liver have been reported to vary by as much as 20-fold. Some references that present data and discuss inter-individual differences in cytochrome P450 levels are:

Lash, L.H., Lipscomb, J.C., Putt, D.A., and Parker, J.C. (1999) Glutathione conjugation of trichloroethylene in human liver and kidney: Kinetics and individual variation. *Drug Metab. Dispos.* **27**, 351-359.

Lipscomb, J.C., Garrett, C.M., and Snawder, J.E. (1997) Cytochrome P450-dependent metabolism of trichloroethylene: Interindividual differences in humans. *Toxicol. Appl. Pharmacol.* **142**, 311-318.

Lipscomb, J.C., Fisher, J.W., Confer, P.D., and Byczkowski, J.Z. (1998) In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. *Toxicol. Appl. Pharmacol.* **152**, 376-387.

Lipscomb, J.C., and Kedderis, G.L. (2002) Incorporating human interindividual biotransformation variance in health risk assessment. *Sci. Total Environ.* **288**, 13-21.

Snawder, J.E., and Lipscomb, J.C. (2000) Interindividual variance of cytochrome P450 forms in human hepatic microsomes: Correlation of individual forms with xenobiotic metabolism and implications in risk assessment. *Regul. Toxicol. Pharmacol.* **32**, 200-209.

Page 243, para. 3: In discussing human population variability, no mention is made of genetic polymorphisms in drug metabolism enzymes, transporters, and receptors, all of which can markedly affect susceptibility to a toxic chemical.

Page 244, Table 5-20: In the top row under "Justification," the table notes that "There is no evidence in humans for hepatic cancer associated with carbon tetrachloride exposure." Later in the same box, it states that "this evidence supports a conclusion that experimental evidence for liver cancer is relevant to humans." These are seemingly contradictory statements that require some explanation and clarification. How can liver cancer data in animals be relevant to humans if liver tumors have never been observed in humans exposed to carbon tetrachloride?

Page 248, last sentence: Who has made the conclusion that carbon tetrachloride be classified as "likely to be carcinogenic in humans by all routes of exposure"? Is this the recommendation of the report writers or has this been concluded by an NTP committee?

Page 251, para. 2: The manner in which the composite uncertainty factor for the RfC is written, it seems like it should be 1000 rather than 100? Three UFs are listed: 1) A factor of 10 to protect susceptible individuals; 2) a factor of 3 ($10^{0.5}$) to extrapolate from rats to humans; and 3) a factor of 3 ($10^{0.5}$) to account for an incomplete database. These three factors, when combined = 10 x 10 x 10 = 1000. If the composite UF of 100 is indeed correct, then I would suggest writing the second two factors as 3 (= $10^{0.5}$) to avoid confusion.

Page 259: Genetic polymorphisms should be included in a consideration of human population variability.

2. Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.

The references listed above on renal vs. hepatic CYP2E1 in rats vs. humans and those listed above on the human inter-individual variability in CYP expression should be considered. While the errors that were made in omitting these references do not really change the validity of the conclusions or calculations of the RfC and RfD values, they are important to include for the sake of correctness and completeness.

Otherwise, a PubMed search on carbon tetrachloride up through 9-15-2008 did not reveal and additional references that need to be considered that directly relate to carbon tetrachloride mode of action. Note that the database search used to prepare the May, 2008 draft assessment went through December, 2007.

3. Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.

The following are suggested areas of research that are needed to strengthen the database and provide better support for the proposed RfC, RfD, and slope factor values:

(i) Epidemiology studies that clarify the occurrence or lack thereof of liver tumors in carbon tetrachloride-exposed humans;
(ii) Additional, low-dose genotoxicity studies to establish whether DNA damage can really occur at doses relevant to environmental or occupational exposure doses;
(iii) More complete human metabolism data in both liver and extrahepatic tissues;
(iv) More complete analysis of human variation, including genetic polymorphisms, in enzymes that metabolize carbon tetrachloride, including CYP2E1 and CYP3A4.
(v) A new cancer bioassay with oral administration of a wide range of doses, including those below which hepatotoxicity occurs, to provide better data for RfD estimation; this would also eliminate the need for route-to-route extrapolation;
(vi) Repeat of studies where control animals exhibits higher rates of liver cancer than historical controls;
(vii) In vitro mechanistic studies on cytotoxicity and potential mutagenicity in human cells at low doses.

4. Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?

The document carefully and methodically considers all the sources of uncertainty in the hazard assessment of carbon tetrachloride. The choices of studies to consider and their limitations are clearly presented. The only question noted relates to uncertainty factors for calculation of the RfD, in which the document concludes (on page 184, para. 2) that the inhalation data do not support a full default UF of 10. A better explanation is needed for this conclusion.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   Selection of the 1986 Bruckner et al. study is scientifically justified and the rationale for selecting it has been clearly explained. The explanation of why no other study is available to serve as the principal study is logical and appropriate. Thus, I conclude that there are no other studies that could be used in place of the Bruckner et al. study.

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

   The rationale for the choice of serum sorbitol dehydrogenase (SDH) as the critical effect for determination of the RfD is presented clearly and logically and seems scientifically justified. The only questions that arise are first, why on page 184, para. 2, does the document conclude that a full default UF of 10 is not supported, and second, why SDH rather than some of the more commonly measured parameters, such as AST (aspartate aminotransferase) or ALT (alanine aminotransferase), is chosen? I presume that this is because the studies that are cited (i.e., Nagano et al., 2007a,b; JBRC, 1998) measured SDH as opposed to these other parameters. Nonetheless, some comment should be made about how typically SDH is used as a metric of hepatic function.

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   The document clearly describes the rationale and basis for choosing the different modeling methods for determining the POD. The criterion of an increase in SDH activity that is twice the control levels is scientifically appropriate and has been transparently and objectively described. Considering the available database, no other approaches seem more appropriate than that used by the EPA.

4. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

Of all the uncertainty factors, I have a concern about the appropriate of this one more than any other. As noted above, the document incorrectly states that there is no quantitative information on the variation in hepatic levels of CYP2E1 or other relevant drug metabolism enzymes. Several references are provided that have quantified variation in these enzymes. For example, besides CYP2E1, CYP3A4 can metabolize carbon tetrachloride. Genetic polymorphisms in CYP3A enzymes are well known. Furthermore, the document also presents data on the differing ability of carbon tetrachloride metabolites to inactivate CYP2E1, such that enzyme activity from rat liver microsomes is inactivated at a rate that is 7.5-fold higher than that from human liver microsomes. Although this species difference does not seem to be completely reflected in the measured kinetic parameters (particularly V_{max}) for metabolism, those parameters and the difference in CYP inactivation rate suggest that risk for humans should be less than that for rats, not more. Hence, I am not convinced that a default UF of 10 is justified.

- **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

Rationale for use of a smaller UF than the default is clearly explained and justified.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.**

In discussing the issue of data gaps, the document states on page 213, para. 3, that "the absence of these types of studies (i.e., an adequate multigeneration study of reproductive toxicity) introduces uncertainty… the magnitude of this uncertainty cannot be quantified." If the magnitude cannot be quantified, then how is it justified to use an UF that differs from the default? I would think that logically, the default is used when the degree of uncertainty is unclear. Such a position would seem to be more consistent with standard EPA practices.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

In general, the document adequately describes the underlying basis for the use of the various UFs. In two cases, however, the rationale does not seem scientifically justified. The first is the case of interspecies variation. Here, it seems that the document does not account for much of the known information on variation and genetic polymorphisms in CYP2E1 and CYP3A4 or for the stated differences in rates of enzyme inactivation in rat and human liver microsomes. Thus, the UF for interspecies variation is not on a firm scientific foundation. The second is the UF for data gaps. If, as the document states, the degree of uncertainty due to missing data is unknown, why would not the default UF of 10 be used rather than an UF of 3?

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   The document clearly and logically presents all the arguments for why this 2-year inhalation study was chosen. The first reason is that there really are no viable alternatives. The second reason, which is the more important one, is that the study was judged as being properly conducted with sufficient doses and good controls. Thus, the criteria for judging this study as robust and valid are transparently and objectively presented.

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   In terms of choosing a pathological change that is an early sign of future tissue injury, fatty degeneration of the liver is an appropriate choice. The document clearly presents the rationale for choosing this as the critical effect. Based on the pathophysiology of toxic liver injury and liver disease, this is a scientifically appropriate choice. While other parameters can be quantified and potentially used as an early sign of adverse liver function, such as changes in bile acid status or enzyme release into plasma, fatty liver degeneration is the best parameter for the intended purposes.

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

   The decision to not use proteinuria as a critical effect is clearly explained in the document. The significant problems with the proteinuria database would compromise the strength of any conclusions that might arise from use of these data. The rationale is thus scientifically sound and has been transparently and objectively described.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   Use of the BMD approach for determining the POD for the RfC is clearly, transparently, and objectively described. The appropriateness of this modeling approach is clearly described on page 199, para. 2, where it is stated that the aim of the modeling is to model dose-response data for an adverse effect (fatty liver changes in this case) and to select a "benchmark dose" at the low end of the observable range to use as a POD. Using this approach, the modeling provides good fit to observed data. No other approach would seem to have any advantages over this one.

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?**

   The document carefully describes the PBPK modeling approach to obtain a POD by extrapolating data from rats to humans and from inhalation to oral dose estimates. The reasons for using this approach are carefully explained and are scientifically justified. In a step-by-step manner, using different assumed V_{max} values, different conversion factors are calculated to give the desired dose metrics. All these values, the model assumptions, and selection of dose metrics are clearly presented and the uncertainties in the use of this approach are clearly described.

6. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

   - **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

   As with the consideration of the intraspecies UF for deriving the RfD discussed above, a default value of 10 is used here for derivation of the RfC. The rationale for using this default value is an absence of quantitative information on the variability of human response to carbon tetrachloride. As stated above, there is information available regarding CYP expression that has not been considered. While it may be correct that there are no direct data on the variation in human toxic response to carbon tetrachloride exposure, there is information that may mitigate some of the variability. The choice of the default UF of 10 is probably reasonable based on the desire to err on the side of conservatism.

   - **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

As with the consideration of interspecies UF for deriving the RfD discussed above, the document does not consider the metabolic differences that are cited (7.5-fold more rapid CYP inactivation in the rat as compared to the human), which would presumably lower risk in humans as compared to rats. The justification does discuss differences in cellular protective mechanisms, for which little data are available. Hence, the UF of 3 is probably appropriate.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study. Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?**

The UF of 3 for database uncertainty seems appropriate, given the nature of the developmental toxicity database. Based on some information about developmental differences in enzyme expression levels, the document concludes that there is little data to support a conclusion that the developing organism is any more susceptible than adults to liver injury from carbon tetrachloride. Furthermore, although additional studies are probably warranted, such as a multigeneration reproductive toxicity study, the document logically argues that addition of such data would not be likely to result in a smaller POD. Hence, the UF of 3 seems scientifically justified.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (**www.epa.gov/iris/backgr-d.htm**), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

The cancer assessment is generally clearly and transparently described, although I think that the document could be clearer about what the designation means. I have some concerns about the overall conclusion that carbon tetrachloride should be considered "likely to be carcinogenic to humans by all routes of exposure," as it is not clear how this relates to the previous assessment from 1991 that it is "probably a human carcinogen." The previous conclusion was based on sufficient evidence in animals whereas the newly proposed designation of "likely to be carcinogenic to humans" would be based on sufficient evidence in animals and humans. The U.S. EPA Guidelines for Carcinogen Risk Assessment, published in 2005, gives the following categories for chemicals:

> *Carcinogenic to Humans:* The Guidelines recommend this descriptor when there is convincing epidemiologic evidence demonstrating causality between human exposure and cancer, or exceptionally when there is strong epidemiological evidence, extensive animal evidence, knowledge of the mode of action, and information that the mode of action is anticipated to occur in humans and progress to tumors.
>
> *Likely to be Carcinogenic to Humans:* The Guidelines recommend this descriptor when the available tumor effects and other key data are adequate to demonstrate carcinogenic potential to humans, but does not reach the weight-of-evidence for the descriptor "carcinogenic to humans."

Suggestive Evidence of Carcinogenic Potential: The Guidelines recommend this descriptor when the evidence from human or animal data is suggestive of carcinogenicity, which raises a concern for carcinogenic effects but is judged not sufficient for a stronger conclusion.

Inadequate Information to Assess Carcinogenic Potential: The Guidelines recommend this descriptor when available data are judged inadequate to perform an assessment.

Not Likely to be Carcinogenic to Humans: The Guidelines recommend this descriptor when the available data are considered robust for deciding that there is no basis for human hazard concern.

While the animal data clearly demonstrate carcinogenesis, the document notes that tumors in humans, particularly in the liver, have not been observed. The document considers the animal data to be relevant to humans because of similarities in mode of action and in the characteristics of similar tumors that occur in humans under other exposure conditions. Considering that liver effects are considered to be primary, it is unclear to me how the absence of liver tumors in humans can be reconciled with the designation of "likely to be carcinogenic in humans."

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

The potential genotoxicity of carbon tetrachloride has been fairly thoroughly investigated with little positive results. The document clearly explains the significance of the findings and justly concludes that carbon tetrachloride is unlikely to be genotoxic by a direct mode of action but rather, that liver injury and proliferation are likely to lead to genotoxicity (i.e., an indirect mechanism). Most of the investigations of mutagenicity and genotoxicity have been performed with relatively higher doses of carbon tetrachloride, presumably because low doses produced no responses. Based on the conclusion that hepatotoxicity is a prerequisite for subsequent genotoxicity, a nonlinear extrapolation approach is proposed. This conclusion and approach derive primarily from the rat liver tumor data. The document also notes that the incidence of hepatocellular adenomas in female mice exposed to low doses in the absence of hepatotoxicity, suggest that the cytotoxicity-proliferation mode of action cannot fully explain carbon tetrachloride-induced liver cancer and that a linear extrapolation approach may be more appropriate. The document also suggests that direct effects on DNA, possibly leading to genotoxicity, may occur at low doses, thereby further justifying the linear extrapolation approach. It is further suggested that in the absence of mode of action information on mouse pheochromocytomas, the default linear extrapolation approach should be used.

With regard to the cytotoxicity-proliferative mechanism, there are ample data to support this. With the exception of the unexplained hepatocellular adenomas in female mice at low doses, there are no data to my knowledge that support any other mechanism of action. Thus, while it is certainly appropriate to suggest additional mechanism to be consistent with unexplained data, I am not sure that the mouse data provide a very strong rationale for an alternate mode of action.

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   As described above, the scientific basis and logical support for the nonlinear extrapolation approach have a good deal of support from the literature. I agree completely that this approach is most consistent with the most reliable data and the deduced MOA involving hepatotoxicity and regenerative hyperplasia. In contrast, the choice of a linear extrapolation approach based on a small, possibly aberrant set of data (i.e., hepatocellular adenomas in female mice at a low dose) is a default approach that has little scientific support. While I think that it is completely appropriate to present the alternative approach for low-dose, cancer risk assessment, the document falls short in not making some judgment as to the relative strength of the two proposed approaches.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

   While the mouse pheochromocytoma data are interesting and certainly cannot be ignored, their relevance to humans is questionable. The document generally presents these results clearly and objectively. Because no MOA information is available for this type of tumor response, default approaches are used. On page 216, first full para., the document states the following conclusion about this tumor target:

   > "Thus, the finding of pheochromocytomas in the mouse may be a species-specific finding and, as such, may present a less certain human cancer risk than does the finding of liver tumors in experimental animals. Nevertheless, the RfD and RfC based on liver toxicity cannot be assumed to be protective for the potential cancer risk associated with carbon tetrachloride-induced pheochromocytomas in the mouse."

   I believe that there are two faults with this analysis. First, pheochromocytomas have been observed at higher doses than those that cause liver tumors. Second, the relevance to humans is questionable as this tumor has not been previously observed in carbon tetrachloride-exposed individuals. Moreover, these tumors are almost always benign, although certainly the possibility of metastasis does exist. Hence, I do not agree that this tumor should override the conclusions based on the use of liver tumors as the primary response.

5. ***Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the**

utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.

I believe that the nonlinear approach for cancer risk assessment is the one that is best supported by the database. All the aspects of the presentation of this approach are clear, transparent, and objective. I also believe that the fairly large database showing negative results for carbon tetrachloride-induced genotoxicity at low doses should provide a reasonable level of confidence that exposures below the level of the RfD/RfC do not pose a significant risk for liver cancer in humans (or experimental animals for that manner).

6. ***Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?**

As a potential alternative approach, the document clearly describes the procedures (i.e., assumptions and modeling) that are done. The choice of a BMR of 5% for female rat liver tumor data and a BMR of 10% for the other tumor data sets make complete sense and are scientifically justified. However, the rationale for choosing anything but a nonlinear extrapolation approach is not, in my opinion, based on scientific facts and logic. Rather, the rationale is to use a default approach because the findings are either unexplained or the MOA is unknown. While I can agree that use of a default approach would err on the side of caution, there are some considerations that argue against using this default approach. These include: (i) The questionable relevance and validity of female mouse liver tumors at a single dose below that which causes hepatotoxicity; (ii) the absence of any data showing direct genotoxic effects of carbon tetrachloride at such low doses; and (iii) the questionable relevance of pheochromocytomas to humans. Thus, as stated above, while I feel that it is entirely appropriate to present this alternate approach, some sort of evaluative statement regarding the likelihood that it is correct as compared with the nonlinear extrapolation approach should be added.

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

   The conclusion that data from oral exposures cannot be used to derive a quantitative cancer risk estimate is based on the limitations of these data. This judgment is completely scientifically justified. The presentation of the rationale for this conclusion has been clearly, transparently, and objectively presented. Use of a PBPK model for the inhalation-to-oral exposure extrapolation has become a standard approach and has significant validation to support its use. Its presentation here is clear and objective.

8. **EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?**

   As stated in multiple places above, I believe that it is entirely appropriate to present a possible, alternative risk assessment approach. However, the document lacks an evaluation of the likelihood of one approach over the other providing an accurate assessment. I believe that there are concerns with both the validity of some of the data and with their relevance to humans that makes the linear approach much less likely to yield accurate estimates of risk.

Madhusudan G. Soni, Ph.D., FACN

Soni and Associates, Inc.

Dr. Madhusudan G. Soni is a principal in the toxicology and regulatory affairs consulting firm Soni and Associates, Inc. He has an advanced degree in Biochemistry (Biochemical Pharmacology & Toxicology) and post-doctoral work in mechanisms of toxicity from the Jichi Medical School in Japan and University of Mississippi. He has over 15 years of experience dealing with regulatory issues related to product safety and risk assessment. Dr. Soni has over 70 peer reviewed publications in scientific journals. His experience includes Director of Research at Vero Beach Hematology Oncology, Senior Toxicologist at a consulting firm, Research Assistant Professor at the University of Louisiana, and Research Scientist at the National Institute of Nutrition in India. Dr. Soni has considerable experience in cancer biomarkers, chemoprevention, drug metabolism, toxicokinetics, toxicodynamics and molecular toxicology. He is a Fellow of American College of Nutrition and is a member of the Society of Toxicology. Dr. Soni received several awards for his research, the notable ones are the Best Paper Award from the Risk Assessment Specialty Section of Society of Toxicology in 1998, and the Board of Publications Award for the Best Paper Published in Toxicology and Applied Pharmacology in 1999.

General Charge Questions:

1. **Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?**

   Reviewer comment: Overall the information presented in this review is clearly written, concise and adequate to illustrate noncancer and cancer hazards of carbon tetrachloride. The report follows a defined path to arrive at a risk assessment.

2. **Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.**

   Reviewer comment: The review covers pertinent scientific information. A database search (PubMed, ToxLine) did not reveal any significant publications that were not considered in the assessment. Recently, Dr. Eastmond (Enviorn Mol Mutagen, 2008) published an article on carbon tetrachloride genotoxicity-mode of action. Only abstract of the article was available. As Dr. Eastmond is also an author of the current Toxicological Review, it is assumed that any significant findings from the publication may have been covered in the current review.

3. **Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.**

   Reviewer comment: The available mode of action data is indicative of cell regeneration and cell death may be responsible for mutations that can lead to cancer. Genotoxicity and mutagenicity at lower doses will add to the database. Cancer bioassays at low doses will be helpful. A multigenerational toxicity study will certainly add the confidence in database and the risk assessment.

4. **Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?**

   Reviewer comment: The uncertainties are well defined and characterized in the review document. The key sources of uncertainties such as intraspecies variations, interspecies differences, subchronic to chronic extrapolation and inadequate database are clearly and adequately described in the review. The discussion related to choices and assumptions made for uncertainty and its impact on the assessment is clear and transparent.

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. **A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.**

   Reviewer comment: The selection of Bruckner et al. (1986) study over other available studies for reference dose (RfD) is well justified. The study appears to be well conducted and good dose-response was observed in the liver, which is the target organ for carbon tetrachloride toxicity. This study provides the no observed adverse effect level (NOAEL) and low observed adverse effect (LOAEL) for the critical effect. In the previous RfD determination as well as in risk assessment by other agencies this study has been used. Although it is mentioned indirectly, a brief statement or clarification regarding, "Bruckner et al. (1986) identified a NOAEL of 1 mg/kg…" (page 176) and, "This study identified a NOAEL of 0.71 mg/kg…" (page 40) may be helpful. A statement regarding duration adjusted NOAEL may be included. In the review, Bruckner et al. (1986) study has been transparently and objectively described along with the criteria and rationale for selection of this study.

2. **An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.**

   Reviewer comment: The NOAEL as well as LOAEL in the Bruckner et al. (1986) study was identified based on significantly elevated sorbitol dehydrogenase activity and mild centrilobular vacuolization in rats exposed 5 days/week for 12 weeks. As hepatotoxicity is the critical effect of oral exposure to carbon tetrachloride and sorbitol dehydrogenase is a sensitive marker of liver injury, use of this enzyme as the most appropriate critical effect for the RfD is well justified and clearly described in the review.

3. **Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   Reviewer comment: The application of Benchmark dose (BMD) modeling method to SDH data to derive the POD is appropriate. The method is objectively and transparently described in the review. Given the availability of data from the Buckner et al. (1986) study, use of BMD approach is appropriate and it provides a more quantitative alternative to identification of a point of departure (POD) than the traditional NOAEL/LOAEL approach. The BMD modeling is appropriately conducted and the use of increase in

SDH activity two times the control for benchmark response (BMR) and deriving the POD is well justified.

4. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

Reviewer Comment: The selection of uncertainty factors (intraspecies variations, interspecies differences, subchronic to chronic extrapolation and incomplete database) are well justified.

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

Reviewer comment: Inter individual variations (age-related or other factors) in levels of metabolism enzymes such as cytochrome P450 may alter susceptibility to carbon tetrachloride. The P450 enzyme, CYP2E1 responsible for carbon tetrachloride metabolism is known for variation in human population (genetic polymorphism). As carbon tetrachloride is eliminated largely by microsomal drug-metabolizing enzymes, differences in half-life may exceed an order of magnitude. The quantitative information on the variability of human response to carbon tetrachloride is lacking and an uncertainty factor of 10 to derive RfD is well justified.

- **A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.**

Reviewer comment: The rational and justification for use of uncertainty factor of 3 rather than 10 for subchronic to chronic extrapolation is clearly described and supported in the review.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigenerational reproductive toxicity study.**

Reviewer comment: The available database and impact of missing database (such as lack of adequate reproductive toxicity data and multigenerational reproductive toxicity study) support the use of uncertainty factor of 3.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

Reviewer comment: The selection criteria and rationale for uncertainty factors (intraspecies variations, interspecies differences, subchronic to chronic extrapolation and incomplete database) are sound and scientifically justified.

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. **The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically**

justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.

Reviewer comment: The selection of chronic inhalation bioassay in rat (JBRC, 1998) as the principal study for the RfC determination is appropriate and well justified. The criteria and rationale for the selection of JBRC study is transparently and objectively presented in the review.

2. **Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.**

   Reviewer comment: The selection of fatty changes in liver as the critical effect for the determination of RfC is appropriate. The rationale for selection of fatty changes in liver as the critical effect is clear, well described and supported by the available data. The reasons for selection of fatty changes in liver instead of liver enzymes or other histopathological changes is extensively discussed in the review and supports the use of fatty changes as a more sensitive endpoint

3. **An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.**

   Reviewer comment: The rationale provided for not selecting proteinuria as a critical effect is scientifically sound and adequately described in the review.

4. **BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.**

   Reviewer comment: As, use of the BMD approach potentially adds consistency and objectivity to the process of deriving RfC values, this method with incidence data for fatty changes in liver to derive POD is appropriate. The BMD modeling appears to be appropriately conducted and clearly described in the review.

5. **PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions,**

parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?

Reviewer comment: The PBPK modeling for rats to humans and route-to-route extrapolation is clearly described in the review. The model appears to properly represent the toxicokinetics of the species with proper application. The model assumptions, parameter values and dose metrics are clearly presented and supported. Application of UF at this stage may be considered.

6. **Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:**

Reviewer response: The selection of uncertainty factors (intraspecies differences, interspecies variations, subchronic to chronic extrapolation and incomplete database) are well justified:

- **An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.**

Reviewer response: Given the lack of data on the variability in human response to carbon tetrachloride, the use of intraspecies uncertainty factor of 10 is appropriate. Application of UF at internal dose may be considered.

- **An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.**

Reviewer response: The use of interspecies uncertainty factor of 3 appears appropriate for lack of pharmacodynamic uncertainty. A discussion on whether the use of uncertainty factor of 3 was adequate for the interspecies extrapolation from rats to hamster may be included. This may provide support for the use of interspecies uncertainty factor.

- **A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigenerational reproductive toxicity study.**

Reviewer response: Given the lack of multigenerational reproductive toxicity study, use of uncertainty factor of 3 is reasonable.

Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

Reviewer response: The selection criteria and rationale for uncertainty factors for human variability in the susceptibility, interspecies extrapolation and incomplete database are well described and justified.

(C) Carcinogenicity of carbon tetrachloride

1. **Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?**

   Reviewer response: In the absence of adequate evidence of carcinogenicity in humans and given the sufficient evidence in animals, the use of cancer weight of evidence approach is appropriate. The weight of evidence approach is well described and synthesized using the available evidence and mode of action data. The analysis and rationale to justify weight of evidence in assessing the cancer risk were clearly and transparently described.

2. **In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.**

   Reviewer response: The mode of action for liver cancer involving metabolism, cytotoxicity, and regenerative cell regeneration is well described and supports the hypothesis. The carcinogenicity of carbon tetrachloride appears to be a result of two scenarios. In one case, genotoxic effects may lead to cancer as a result of direct covalent binding of reactive metabolites or lipid peroxidation products with DNA. These responses are noted at high doses and such responses at low doses that induce tumors in long term studies remain uncertain. Additionally, carbon tetrachloride overall has not been found to be a potent mutagen. In the second scenario, a non-genotoxic action such as hepatic cytotoxicity, necrosis and cellular regeneration may lead to carcinogenesis. The available evidence supporting this second mode of action is more convincing and well presented in the review, but both modes appear to contribute.

3. **Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.**

   Reviewer response: Both non-linear and linear approaches are well described in the document. A well balanced explanation on the support and deficiencies for both the methods is clearly presented in the

review. The non-linear extrapolation approach for cancer risk appears more consistent with the mode of action involving hepatocellular cytotoxicity and regenerative hyperplasia. Given the uncertainty in understanding the cancer mode of action at low doses, the default linear may also be considered. Elaborate description on implications of both approaches may be included.

4. **Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?**

   Reviewer response: The characterization of mouse pheochromocytomas along with its relevance to cancer risk is clearly described in the review. As this tumor was noted in mice without any incidence in rats indicates species specificity. Additionally, human epidemiological observations did not indicate such tumor formation. These lines of evidence indicate that the linear approach may not be appropriate.

5. ***Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.**

   Reviewer response: The use of RfD and RfC for the potential risk of liver cancer is well described and justified. Experimental data at high levels of carbon tetrachloride exposure indicate the association between hepatocellular cytotoxicity and cellular regeneration and the induction of liver cancer. The genotoxicity data indicate that carbon tetrachloride is more likely an indirect than direct mutagenic agent. Hepatotoxicity is a key event for the hypothesized nonlinear mode of action. Overall the available evidence from experimental data and mode of action supports the use of non-linear approach. The mechanism of action, genotoxicity data and other evidence from experimental studies support the notion for lack of cancer risk for exposures below the level of RfD/RfC. This method seems more appropriate.

6. ***Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition, a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific**

justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?

Reviewer response: Given that some bioassay data at lower exposure levels is inadequate to explain the role of hepatocellular cytotoxicity and regenerative hyperplasia in the development of liver tumors, it is possible that other mode of action may exist at low dose exposure. Additionally, information related to general reactivity and genotoxicity of carbon tetrachloride also indicate possible other modes of action at low levels. Thus it appears that the mode of action at relatively higher doses may be different from that of lower dose levels. This information supports the use of low-dose linear extrapolation approach for cancer risk (for liver tumors and pheochromocytomas). However, based on available evidence the non-linear method seems more appropriate. The alternative linear extrapolation may be considered for cross check. The use of data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor appears sound and provides highest risk estimates. The rationale for low dose linear extrapolation and use of BMR of 5 and 10% for female and other tumor data is clearly described in the review.

7. **The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?**

   Reviewer response: The available human and animal carcinogenicity data on carbon tetrachloride following oral exposure is inadequate for cancer risk assessment using low dose linear approach. The description provided in the review is clear and supports this view. Extrapolation of inhalation data to oral exposure using PBPK modeling is appropriate. As the liver tumor and pheochromocytomas have been noted in animal studies following oral and inhalation exposures, use of data sets for inhalation unit risks for oral slope factor seems appropriate and reasonable.

8. **EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?**

   Reviewer response: The inconsistencies and uncertainties at the low end of the animal exposure range indicate that alternative mode of action may be operative in the carbon tetrachloride carcinogenesis at low exposure levels. The use of linear low dose extrapolation approach is adequately described in the review. As described earlier, the data lacks for support of a linear approach.

Appendix A. Reviewer List

Peer Review Workshop for EPA's Draft Toxicological Review of Carbon Tetrachloride

Navy League Building
Arlington, VA
October 14, 2008

Reviewer List

Janusz Byczkowski
Consultant
212 N. Central Avenue
Fairborn, OH 45324
937-878-5531
Email: jbyczkowski@netscape.net

Gary Ginsberg
Senior Toxicologist
Connecticut Department of Public Health
79 Elm Street
Hartford, CT 06106-5127
860-205-8286
Email: hologram7942@yahoo.com

Dale Hattis
Research Professor
Clark University
George Perkins Marsh Institute
950 Main Street
Worcester, Mass. 01610
617-283-2521
Email: DHattis@clarku.edu

Lisa Kamendulis*
Assistant Professor
Department of Pharmacology and Toxicology
Indiana University
635 Barnhill Drive, MS A509
Indianapolis, IN 46202
317-274-7824
Email: lkamendu@iupui.edu

Lawrence Lash (Chair)
Professor
Department of Pharmacology
Wayne State University
School of Medicine
540 East Canfield Avenue
Detriot, MI 48201
313-577-0475
Email: l.h.lash@wayne.edu

Madhusudan Soni
Consulting Toxicologist
Soni & Associates
749 46th Square
Vero Beach, FL 32968
772-299-4572
Email: sonim@bellsouth.net

*Due to a family emergency, Lisa Kamendulis was unable to attend the peer review workshop.

Appendix B. Charge to Reviewers

Charge to External Reviewers for the Toxicological Review of Carbon Tetrachloride

The U.S. Environmental Protection Agency (EPA) is seeking an external peer review of the scientific basis supporting the human health assessment of carbon tetrachloride that will appear on the Agency's online database, the Integrated Risk Information System (IRIS). IRIS is prepared and maintained by the EPA's National Center for Environmental Assessment (NCEA) within the Office of Research and Development (ORD). An existing IRIS assessment of carbon tetrachloride was posted to the database in 1987.

The current draft Toxicological Review includes a chronic Reference Dose (RfD) and Reference Concentration (RfC) and a carcinogenicity assessment. Below is a set of charge questions that address scientific issues in the assessment of carbon tetrachloride. Please provide detailed explanations for responses to the charge questions.

General Charge Questions:

1. Is the Toxicological Review logical, clear and concise? Has EPA accurately, clearly and objectively represented and synthesized the scientific evidence for noncancer and cancer hazards?

2. Please identify any additional studies that should be considered in the assessment of the noncancer and cancer health effects of carbon tetrachloride.

3. Please discuss research that you think would be likely to increase confidence in the database for future assessments of carbon tetrachloride.

4. Please comment on the identification and characterization of sources of uncertainty in Sections 5 and 6 of the Toxicological Review. Please comment on whether the key sources of uncertainty have been adequately discussed. Have the choices and assumptions made in the discussion of uncertainty been transparently and objectively described? Has the impact of the uncertainty on the assessment been transparently and objectively described?

Chemical-Specific Charge Questions:

(A) Oral reference dose (RfD) for carbon tetrachloride

1. A 12-week oral gavage study in the rat by Bruckner et al. (1986) was selected as the basis for the RfD. Please comment on whether the selection of this study as the principal study is scientifically justified. Has this study been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.

2. An increase in serum sorbitol dehydrogenase (SDH) activity was selected as the most appropriate critical effect for the RfD because it is considered by EPA to be an indicator of hepatocellular injury and a biomarker of an adverse effect. Please comment on whether the rationale for the selection of

this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please provide a detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

3. Benchmark dose (BMD) modeling methods were applied to SDH data to derive the point of departure (POD) for the RfD. Please comment on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Is the benchmark response (BMR) selected for use in deriving the POD (i.e., an increase in SDH activity two times the control mean) scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including the selection of the BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.

4. Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfD. For instance, are they scientifically justified and transparently and objectively described in the document? If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:

   - An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfD because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.
   - A subchronic to chronic uncertainty factor of 3, rather than a default of 10, was used in light of limited chronic oral study data and more extensive inhalation study data that informed the progression of toxicity from subchronic to chronic exposure durations.
   - A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

(B) Inhalation reference concentration (RfC) for carbon tetrachloride

1. The JBRC et al. (1998) 2-year inhalation bioassay in the rat was selected as the basis for the RfC. Please comment on whether the selection of this study as the principal study is scientifically justified. Has the rationale for this selection been transparently and objectively described in the Toxicological Review? Are the criteria and rationale for this selection transparently and objectively described in the document? Please identify and provide the rationale for any other studies that should be selected as the principal study.

2. Fatty changes in the liver was selected as the critical effect for the RfC because it is considered by EPA to be an adverse effect. Please comment on whether the selection of this critical effect is scientifically justified. Are the criteria and rationale for this selection transparently and objectively described in the Toxicological Review? Please comment on whether EPA's rationale about the adversity of the critical effect has been adequately and transparently described and is supported by the available data. Please provide a detailed explanation. Please identify and provide the rationales for any other endpoints that should be considered in the selection of the critical effect.

3. An increase in the severity (but not incidence) of proteinuria in low-dose male and female rats was reported in the 2-year JBRC (1998) bioassay. Because the biological significance of this finding in F344/DuCrj rats was considered unclear (see Section 4.6.2 of the Toxicological Review), proteinuria was not used as the critical effect for the RfC. Please comment on whether the decision not to use proteinuria as the critical effect is scientifically sound and has been transparently and objectively described in the Toxicological Review.

4. BMD methods were applied to incidence data for fatty changes in the liver to derive the POD for the RfC. Please provide comments on whether BMD modeling is the best approach for determining the POD. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the BMR selected for use in deriving the POD (i.e., 10% extra risk of fatty liver) been scientifically justified? Has it been transparently and objectively described? Please identify and provide rationales for any alternative approaches (including BMR, model, etc.) for the determination of the POD and discuss whether such approaches are preferred to EPA's approach.

5. PBPK modeling was used to extrapolate the POD from rats to humans and from inhalation to oral dose estimates. Please comment on whether the PBPK modeling for interspecies and route-to-route extrapolation is scientifically justified. Has the modeling been transparently and objectively described in the Toxicological Review? Does the model properly represent the toxicokinetics of the species under consideration? Was the model applied properly? Are the model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Has the sensitivity analysis been clearly presented, and appropriately characterized and considered? Has the uncertainty been accurately captured and considered?

6. Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfC. If changes to the selected uncertainty factors are proposed, please identify and provide a rationale(s). Please comment specifically on the following uncertainty factors:

   - An intraspecies (human variability) uncertainty factor of 10 was applied in deriving the RfC because the available quantitative information on the variability in human response to carbon tetrachloride is considered insufficient to move away from the default uncertainty factor of 10.
   - An interspecies uncertainty factor of 3 was used to address pharmacodynamic uncertainty only, because PBPK modeling was used to address pharmacokinetic extrapolation from rodents to humans. This contrasts with using the full default interspecies uncertainty factor of 10 for the RfD where an oral PBPK model to support interspecies extrapolation is not available.
   - A database uncertainty factor of 3 was used to account for lack of adequate reproductive toxicity data for carbon tetrachloride, and in particular absence of a multigeneration reproductive toxicity study.

   Are the criteria and rationale for the selection of these uncertainty factors transparently and objectively described in the document? Please comment on whether the application of these uncertainty factors has been scientifically justified?

(C) Carcinogenicity of carbon tetrachloride

1. Under EPA's 2005 *Guidelines for Carcinogen Risk Assessment* (www.epa.gov/iris/backgr-d.htm), the Agency concluded that carbon tetrachloride is likely to be carcinogenic to humans by all routes of exposure. Please comment on the cancer weight of evidence characterization. Has the scientific

justification for the weight of evidence descriptor been sufficiently, transparently and objectively described? Do the available data for both liver tumors in rats and mice and pheochromocytomas in mice support the conclusion that carbon tetrachloride is a likely human carcinogen? Has the scientific justification for deriving a quantitative cancer assessment been transparently and objectively described?

2. In the Toxicological Review, EPA discussed a mode of action (MOA) for liver cancer involving metabolism, cytotoxicity, and regenerative proliferation leading to tumor induction as key events occurring at relatively high exposure levels. EPA also discussed that carbon tetrachloride carcinogenicity may not be explained by a cytotoxic-proliferative MOA only and that a MOA involving genetic damage may also be operative at high exposure levels and may predominate at noncytotoxic (low) exposures. Please provide detailed comments on whether this analysis regarding carbon tetrachloride's MOA(s) is scientifically justified. In particular, please provide comments on EPA's evaluation of the carbon tetrachloride genotoxicity database and EPA's judgments about potential low-dose genotoxicity given the limited information at low doses. Has the MOA for liver cancer been transparently and objectively described in the document? Considerations should include the scientific support regarding the plausibility for each of the hypothesized MOAs, and the characterization of uncertainty regarding these MOAs.

3. Regarding liver cancer, two approaches to dose-response assessment for the inhalation exposure route are presented in the Toxicological Review—a nonlinear low-dose approach and a linear low-dose extrapolation approach. Do you agree with EPA regarding the support for a nonlinear extrapolation approach consistent with a MOA involving hepatocellular cytotoxicity and regenerative hyperplasia? Do you agree with EPA regarding the support for applying the default linear extrapolation approach due to uncertainty in understanding the cancer MOA at low doses? Please provide detailed comments on whether the inclusion of both approaches to dose-response assessment is scientifically sound and transparently and objectively described in the document.

4. Is EPA's characterization of mouse pheochromocytomas, including their relevance to human cancer risk, transparently and objectively described in the Toxicological Review? EPA applied a linear extrapolation approach to pheochromocytoma data from the JBRC inhalation bioassay in mice in the absence of MOA information. Please comment on the scientific justification for quantification of cancer risk for this tumor type, considering relevance to humans. Has the dose-response modeling been appropriately and objectively conducted? Are the results objectively and transparently described?

5. *Nonlinear approach*: The Toxicological Review finds that the RfD of 0.004 mg/kg-day and the RfC of 0.1 mg/m^3 be used to assess liver cancer risk for carbon tetrachloride under the assumption of a MOA consistent with low-dose nonlinearity. Please provide detailed comments on whether this nonlinear approach is scientifically justified. Has this approach been transparently and objectively described in the document? Are there other nonlinear approaches to evaluating liver cancer risk for carbon tetrachloride that should be presented in the Toxicological Review? Please comment on the utility of including these alternative nonlinear approaches. Please comment on the confidence that EPA should have that there is not a cancer risk for exposures below the level of the RfD/RfC.

6. *Linear extrapolation*: The Toxicological Review describes the alternative approaches for incorporating low-dose linearity that were applied to four tumor datasets from JBRC (1998) (female rat and mouse liver tumors and male and female mouse pheochromocytomas). These included (1) POD-based straight line risk calculations and (2) similar risk calculations (for liver tumor data sets only) that examined the effect on risk estimates of using only data on carbon tetrachloride cancer response at exposure levels below those for which increased cell replication was reported. In addition,

a Bayesian approach was applied to male mouse pheochromocytoma data to investigate the distribution of the slope parameter in the log-probit model. Please comment on whether the linear extrapolation approaches are scientifically plausible given potential for a cytotoxic MOA at higher doses and other MOAs at lower doses. Please comment on EPA's choice of using data for pheochromocytomas in the male mouse as the basis for the inhalation unit risk and data for female mouse liver tumors as the basis for the oral slope factor. Has the rationale for including a low-dose linear extrapolation been transparently and objectively described in the document? In the above analyses, a BMR of 5% was used for the female rat liver tumor data set, and a BMR of 10% was used for the other tumor data sets. Please comment on the scientific justification for the selection of these BMRs. Is the rationale transparently and objectively described in the document?

7. The conclusion was reached that studies of carbon tetrachloride carcinogenicity by the oral exposure route are not sufficient to derive a quantitative estimate of cancer risk using oral cancer response data and low-dose linear approaches. Please provide detailed comments on whether this judgment is scientifically justified. Has EPA's judgment been transparently and objectively described in the Toxicological Review? EPA used a PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate. Please comment on EPA's application of a PBPK model for route-to-route extrapolation to derive an oral cancer risk estimate from the inhalation data. Please provide detailed comments on whether this approach is scientifically justified. Has EPA's judgment been transparently and objectively described in the document?

8. EPA's 2005 *Guidelines for Carcinogen Risk Assessment* provides guidance on choosing an approach for dose-response extrapolation below the observed data. Relevant language related to choosing an extrapolation approach is provided in Section 5.4.3 of the Toxicological Review. In this section of the Toxicological Review, a linear low-dose extrapolation approach is recommended for assessing carbon tetrachloride cancer risk over a nonlinear approach due to uncertainty in understanding the cancer MOA as well as some bioassay evidence inconsistent with a nonlinear MOA at low exposure levels. Please comment on the scientific justification for this recommendation. Has this recommendation been transparently and objectively described in the document?

Appendix C. Observer List

Peer Review Workshop for EPA's Draft Toxicological Review of Carbon Tetrachloride

Navy League Building
Arlington, VA
October 14, 2008

Observer List

Xabier Arzuaga
Toxicologist
U.S. Environmental Protection Agency
ORD/NCEA/IRIS
U.S. EPA Two Potomac Yard, Suite N-7845
2733 S. Crystal Drive
Arlington, VA 22202
703-347-8634
Arzuaga.Xabier@epamail.epa.gov

Ted Berner
Statistician
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460
703-347-8583

Jan Connery (Facilitator)
Vice President, ERG
110 Hartwell Avenue
Lexington, MA 02421
781-674-7322
jan.connery@erg.com

Johanna Droubay
ERG
2300 Wilson Boulevard, Suite 350
Arlington, VA 22201-5636
703-841-0500
johanna.droubay@erg.com

Emily Hintzen
Associate
ENVIRON Internation Corporation
4350 North Fairfax Drive, Ste 300
Arlington, VA 22203
703-516-2416
ehintzen@environcorp.com

Abdel-Razak Kadry
Director, IRIS Program
U.S. Environmental Protection Agency, ORD
1200 Pennsylvania Avenue, NW (8601P)
Washington, DC 20460
703-347-8545

Aaron Lovell
Associate Editor
Inside EPA's Risk Policy Report
1919 South Eads Street, Suite 201
Arlington, VA 22202
703-416-8541
alovell@iwpnews.com

Kathleen Newhouse
U.S. Environmental Protection Agency
1200 Pennsylvania Ave NW (8601P)
Washington, DC 20460
703-347-8641
newhouse.kathleen@epa.gov

Susan Rieth
Toxicologist
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW (8601P)
Washington, DC 20460
703-347-8582
rieth.susan@epa.gov

Reeder Sams
Environmental Health Scientist
U.S. Environmental Protection Agency
MC: B-243-01
RTP, NC 27711
919-541-0661
sams.reeder@epa.gov

Appendix D. Workshop Agenda

Peer Review Workshop for EPA's Draft Toxicological Review of Carbon Tetrachloride

Navy League Building
Arlington, VA
October 14, 2008

Agenda

8:00 a.m. **Registration**

8:30 a.m. **Welcome, Introductions, Meeting Purpose & Agenda** ..... *Jan Connery, ERG*

8:40 a.m. **EPA Welcome Remarks**..... *EPA/NCEA Management Representative*

8:50 a.m. **Public Comment**..... *Jan Connery*

9:00 a.m. **Discussion Process and Overarching Comments**..... *Larry Lash (Chair) & Panel*

9:15 a.m. **Oral RfD for Carbon Tetrachloride** ..... *Larry Lash & Panel*

A1) Use of Bruckner et al. (1986) as the basis for the RfD. Scientifically justified as principal study? Criteria and rationale for selection transparently and objectively described? Should any other studies be selected as the principal study?
A2) Selection of SDH activity as critical effect. Selection scientifically justified? Criteria and rationale for selection transparently and objectively described? Should any other endpoints be considered for the critical effect?
A3) Use of BMD modeling to derive the POD. Is BMD modeling the best approach? Has it been appropriately conducted and objectively and transparently described? Was the BMR selection scientifically justified and transparently and objectively described? Should EPA consider any alternative approaches for deriving the POD?
A4) Uncertainty factors applied to the POD (intraspecies UF of 10; subchronic-to-chronic UF of 3; database UF of 3). Is their selection and application scientifically justified and transparently and objectively described? Do you suggest any changes to the selected uncertainty factors?

10:30 a.m. BREAK

10:45 a.m. **Inhalation RfC for Carbon Tetrachloride** ..... *Larry Lash & Panel*

B1) **Use of JBRC et al. (1998) as basis for the RfC**. Scientifically justified as principal study? Criteria and rationale for selection transparently and objectively described? Should any other studies be selected as the principal study?
B2) **Use of fatty changes in liver as the critical effect for the RfC.** Scientifically justified? Criteria and rationale for selection transparently and objectively described? Should any other endpoints be considered for the critical effect? Rationale about the adversity of this effect adequately and transparently described and supported by available data?

Agenda (cont.)

Inhalation RfC for Carbon Tetrachloride *(continued)*..... *Larry Lash & Panel*

B3) **Decision not to use proteinuria as the critical effect**. Scientifically sound and transparently and objectively described?

B4) **Use of BMD modeling to derive the POD.** Is this the best approach? Has it been appropriately conducted and objectively and transparently described? Was the BMR selection scientifically justified and transparently and objectively described? Should EPA consider any alternative approaches for deriving the POD?

B5) **Use of PBPK modeling for interspecies and route-to-route extrapolation.** Scientifically justified? Transparently and objectively described? Properly represents toxicokinetics of species under consideration? Applied properly? Model assumptions, parameter values, and selection of dose metrics clearly presented and scientifically supported? Sensitivity analysis clearly presented, and appropriately characterized? Uncertainty accurately captured and considered?

B6) **Uncertainty factors applied to the POD (intraspecies UF of 10; interspecies UF of 3; database UF of 3).** Is their selection and application scientifically justified and transparently and objectively described? Do you suggest any changes to the selected UFs?

Noon LUNCH

1:00 p.m. **Carcinogenicity of Carbon Tetrachloride** ..... *Larry Lash & Panel*

C1) **Cancer weight-of-evidence characterization.** Scientific justification sufficiently, transparently and objectively described? Available data support the conclusion that carbon tetrachloride is a likely human carcinogen? Scientific justification for deriving a quantitative cancer assessment transparently and objectively described?

C2) **Mode of action analysis.** Scientifically justified and transparently and objectively described? Comment on evaluation of genotoxicity database and judgment about potential low-dose genotoxicity. MOA for liver cancer transparently and objectively described, considering scientific support for plausibility of and uncertainty characterization for each hypothesized MOA?

C3) **Two approaches to liver cancer dose-response assessment for inhalation exposure route.** Agree with support for a nonlinear extrapolation approach and application of default linear extrapolation approach? Inclusion of both approaches scientifically sound and transparently and objectively described?

C4) **Characterization of mouse pheochromocytomas and their relevance to human cancer risk?** Transparently and objectively described? Quantification of cancer risk for this tumor type scientifically justified considering its relevance to humans? Dose-response modeling appropriately and objectively conducted? Results objectively and transparently described?

C5) **Nonlinear approach** scientifically justified and transparently and objectively described? Should other nonlinear approaches be presented? Why? What confidence should EPA have that there is not a cancer risk for exposures below the level of the RfD/RfC?

C6) **Linear extrapolation approaches.** Scientifically plausible given the potential for a cytotoxic MOA at higher doses and other MOAs at lower doses? Comment on EPA's choice of data for the inhalation unit risk and oral slope factor. Rationale for including a low-dose linear extrapolation transparently and objectively described? Selection of BMRs scientifically justified and transparently and objectively described?

C7) **Conclusion that oral cancer response data are insufficient for quantification of oral cancer risk using low-dose linear approaches.** Scientifically justified and transparently and objectively described? Use of PBPK model to extrapolate inhalation data to derive an oral cancer risk estimate scientifically justified and transparently and objectively described?

Agenda (cont.)

Carcinogenicity of Carbon Tetrachloride *(continued)*..... *Larry Lash & Panel*

C8) **Recommendation to use linear low-dose extrapolation approach to assess cancer risk** scientifically justified and transparently and objectively described?

2:45 p.m. BREAK

3:00 p.m. **General Questions** ..... *Larry Lash & Panel*

1) Document logical, clear and concise? Scientific evidence for the noncancer and cancer hazards accurately, clearly and objectively represented?
2) What additional studies should EPA consider for assessing noncancer and cancer health effects?
3) What research would likely increase confidence in the database for future assessments?
4) Sections 5 and 6, sources of uncertainty: Key sources adequately discussed? Choices and assumptions transparently and objectively described? Impact of uncertainty transparently and objectively described?

4:40 p.m. **Reviewer Final Comments** ..... *Larry Lash & Panel*

4:55 p.m. **Closing Remarks** .....*Jan Connery & EPA/NCEA*

5:00 p.m. ADJOURN