External Peer Review

U. S. Environmental Protection Agency
Toxicological Review for the
Bromobenzene Human Health Assessment

Final Compilation of Reviewer Comments
And Responses to Charge Questions

Prepared for
Integrated Risk Information System (IRIS) Program
Office of Research and Development
National Center for Environmental Assessment
U.S. Environmental Protection Agency

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EPA BROMOBENZENE REVIEW

TABLE OF CONTENTS

EXTERNAL PEER REVIEWERS 5

PEER REVIEW PROJECT 6

CHARGE TO EXTERNAL REVIEWERS 7

REVIEWER RESPONSES TO GENERAL CHARGE QUESTIONS 10

QUESTION 1 10

Response from John L. Farber 10
Response from David William Gaylor 10
Response from David Jollow 10
Response from José E. Manautou 12
Response from Jian Zheng 12

QUESTION 2 13

Response from John L. Farber 13
Response from David William Gaylor 13
Response from David Jollow 13
Response from José E. Manautou 13
Response from Jian Zheng 13

CHEMICAL-SPECIFIC CHARGE QUESTIONS 14

(A) ORAL REFERENCE DOSE (RfD) FOR BROMOBENZENE 14

QUESTION A1 14

Response from John L. Farber 14
Response from David William Gaylor 14
Response from David Jollow 14
Response from José E. Manautou 14
Response from Jian Zheng 15

QUESTION A2 16

Response from John L. Farber 16
Response from David William Gaylor 16
Response from David Jollow 17
Response from José E. Manautou 19
Response from Jian Zheng 19

QUESTION A3 20

Response from John L. Farber 20
Response from David William Gaylor 20
Response from David Jollow 21
Response from José E. Manautou 21
Response from Jian Zheng 21

QUESTION A4 22

Response from John L. Farber 22
Response from David William Gaylor 22
Response from David Jollow 22
Response from José E. Manautou 22
Response from Jian Zheng 22

QUESTION A5 23

Response from John L. Farber 23
Response from David William Gaylor 23
Response from David Jollow 23
Response from José E. Manautou 23
Response from Jian Zheng 23

(B) INHALATION REFERENCE CONCENTRATION (RfC) FOR BROMOBENZENE 24

QUESTION B1 24

Response from John L. Farber 24
Response from David William Gaylor 24
Response from David Jollow 24
Response from José E. Manautou 24
Response from Jian Zheng 24

QUESTION B2 ..... **25**

Response from John L. Farber 25
Response from David William Gaylor 25
Response from David Jollow 26
Response from José E. Manautou 26
Response from Jian Zheng 26

QUESTION B3 ..... **27**

Response from John L. Farber 27
Response from David William Gaylor 27
Response from David Jollow 28
Response from José E. Manautou 28
Response from Jian Zheng 28

QUESTION B4 ..... **29**

Response from John L. Farber 29
Response from David William Gaylor 29
Response from David Jollow 29
Response from José E. Manautou 30
Response from Jian Zheng 30

QUESTION B5 ..... **31**

Response from John L. Farber 31
Response from David William Gaylor 31
Response from David Jollow 31
Response from José E. Manautou 32
Response from Jian Zheng 32

(C) CARCINOGENICITY OF BROMOBENZENE **33**

QUESTION C1 ..... **33**

Response from John L. Farber 33
Response from David William Gaylor 33
Response from David Jollow 33
Response from José E. Manautou 33
Response from Jian Zheng 33

ADDITIONAL COMMENTS **34**

José E. Manautou 34
Jian Zheng 35

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The ORISE IRIS Technical Assistance Team has neither altered nor edited these comments for grammatical or other errors.

PEER REVIEW PROJECT

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CHARGE TO EXTERNAL REVIEWERS
FOR THE IRIS ASSESSMENT OF BROMOBENZENE

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 hazard?

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

Chemical-Specific Charge Questions

(A) Oral reference dose (RfD) for bromobenzene

1. A subchronic and chronic RfD for bromobenzene have been derived from the 90-day oral gavage study (NTP, 1985b) in mice. Please comment on whether the selection of this study as the principal study has been scientifically justified and 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. Liver toxicity (including increased liver weight and liver lesions) was selected as the most appropriate critical effect. Please comment on whether the selection of this critical effect has been scientifically justified and transparently and objectively described in the document. Please provide detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

3. The subchronic and chronic RfDs have been derived utilizing benchmark dose (BMD) modeling to define the point of departure (POD). All available models were fit to the data for the combined incidence of animals with one or more of the histopathologic liver lesions (centrilobular cytomegaly, necrosis, inflammation, mineralization), liver weight, and SDH levels. Please comment on the appropriateness and scientific justification presented for combining the incidence of liver effects to obtain a data set for BMD modeling. Please provide comments with regards to whether BMD modeling is the best approach for determining the point of departure. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the benchmark response selected for use in deriving the POD been scientifically justified and transparently and objectively described? Please comment on the appropriateness of averaging the benchmark doses for increased liver weight and liver lesions to derive the POD. Please identify and provide rationale for any alternative approaches (including the selection of BMR, model, etc.) for the determination of the point of departure, and if 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 RfDs. For instance, are they scientifically justified and transparently and objectively described in the document?

5. EPA used the data available for chlorobenzene to inform the selection of the subchronic to chronic uncertainty factor for the derivation of the chronic RfD for bromobenzene. Please comment on the scientific justification for this use of data from chlorobenzene. Has the scientific justification for this selection been transparently and objectively presented?

(B) Inhalation reference concentration (RfC) for bromobenzene

1. A subchronic and chronic RfC for bromobenzene has been derived from the 13 week inhalation study (NTP, 1985d) in mice. Please comment on whether the selection of this study as the principal study has been scientifically justified and 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. Liver cytomegaly in female mice was selected as the critical toxicological effect. Please comment on whether the selection of this critical effect has been scientifically justified and transparently and objectively described in the document. Specifically, please address whether the selection of increased incidence of cytomegaly as the critical effect instead of increased liver weight has been adequately and transparently described. Please provide detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

3. The subchronic and chronic RfCs have been derived utilizing benchmark dose modeling to define the point of departure. Please provide comments with regards to whether BMD modeling is the best approach for determining the point of departure. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the benchmark response selected for use in deriving the POD been scientifically justified and transparently and objectively described? Please comment on the justification for not utilizing the 100 ppm dose identified in the NTP (1985d) study as a NOAEL. Please identify and provide rationale for any alternative approaches (including the selection of BMR, model, etc.) for the determination of the point of departure, and if 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 RfCs. For instance, are they scientifically justified and transparently and objectively described in the document.

5. EPA used the data available for chlorobenzene to inform the selection the subchronic to chronic uncertainty factor for the derivation of the chronic RfC for bromobenzene. Please comment on the scientific justification for this use of data from chlorobenzene. Has the scientific justification for this selection been transparently and objectively presented?

(C) Carcinogenicity of bromobenzene

1. Under the EPA's 2005 *Guidelines for carcinogen risk assessment* (www.epa.gov/iris/backgr-d.htm), *data are inadequate for an assessment of the human carcinogenic potential* of bromobenzene. Please comment on the scientific justification for the cancer weight of the evidence characterization. A quantitative cancer assessment was not derived for bromobenzene. Has the scientific justification for not deriving a quantitative cancer assessment been transparently and objectively described?

REVIEWER RESPONSES TO GENERAL CHARGE QUESTIONS

QUESTION 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 hazard?

Response from John L. Farber

I find the Toxicological Review of Bromobenzene to be logical and clear. EPA has objectively represented and synthesized the scientific evidence for the noncancerous and cancerous hazard potentially represented by bromobenezene.

Response from David William Gaylor

The Toxicological Review is clear and concise. The use of the benchmark dose approach for determining a point of departure (POD) for establishing reference doses (RfDs) and reference concentrations (RfCs) is fully supported.

Response from David Jollow

This document is generally well written and laid out in a logical and concise manner. The scientific evidence for non-cancer and cancer end- points is presented clearly. However, there appears to be several areas where some attention and additional discussion may improve the document. These are:

Metabolism (section 3.3) and Elimination (section 3.4). General comments.

The metabolism of bromobenzene in mammals has been extensively studied for many years, not only in terms of the types of metabolites produced but also in terms of the reaction routes by which the metabolites are generated. The metabolic pathway, as shown in Fig. 3-1, is complex. In addition to the secondary metabolism (e.g., further oxidation of phenolic metabolites), it is now appreciated that some phenolic metabolites may arise by more than one pathway and that the contribution of the different routes may vary between species. The authors are to be commended for weaving their way through these complications in a concise manner.

However, in laying out all of the possible biotransformations, these sections seem to have lost focus in terms of the role of metabolism in tissue toxicity. An important omission is the lack of discussion (and illustration in the figure) of the role of glucuronidation and sulfation of the primary phenolic metabolites as a determinant of the extent of secondary oxidation. Clearly, to the extent that phenols arising from the primary oxidation are rapidly conjugated, they will not be available for the secondary CYP oxidations. The fraction available for secondary oxidation may be expected to increase as the dose of bromobenzene increases, due to capacity limitation of the PAPS-dependent sulfuryltransferase activity as PAPS becomes depleted in the hepatocyte. Glucuronyltransferase activity can also become limited by fall in UDPGA levels under some

conditions (e.g., an overnight fast of the experimental rodents, perhaps associated with treatment induced morbidity). It is appreciated that the early studies on the primary metabolites of bromobenzene did not quantitate the urinary glucuronide and sulfate conjugates, *per se*. That they occurred as major elimination products is clear from the fact that it was essential to subject the urinary metabolites to hydrolysis by glucuronidase/sulfatase (plus lowering the pH) before the (radioactive) metabolites could be extracted and analyzed (for example, see Zampaglione et al 1973). It is reasonable to expect that an increase in secondary oxidation as the dose of bromobenzene is increased may contribute to the higher dose needed to induce kidney lesions as compared with the liver lesions.

The modification suggested is more a matter of emphasis rather than of extensive rewriting. It might be helpful to present the information in three categories: 1), the primary oxidation by CYPs into the two arene oxide pathways as the "sole" initial event in bromobenzene elimination followed by subsequent reactions and rearrangements, plus UDPGA- and PAPS-dependent conjugative pathways. It should be made clear that these events account for the major part of the metabolic transformation and elimination of bromobenzene at all doses. 2), the secondary oxidation of phenols by CYP and the possible role of capacity limitation of phenol clearance due to decreased availability of PAPS and (possibly) UDPGA within the hepatocyte. Extra-hepatic transformations could be included in this part of the discussion. 3), The existence of multiple routes to generate individual metabolites with the possibility of differences in transient reactive metabolites may occur. In that there appears to be species differences in the extent to which the individual routes contribute to the formation of the phenols, it is conceivable that there may be differences in species susceptibility to renal cell injury. However, it needs to be clear that this is only a theoretical possibility and that there is at present, no evidence to suggest that this complication is important in the risk assessment of bromobenzene.

Minor points:

i) Section 3.3 Metabolism, page 9, line 1. The clause "as well as phenobarbital-induced CYP isozymes" does not seem to fit. Please expand/explain or omit. The references Girault et al and Krusekopf et al are concerned with fundamental issues of CYP induction and their direct relevance to bromobenzene metabolism is not self-explanatory. It is appreciated that some overlap can occur between phenobarbital-type and 3MC-type inducers, but is that relevant in this context? Is it known which CYPS are involved in the secondary oxidations? Does exposure of the rodents to inducers alter the extent of secondary oxidation of phenols and renal toxicity?

   Please add Zampaglione et al '73 to the references re the formation of the 2,3 arene oxide in 3MC induced rats. I believe this paper was the first to document this pathway and discuss its significance in the protection of rats to bromobenzene-induced hepatotoxicity.

ii) Page 10, paragraph 3. It is unclear what is meant by the sentence "Metabolism of bromobenzene in the liver appears to be capacity-limited". Early studies (Zampaglione et al '73) showed that the whole body half-life of an *iv* dose of ^{14}C-bromobenzene was short and not changed by "simultaneous" *ip* administration of bromobenzene in oil. The prolonged tissue levels seen after *ip* administration reflects the continued absorption of the bromobenzene from the oily mixture. In the absence of oil, absorption was extremely rapid and, at the doses used to elicit hepatic necrosis, caused rapid onset CNS depression and death.

The longer half-lives seen after hepatotoxic doses (i.e., of the terminal elimination phase) was considered to reflect back diffusion from extrahepatic tissues, especially adipose. Of interest, even though the (*iv*) half-life of bromobenzene in the normal rats was short (*ca* 10 min.), it was not a first-pass elimination as indicated by the fact that phenobarbital pretreatment resulted in enhancement of the intrinsic hepatic clearance of this compound.

Is the reference given in this paragraph (Lertratanangkoon and Horning '87) correct? This paper deals predominately with the pathways of elimination of pre-mercapturic acids of bromobenzene rather than of bromobenzene itself. The methods section does indicate that bromobenzene was administered to the rats in oil and hence it is likely that delayed elimination of the toxic dose reflects delayed absorption rather than capacity-limited metabolism.

iii) Figure 3-1. The arrow leading from the 3-4 dihydrodiol to 4-bromophenol would best be described as "dehydration" rather than as "spontaneous rearrangement". Please add some notation to indicate the glucuronidation and sulfation of the phenolic metabolites.

Response from José E. Manautou

The Toxicological Review of Bromobenzene by EPA is indeed a clearly written and concise document. Overall, the scientific data has been summarized in an objective manner and the authors did their best with the limited data available on the carcinogenicity and non-cancer toxicological endpoints of bromobenzene.

Response from Jian Zheng

Overall the review is clearly written with a logical flow, and there is no difficulty to follow. Particularly, the metabolism and hepatotoxicity studies on bromobenezene have been extensively performed in last 30 years. The authors made a great effort to do literature search and well presented and synthesized scientific data published.

QUESTION 2

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

Response from John L. Farber

I am aware of no additional studies it should consider in its assessment.

Response from David William Gaylor

No comment.

Response from David Jollow

No additional studies are suggested.

Response from José E. Manautou

This reviewer is not aware of additional data or studies on the subject.

Response from Jian Zheng

It is worthwhile evaluating bromobenzene cytotoxicity using rat and human hepatocytes which are commercially available now. Although the data from the *in vitro* studies do not necessarily be extrapolated to what exactly happens *in vivo*, at least the data are obtained from human tissue studies. It is better than nothing.

CHEMICAL-SPECIFIC CHARGE QUESTIONS

(A) ORAL REFERENCE DOSE (RfD) FOR BROMOBENZENE

QUESTION A1

A subchronic and chronic RfD for bromobenzene have been derived from the 90-day oral gavage study (NTP, 1985b) in mice. Please comment on whether the selection of this study as the principal study has been scientifically justified and 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 from John L. Farber

The selection of the 90-day oral gavage study in mice as the principal study was justified and objectively described. I am not aware of any other studies that should be used as the principal study.

Response from David William Gaylor

Actually, two 90-day oral gavage studies were examined in detail, NTP (1985a) in rats and NTP (1985b) in mice. Based on the lower 95% confidence limit estimate of the benchmark dose corresponding to an extra risk of 10% ($BMDL_{10}$), female mice were the most sensitive. An objective and transparent explanation of this process is provided in the document.

Response from David Jollow

The selection hepatic necrosis as the critical effect for determination of the risk assessment is highly appropriate. However, for the reasons given below, I feel that the RfD derived by the use of the combined index yields an inaccurate estimation of the hepatotoxicity of bromobenzene.

Response from José E. Manautou

The selection of the study was justified in light of the absence of other studies assessing toxicological outcomes of chronic and sub-chronic bromobenzene exposures. However, this reviewer feels that the design, implementation and interpretation of data from the NTP studies conducted in the mid-1980's are rather poor. The main concern is the number of animals used per treatment group in the studies (n=10). This is unrealistically small. A bigger concern is the data for some of the endpoints at the highest dose of bromobenzene used in these studies. For example, the majority of animals in the 90 days gavage study receiving 600 mg/kg/day died. The way data for those dosing regimens have been presented can be misleading. Despite this, the authors did their best synthesizing the existing data.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION A2

Liver toxicity (including increased liver weight and liver lesions) was selected as the most appropriate critical effect. Please comment on whether the selection of this critical effect has been scientifically justified and transparently and objectively described in the document. Please provide detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

Response from John L. Farber

Liver toxicity is the most appropriate critical effect. The only meaningful indicator of liver toxicity, however, is liver cell necrosis. In particular, liver weight is not an acceptable endpoint with which to assess liver toxicity. The liver can increase in weight by a variety of mechanisms. Thus, simply measuring liver weight does not specify the particular reason for the increase in weight. Importantly, some causes of a liver weight increase may reflect a toxic endpoint, e.g. steatosis, hydropic swelling, whereas others do not. For instance, many compounds that are metabolized by the liver cause an increase in liver weight, owing to an increase in the machinery (endoplasmic reticulum) that carries out this metabolism. Such a cause of increased liver weight would be misleading to label as toxicity.

Response from David William Gaylor

Liver weight and liver lesions were selected by the EPA as the most critical effects, based on producing the lowest values for $BMDL_{10}$. This process was objectively and transparently described in the document. Full details were provided in Appendix B of the document.

Combining cytomegaly, inflammation, mineralization, and necrosis to obtain an incidence of combined liver lesions and the use of liver weight gain as an indication of toxicity have been questioned by other review panel members. Hence, only liver necrosis is used here to estimate the point-of-departure (lower confidence limit on the dose estimated to produce an extra risk of 10%, $BMDL_{10}$) for calculating a reference dose. The log-logistic model generally provided excellent fits for the incidence of liver necrosis. The estimates of $BMDL_{10}$ for oral exposures are presented in Table 1. Male rats were the most sensitive for liver necrosis with $BMDL_{10}$ = 93 mg/kg-day. This is almost four times greater than the $BMDL_{10}$ = 25 mg/kg-day for combined liver lesions obtained in female mice based on the Weibull model.

Table 1. Lower 95% confidence limits for the oral dose estimated to produce an extra risk of 10% for liver necrosis based on the log-logistic model.

Species	Sex	Chi-square goodness-of-fit P-value	$BMDL_{10}$ (mg/kg-day)
Rats	Male	0.82	93
Rats	Female	0.91	171
Mice	Male	0.98	135
Mice	Female	0.38	288

Response from David Jollow

I am not comfortable with the use of a combined index of liver injury, i.e., the sum of cytomegaly, necrosis, inflammation and mineralization to determine a LOAEL.

My concerns are based on both the data presented and on fundamental considerations. The argument for the use of the combined index is that: i), all four responses occur in the centrilobular region of the liver, ii), that they show dose/response relationships to the administered bromobenzene in which statistically significant increases in necrosis or inflammation were observed only with doses equal to or greater than those that elicit cytomegaly, and iii), inflammation and mineralization were considered to be direct results of hepatocellular necrosis in the NTP report (NTP 1985a). It is inherent in the use of this concept that all four cellular responses are different manifestations of same toxic insult and that cytomegaly, inflammation and mineralization are all part of the overall pathological process that leads to the observed hepatocellular necrosis. That is, that cytomegaly, inflammation and mineralization are not just adaptive responses to cellular stress, but are part of the actual cellular injury.

If this was true, one might expect some sort of more-or-less constant relationship between the surrogates and centrilobular necrosis. Inspection of the data in Fischer 344/N rats (Table 4-2) and B6C3F1 mice (Table 4-4) does not seem to me to support such a relationship. Although there is significant inflammation when frank necrosis is present, there appears to be no reason to relate this exacerbated response to the mild to modest inflammation seen in the controls and after sub-toxic doses. Mild to modest inflammation is a common observation in the livers of experimental rodents, especially during chronic and subchronic studies and may be attributable to the stress of frequent handling and dosing. Cytomegaly and the associated increase in liver weight are also non-specific responses in that they occur after exposure to many compounds that are not classical hepatotoxins. Phenobarbital is a well-known example. Both effects are normally considered to be adaptive changes rather than pathological responses, *per se*.

The importance of distinguishing between adaptive and toxic responses is well illustrated by the studies of Heijne et al (2004). As discussed in section 4.5.3, these workers administered toxic and non-toxic doses of bromobenzene to rats and examined the time- and dose-related genomic

changes at the transcriptional level. Numerous alterations in gene expression were observed but none could be related to the pathological sequence of events leading up to cell death.

Of particular concern is the data of Table 4-4. The statistically significant response in the combined index for both male and female mice occurs at 200mg/kg/day and is largely due to cytomegaly. In contrast, statistically significant frank necrosis occurs at 400mg/kg/day in the male mice but only after 600mg/kg/day in the females. This lack of correlation between the index response and actual necrosis in male *vs.* female mice is disconcerting and does not argue well for the use of the index for the purpose of assessing the risk to human health posed by bromobenzene. In particular, since there are statistically significant increases in inflammation in the male mice at a dose (200mg/kg/day) without a similar statistically significant increase in the incidence of liver necrosis, the second criteria noted above for the justification of the combined index, does not seem to apply.

Perhaps my major concern in the use of the combined index lies in what we know about the mechanism underlying bromobenzene-induced hepatocellular necrosis. Our mechanistic understanding is summarized in section (4.5.1). While the precise steps that lead to cell death are still undefined, there is ample evidence for the crucial role played by glutathione in protecting the liver cell against the toxic metabolite and that the toxic "hit" occurs only after glutathione has been depleted from the liver cell. This "threshold' nature of the toxic mechanism is well accepted for bromobenzene and explains the very sharp dose-response curve seen in acute animal studies.

The importance of this threshold is well illustrated by the analogous situation with acute-overdose acetaminophen; that this drug has a threshold dose, below which hepatocellular necrosis does not occur, is well accepted. That necrosis can occur when the threshold is reduced by fasting (decreased thio-amino acid intake leads to decreased hepatic glutathione) and/or production of the toxic metabolite is increased by ethanol induction, emphasizes the importance of the threshold concept of the dose-response curve for this type of hepatotoxin. Additional stress is placed on the glutathione protective threshold of the liver cell by reduction in sulfotranferase (decreased PAPS capacity) and glucuronyltransferase (decreased glycogen and hence decrease UDPGA production capacity). Whether fasting or extreme dieting could exacerbate bromobenzene-induced hepatic necrosis in humans is not known but could conceivably lead to hyper-responsiveness for this lesion after exposure to the chemical. However, the extent of exacerbation of liver necrosis would be expected to be much less than that seen with acetaminophen in that, for acetaminophen, the sulfotransferase and glucuronyltransferase pathways are competitive with the CYP-mediated toxic pathway (to NAPQI). Diminution of the conjugation reactions thus enhances the proportion of the dose converted to NAPQI and its toxicity. For bromobenzene, the conjugation occurs with the phenolic metabolites arising after the collapse of the toxic arene oxide metabolite and hence diminution of conjugation should have minimal effect on the extent of formation of the arene oxide and on the severity of hepatic necrosis. Whether diminution of conjugation could enhance the CYP-mediated further metabolism of the phenolic metabolites and renal toxicity is an open question.

The effect of subchronic thiol-amino acid deficiency on hepatic glutathione levels and acetaminophen hepatotoxicity has been studied in rats (Price and Jollow TAP 101(2), 356-

369,1989). Extensive deficiency was required to significantly lower hepatic glutathione in these animals. The equivalent situation in humans is unclear.

On the basis of our mechanistic understanding, the only reliable indicator of bromobenzene-induced hepatocellular necrosis measured in the NTP studies is the cytological assessment of the necrotic lesion in the liver sections. The sharp transition between a NOAEL and a LOAEL for necrosis recorded in the NTP Tables is completely consistent with everything we know about this type of glutathione-threshold mechanism. The proposed surrogates do not fit the pattern and can only lead to an inappropriate determination of the dose–response curve for bromobenzene-induced hepatocellular necrosis. It is strongly recommended that the index not be used and that the risk assessment calculations be based on the actual incidence of centrilobular necrosis as observed under the microscope.

Thus the LOAEL becomes 400mg/kg/day rather than 200mg/kg/day.

Response from José E. Manautou

There are concerns with the endpoint selection criteria. Chemicals can produce increases in liver weight in the absence of toxicity. Generalizations and assumptions were made based on the well-documented hepatotoxic potential of bromobenzene. It would be advantageous to consider selecting endpoints more closely related to the mode of action of toxicants that generate reactive intermediates, such as changes in expression of stress genes and markers of oxidative stress. This reviewer recognizes that this analysis might not be possible since such data was not generated in the NTP studies.

Response from Jian Zheng

The parameters monitored as end-points in bromobenzene liver toxicity studies are acceptable except for increased liver weight. Elevation of liver weight does not necessarily mean the development of liver injury. For example, phenobarbital is a known cytochrome P450 inducer. Exposure to phenobarbital can cause significant elevated liver weight but does not necessarily produce liver toxicity.

QUESTION A3

The subchronic and chronic RfDs have been derived utilizing benchmark dose (BMD) modeling to define the point of departure (POD). All available models were fit to the data for the combined incidence of animals with one or more of the histopathologic liver lesions (centrilobular cytomegaly, necrosis, inflammation, mineralization), liver weight, and SDH levels. Please comment on the appropriateness and scientific justification presented for combining the incidence of liver effects to obtain a data set for BMD modeling. Please provide comments with regards to whether BMD modeling is the best approach for determining the point of departure. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the benchmark response selected for use in deriving the POD been scientifically justified and transparently and objectively described? Please comment on the appropriateness of averaging the benchmark doses for increased liver weight and liver lesions to derive the POD. Please identify and provide rationale for any alternative approaches (including the selection of BMR, model, etc.) for the determination of the point of departure, and if such approaches are preferred to EPA's approach.

Response from John L. Farber

I have concern with regard to using a combined incidence of liver lesions – that is the number of animals with one or more liver lesions (cytomegaly, necrosis, inflammation, mineralization). As stated above, alterations other than necrosis are not necessarily indicators of liver toxicity. Thus, combining animals with lesions with differing toxicological significance has the potential to distort the derived incidence of toxic lesions (necrosis) in the subchronic and chronic studies.

Response from David William Gaylor

BMD modeling is far superior to using NOAELs or LOAELs for the POD. NOAELs and LOAELs are primarily statistical effects that depend heavily on experimental sample sizes and are limited to the experimental dose levels employed. Whereas, BMDs provide estimates of risk of biological effects and utilize dose response relationships to provide interpolation of biological effects between experimental doses.

For continuous data, no discussion or reference is provided to support the choice of a one standard deviation shift in the mean for the BMD. No statement is explicitly presented that $BMD_{1sd} = BMD_{10}$. This is only very subtly surmised by noting that the title of Table 5-5 (page 57) refers to BMD_{10} and $BMDL_{10}$; whereas, the results listed in the table refer to BMD_{1sd} and $BMDL_{1sd}$. The choice of a one standard deviation shift in the mean for an effect measured on a continuous scale needs to be presented. For biological effects measured on a continuous scale, e.g., organ weights and clinical chemistry, there generally is not a specific value that identifies when an adverse effect will result. In the absence of such a value, particularly for laboratory animals, extreme percentiles of the effect measured in unexposed controls may be selected to indicate abnormal responses. For effects that are normally distributed, selection of the 1.5^{th} and 98.5^{th} percentiles to define the normal range result in an extra risk of abnormal levels outside this range of approximately 10% at the dose where the mean of the responses is changed by one standard deviation from the control mean. This assumes no change in the standard deviation

with dose. Levels in the abnormal range may or may not produce adverse biological effects, but such extreme values possibly may be of concern.
Table 5-8 (Page 58). P-value cannot exceed one.

In the absence of a biological reason for selecting a particular dose response model for BMD estimation of a biological effect, a weighted average of BMDLs across models could have been used. Model averaging weights each BMDL by a function of the goodness of fit utilizing the Akaike Information Criterion (AIC). See, e.g.,{Kang, SH, Kodell, RL, and Chen, JJ. Incorporating model uncertainties along with data uncertainties in microbial risk assessment. *Regulatory Toxicol Pharmacol* 32: 68-72, 2000}. Also, for a specific biological effect, BMDLs may be averaged across studies, e.g., male and female rats and mice, in order to obtain a more representative value. There is no logic, and hence no precedent, for averaging BMDLs across different biological effects. What is the justification for only averaging the BMDLs for liver lesions and weight and not including liver-to-body-weight ratio and SDH? Absolutely, do not average across biological endpoints!

Response from David Jollow

As noted above, it is strongly recommended that incidence data for the combined index (cytomegaly etc) not be used. Cytomegaly and increase in liver weight are well-recognized adaptive responses that occur in the absence of frank necrosis. Inflammation and mineralization are results of liver injury and cannot be considered here as causal and/or as more sensitive markers of that injury. At best, the combined index is a multiplier of the observed necrosis and tends to obscure accurate quantitation of the chemical-induced tissue lesion. The only reliable index is the actual observations on the incidence of necrosis as measured microscopically. As noted above, increased precision

The modeling procedure itself appears appropriate and is unobjectionable. Confidence in the precision of the LOAEL/benchmark dose would be considerably enhanced by repetition of the NTP studies with additional dose levels around the glutathione-threshold. Of importance, standard morphometric analysis should be employed to quantitate the extent of liver necrosis with multiple sections and fields per animal.

Response from José E. Manautou

The clustering of histopathologic lesion endpoints is also problematic. The document as written does not provide proper justification for combining the endpoints for the purpose of developing an incidence/severity index.

In regards to BMD modeling, this reviewer is not suitable for providing a critical analysis of these modeling exercises since this is beyond his area of expertise. On the other hand, modeling exercise that included data from treatment groups with high mortality rates or inclusion of animals showing extreme distress (moribund animals euthanized prior to completion of the study) should be reconsidered and discussed.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION A4

Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfDs. For instance, are they scientifically justified and transparently and objectively described in the document?

Response from John L. Farber

The uncertainty factors applied to the POD for the derivation of the RfCs are scientifically justified. They are described in the document both transparently and objectively.

Response from David William Gaylor

The choice of uncertainty factors appear to be appropriate.

Response from David Jollow

The three areas of uncertainty: interspecies extrapolation; inter-individual human variability; and database deficiencies, are all appropriate. The use of a factor of ten for interspecies extrapolation and data base deficiencies is acceptable. In view of what we know about the MOA for liver toxicity and the strong analogy that can be drawn with acetaminophen in regards to the glutathione protective capacity of the liver, a factor of ten for inter-individual could be considered to be too high. A factor of five should provide adequate protection for sensitive sub-populations.

A factor of three for extrapolation from subchronic to chronic is appropriate.

Response from José E. Manautou

It appears that standard procedures for identifying and selecting uncertainty factors were applied. The document describes an intriguing phenomenon observed in rodents exposed to bromobenzene. That is the development of tolerance to hepatotoxicity with repeated bromobenzene treatment. Is this a specie-specific effect and does it warrant the inclusion of an additional uncertainty factor? Perhaps not, the inclusion of uncertainty factors for interspecies extrapolation and interindividual variability may account for this.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION A5

EPA used the data available for chlorobenzene to inform the selection of the subchronic to chronic uncertainty factor for the derivation of the chronic RfD for bromobenzene. Please comment on the scientific justification for this use of data from chlorobenzene. Has the scientific justification for this selection been transparently and objectively presented?

Response from John L. Farber

Data available suggest that chlorobenzene may be somewhat less hepatotoxic than bromobenzene. This point, however, is not relevant to using the data available for chlorobenzene to inform the selection of the subchronic to chronic undertainty factor in the derivation of the chronic RfC for bromobenzene. Such a use of data from chlorobenze is scientifically justified. In turn, the scientific justification for this selection has been transparently and objectively presented.

Response from David William Gaylor

The selection of an uncertainty factor of three for subchronic to chronic extrapolation appears reasonable.

Response from David Jollow

All available data on absorption, distribution, biotransformation and mechanism of hepatotoxicity for the two halobenzenes are similar, and provide strong support for the use of chlorobenzene data to extend the data base for bromobenzene.

Response from José E. Manautou

There are clear differences in susceptibility to hepatotoxicity between bromobenzene and chlorobenzene, with the latter being less hepatotoxic. However, the use of chlorobenzene data for the selection of uncertainly factors in the derivation of RfD for bromobenzene is appropriate based on similarities in ADME and toxicodynamic pathways between the two compounds.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

(B) INHALATION REFERENCE CONCENTRATION (RfC) FOR BROMOBENZENE

QUESTION B1

A subchronic and chronic RfC for bromobenzene has been derived from the 13 week inhalation study (NTP, 1985d) in mice. Please comment on whether the selection of this study as the principal study has been scientifically justified and 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 from John L. Farber

The selection of the 13-week inhalation study in mice as the principal study was justified and objectively described. I am not aware of any other studies that should be used as the principal study.

Response from David William Gaylor

The 13-week inhalation in female mice appears to be an appropriate choice for determination of an RfC.

Response from David Jollow

The use of the 13 week inhalation study (NTP 1985d) is scientifically justified and is discussed objectively. As noted above, it is strongly recommended that the incidence of cytomegaly and increased liver weight not be used as an index of toxicity. If overt liver necrosis is evident, cytomegaly and organ weight increase is superfluous; if necrosis is not evident, the use of these adaptive responses is (as noted in the document [section 5.2.1.1, page 62, lines 31-33]) inappropriate.

Response from José E. Manautou

The same concerns expressed above regarding the design of the NTP studies also apply here (selection of just 10 animals per treatment group). A discussion on whether or not NTP conducted a statistical power analysis to justify the number of animals selected per treatment group would be useful.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION B2

Liver cytomegaly in female mice was selected as the critical toxicological effect. Please comment on whether the selection of this critical effect has been scientifically justified and transparently and objectively described in the document. Specifically, please address whether the selection of increased incidence of cytomegaly as the critical effect instead of increased liver weight has been adequately and transparently described. Please provide detailed explanation. Please identify and provide the rationale for any other endpoints that should be considered in the selection of the critical effect.

Response from John L. Farber

The same comment regarding the use of any index of toxicity other than necrosis, i.e., cytomegaly, applies here as well.

Response from David William Gaylor

Page 65, line 31. Change 98^{th} to 98.5^{th} or $98\text{-}99^{th}$. Change 2^{nd} to 1.5^{th} or 1^{st} -2^{nd}.

Contrary to the discussion in Section 5.2.1.2, liver necrosis (incidence displayed in Table 5-9) demonstrates a highly statistically significant ($P<0.011$) dose response based on the Cochran-Armitage trend test. For the multistage model, BMC_{10} = 126 ppm (above the range where necrosis was considered minimal) and $BMCL_{10}$ = 36.8 ppm, for liver necrosis in female mice. Footnote b in Table 5-12 should be replaced with:

Statistically significantly different from controls ($p<0.001$) based on Student's two-tailed t-test. Page 68, line 10, the statement of the absence of necrosis is incorrect.

The review panel was opposed to using liver weight gain as an indication of toxicity and opposed to combining liver lesions and recommended considering just liver necrosis. Estimates of the $BMCL_{10}$ for inhalation exposures based on the incidence of liver necrosis are presented in Table 2. Female mice were the most sensitive for liver necrosis with $BMCL_{10}$ = 28 ppm. This is about one-half the $BMCL_{10}$ = 59 ppm for combined liver lesions obtained in female mice based on the log-logistic model. The incidence of liver necrosis in female mice should be considered for calculating RfCs.

Table 2. Lower 95% confidence limits for the inhalation concentration estimated to produce an extra risk of 10% for liver necrosis based on the log-logistic model.

Species	Sex	Chi-square goodness-of-fit P-value	$BMCL_{10}$ (ppm)
Rats	Male	No dose response trend	
Rats	Female	No dose response trend	
Mice	Male	1.00	33
Mice	Female	0.32	28

Response from David Jollow

As noted above, this adaptive response should not be used as an index of a toxic response.

Response from José E. Manautou

There are contradictory statements in the document regarding this. On page 19, it is stated that "by themselves, increased liver weight and increased incidence of cytomegaly can be considered to be of questionable toxicological significance". On the other hand, this endpoint is used in other instances as a key toxicological effect. The document is rather superficial and inconsistent in the use and justification of this endpoint.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION B3

The subchronic and chronic RfCs have been derived utilizing benchmark dose modeling to define the point of departure. Please provide comments with regards to whether BMD modeling is the best approach for determining the point of departure. Has the BMD modeling been appropriately conducted and objectively and transparently described? Has the benchmark response selected for use in deriving the POD been scientifically justified and transparently and objectively described? Please comment on the justification for not utilizing the 100 ppm dose identified in the NTP (1985d) study as a NOAEL. Please identify and provide rationale for any alternative approaches (including the selection of BMR, model, etc.) for the determination of the point of departure, and if such approaches are preferred to EPA's approach.

Response from John L. Farber

My comments detailed above under the Oral Reference Dose of Bromobenzene apply here as well.

A1 – The selection of the 90-day oral gavage study in mice as the principal study was justified and objectively described. I am not aware of any other studies that should be used as the principal study.

A2 – Liver toxicity is the most appropriate critical effect. The only meaningful indicator of liver toxicity, however, is liver cell necrosis. In particular, liver weight is not an acceptable endpoint with which to assess liver toxicity. The liver can increase in weight by a variety of mechanisms. Thus, simply measuring liver weight does not specify the particular reason for the increase in weight. Importantly, some causes of a liver weight increase may reflect a toxic endpoint, e.g. steatosis, hydropic swelling, whereas others do not. For instance, many compounds that are metabolized by the liver cause an increase in liver weight, owing to an increase in the machinery (endoplasmic reticulum) that carries out this metabolism. Such a cause of increased liver weight would be misleading to label as toxicity.

A3 – I have concern with regard to using a combined incidence of liver lesions – that is the number of animals with one or more liver lesions (cytomegaly, necrosis, inflammation, mineralization). As stated above, alterations other than necrosis are not necessarily indicators of liver toxicity. Thus, combining animals with lesions with differing toxicological significance has the potential to distort the derived incidence of toxic lesions (necrosis) in the subchronic and chronic studies.

Response from David William Gaylor

A dose of 100 ppm is not a NOAEL. There is a statistically significant increase in liver weight in female mice at this dose. With only 10 animals per dose group, the incidence of liver cytomegaly or necrosis at 100 ppm in female mice is highly uncertain. The BMC approach that makes full use of the dose response data is far superior to selecting a single dose for a NOAEL that may pose a high risk.

Response from David Jollow

The procedure appears to be adequately described and justified. As noted above, additional NTP studies with more doses/more animals per dose, and a more rigorous morphometric assessment of liver necrosis would be helpful.

Response from José E. Manautou

Modeling for benchmark dose determination is beyond the scope of this reviewer's expertise.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION B4

Please comment on the selection of the uncertainty factors applied to the POD for the derivation of the RfCs. For instance, are they scientifically justified and transparently and objectively described in the document.

Response from John L. Farber

My comments detailed above under the Oral Reference Dose of Bromobenzene apply here as well.

A1 – The selection of the 90-day oral gavage study in mice as the principal study was justified and objectively described. I am not aware of any other studies that should be used as the principal study.

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Response from David William Gaylor

The POD = 28 ppm for the incidence of liver necrosis in female mice is converted to a continuous exposure of 32 mg/m^3. The uncertainty factors appear to be appropriate. Hence, the sub-chronic RfC = 32 / 300 = 0.11 mg/m^3.

Response from David Jollow

As discussed above, the uncertainty factors selected appear appropriate except for possible over estimation of inter individual variation.

Response from José E. Manautou

See comments above (under oral RfD).

A1 – The selection of the study was justified in light of the absence of other studies assessing toxicological outcomes of chronic and sub-chronic bromobenzene exposures. However, this reviewer feels that the design, implementation and interpretation of data from the NTP studies conducted in the mid-1980's are rather poor. The main concern is the number of animals used per treatment group in the studies (n=10). This is unrealistically small. A bigger concern is the data for some of the endpoints at the highest dose of bromobenzene used in these studies. For example, the majority of animals in the 90 days gavage study receiving 600 mg/kg/day died. The way data for those dosing regimens have been presented can be misleading. Despite this, the authors did their best synthesizing the existing data.

A2 – There are concerns with the endpoint selection criteria. Chemicals can produce increases in liver weight in the absence of toxicity. Generalizations and assumptions were made based on the well-documented hepatotoxic potential of bromobenzene. It would be advantageous to consider selecting endpoints more closely related to the mode of action of toxicants that generate reactive intermediates, such as changes in expression of stress genes and markers of oxidative stress. This reviewer recognizes that this analysis might not be possible since such data was not generated in the NTP studies.

A3 – The clustering of histopathologic lesion endpoints is also problematic. The document as written does not provide proper justification for combining the endpoints for the purpose of developing an incidence/severity index.

In regards to BMD modeling, this reviewer is not suitable for providing a critical analysis of these modeling exercises since this is beyond his area of expertise. On the other hand, modeling exercise that included data from treatment groups with high mortality rates or inclusion of animals showing extreme distress (moribund animals euthanized prior to completion of the study) should be reconsidered and discussed.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

QUESTION B5

EPA used the data available for chlorobenzene to inform the selection the subchronic to chronic uncertainty factor for the derivation of the chronic RfC for bromobenzene. Please comment on the scientific justification for this use of data from chlorobenzene. Has the scientific justification for this selection been transparently and objectively presented?

Response from John L. Farber

My comments detailed above under the Oral Reference Dose of Bromobenzene apply here as well.

A1 – The selection of the 90-day oral gavage study in mice as the principal study was justified and objectively described. I am not aware of any other studies that should be used as the principal study.

A2 – Liver toxicity is the most appropriate critical effect. The only meaningful indicator of liver toxicity, however, is liver cell necrosis. In particular, liver weight is not an acceptable endpoint with which to assess liver toxicity. The liver can increase in weight by a variety of mechanisms. Thus, simply measuring liver weight does not specify the particular reason for the increase in weight. Importantly, some causes of a liver weight increase may reflect a toxic endpoint, e.g. steatosis, hydropic swelling, whereas others do not. For instance, many compounds that are metabolized by the liver cause an increase in liver weight, owing to an increase in the machinery (endoplasmic reticulum) that carries out this metabolism. Such a cause of increased liver weight would be misleading to label as toxicity.

A3 – I have concern with regard to using a combined incidence of liver lesions – that is the number of animals with one or more liver lesions (cytomegaly, necrosis, inflammation, mineralization). As stated above, alterations other than necrosis are not necessarily indicators of liver toxicity. Thus, combining animals with lesions with differing toxicological significance has the potential to distort the derived incidence of toxic lesions (necrosis) in the subchronic and chronic studies.

Response from David William Gaylor

Using the POD = 28 ppm for the incidence of liver necrosis in female mice gives a continuous exposure of 32 mg/m^3. This gives a chronic RfC = 32/ 1000 = 0.03 mg/m^3.

Response from David Jollow

As noted above, this procedure appears appropriate and is adequately justified.

Response from José E. Manautou

See comments above (under oral RfD).

A1 – The selection of the study was justified in light of the absence of other studies assessing toxicological outcomes of chronic and sub-chronic bromobenzene exposures. However, this reviewer feels that the design, implementation and interpretation of data from the NTP studies conducted in the mid-1980's are rather poor. The main concern is the number of animals used per treatment group in the studies (n=10). This is unrealistically small. A bigger concern is the data for some of the endpoints at the highest dose of bromobenzene used in these studies. For example, the majority of animals in the 90 days gavage study receiving 600 mg/kg/day died. The way data for those dosing regimens have been presented can be misleading. Despite this, the authors did their best synthesizing the existing data.

A2 – There are concerns with the endpoint selection criteria. Chemicals can produce increases in liver weight in the absence of toxicity. Generalizations and assumptions were made based on the well-documented hepatotoxic potential of bromobenzene. It would be advantageous to consider selecting endpoints more closely related to the mode of action of toxicants that generate reactive intermediates, such as changes in expression of stress genes and markers of oxidative stress. This reviewer recognizes that this analysis might not be possible since such data was not generated in the NTP studies.

A3 – The clustering of histopathologic lesion endpoints is also problematic. The document as written does not provide proper justification for combining the endpoints for the purpose of developing an incidence/severity index.

In regards to BMD modeling, this reviewer is not suitable for providing a critical analysis of these modeling exercises since this is beyond his area of expertise. On the other hand, modeling exercise that included data from treatment groups with high mortality rates or inclusion of animals showing extreme distress (moribund animals euthanized prior to completion of the study) should be reconsidered and discussed.

Response from Jian Zheng

The available toxicity data obtained from chlorobenzene studies may be acceptable and relevant to bromobenzene toxicity.

(C) CARCINOGENICITY OF BROMOBENZENE

QUESTION C1

Under the EPA's 2005 Guidelines for carcinogen risk assessment (www.epa.gov/iris/backgr-d.htm), data are inadequate for an assessment of the human carcinogenic potential of bromobenzene. Please comment on the scientific justification for the cancer weight of the evidence characterization. A quantitative cancer assessment was not derived for bromobenzene. Has the scientific justification for not deriving a quantitative cancer assessment been transparently and objectively described?

Response from John L. Farber

The report is clear in its justification for not deriving a quantitative cancer assessment for bromobenzene.

Response from David William Gaylor

It appears that there is inadequate information to assess the carcinogenic potential of bromobenzene.

Response from David Jollow

The scientific justification for not deriving a quantitative assessment has been transparently and objectively described.

Response from José E. Manautou

In light of the absence of data, the scientific justification for not carrying a quantitative cancer assessment is appropriate.

Response from Jian Zheng

I am unable to provide any critical evaluation, since I do not have the expertise.

ADDITIONAL COMMENTS

José E. Manautou

1. Some of the tables in the document can be misleading. Reporting incidence of lesions at the higher doses of bromobenzene for the 90-day studies seems inappropriate due to high mortality numbers.

2. The description of renal tubular degeneration in the absence of necrosis should be better explained.

3. Whether or not hepatocellular cytomegaly should be considered a pathological endpoint is also not clear and there are inconsistencies on this subject.

4. The description and survey of mechanistic studies for bromobenzene is rather superficial. Equally superficial and non-descriptive are the more recent genomic studies.

5. Table 4-11 is missing glutathione depletion values from untreated animals.

6. In regards to development of tolerance to bromobenzene hepatotoxicity, does tolerance develop at all dose levels? Is there a dose threshold for this effect? The authors attributed this tolerance to more rapid replenishment of glutathione. Other models of auto-resistance demonstrate that multiple cellular events contribute to toxicity tolerance.

7. Also regarding gene expression studies, a table listing altered genes, cellular function and trends (up or down regulation) should be included.

8. The term "genes involved in glutathione depletion" is problematic. Genes involved in GSH homeostasis are either involved in generation and utilization. Depletion can be interpreted at exhaustion of stores.

9. There authors also described some interpretative challenges with the NTP 2-year toxicity and carcinogenicity study for chlorobenzene. This casts doubts on the utility of the chlorobenzene data for establishing comparisons with bromobenzene toxic potential. Caution should be used when utilizing these data.

In summary, this is a straight forward, well-written document. The existing data on bromobenzene have been properly presented. There are no over- or misinterpretations of data. Two important points to consider for the final draft of the document are:

1. The inadequacy and lack of justification for the clustering of endpoints for bench mark dose modeling.

2. The need for a statement highlighting the poor quality of the existing subchronic toxicity studies on bromobenzene.

Jian Zheng

1. Page 3, line 17: Bromine naturally exists in its isotopes of 79 and 81. Mass spectrometry of bromobenzene shows its molecular weight of 156 and 158 not 157.

2. Page 8, figure 3-1: (1) Figure 3-1 is a little too complicated, and I prepared two separated figures to describe the metabolism of bromobenzene as shown below (Figures 1 and 2); (2) Metabolites 4- and 2-bromophenols are formed mainly from spontaneous rearrangement (NIH shift) of the corresponding bromobenzene oxides; and (3) bromobenzene dihydrodilos may be transformed to the corresponding bromophenols by dehydration but **not** rearrangement.

Figure 1

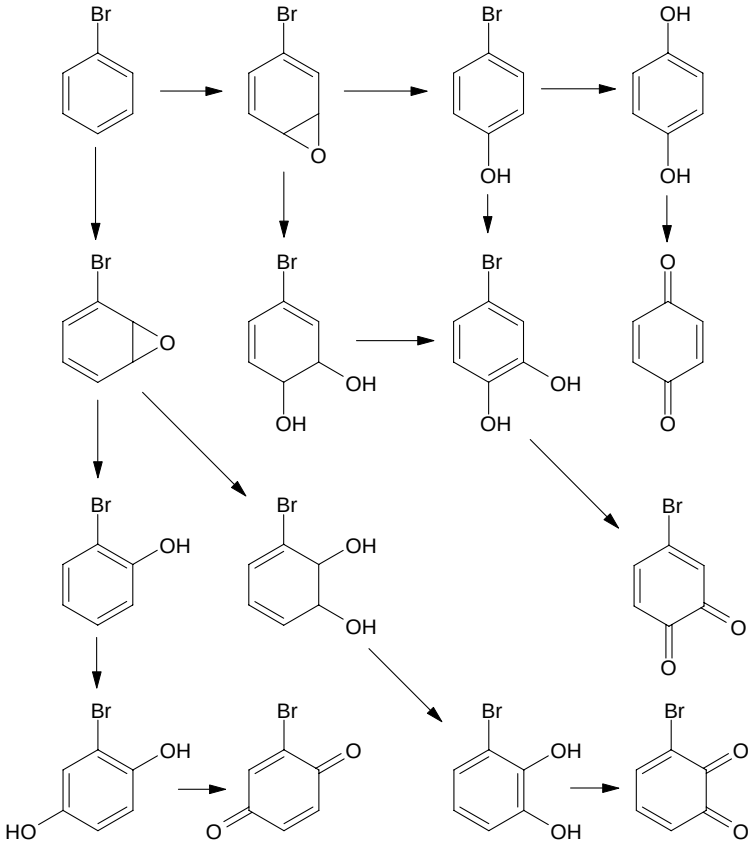
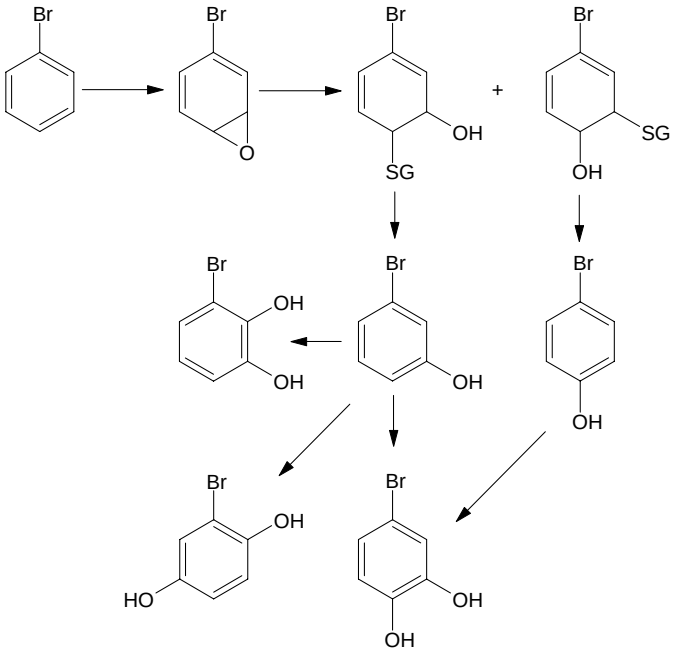


Figure 2



3. Page 9, lines 18 and 21: Refer to 2 above.

4. Page 9, lines 21-22: The dehydration of the 3,4-dihydrodiol to the 4-bromophenol is a minor pathway for the formation of 4-bromophenol in either rat or guinea pig. The dehydration process takes place spontaneously without enzyme involvement. It does not make sense that the rate of the spontaneous process (dehydration) differs in the two species at the same pH environment. Apparently, Lertratanangkoon's papers did not indicate that spontaneous dehydration of the 3,4-dihydrodiols is the major pathway responsible for the formation of 4-bromophenol in rat.

5. Page 9, lines 31: GT is a typo, and it should be replaced by GST for glutathione S-transferase.

6. Page 34, lines 13-15: Boromobenzene dihydrodiols should not be considered as reactive intermediates. Reactive intermediates often refer those molecules which can react with others spontaneously. The diols are not considered to be chemically reactive, and the chemical reaction occurs intramolecularly by dehydration.

7. Page 43, lines 26-27: Oxidative debromination has been reported in metabolism of bromobenzene (refer to Zheng and Hanzlik's paper).

8. Page 50: The details in bromobenzene bioactivation and protein modification by reactive metabolites of bromobenzene have been provided on pages 37 and 42, respectively. It seems that the discussion is repeated.