SUMMARY REPORT

OF THE EXTERNAL PEER REVIEW OF THE DRAFT

TOXICOLOGICAL PROFILE FOR

ETHYLBENZENE

Submitted to:

The Agency for Toxic Substances and Disease Registry
Division of Toxicology
1600 Clifton Road NE, MS F-32
Atlanta, GA 30333

Submitted by:

Eastern Research Group, Inc.
110 Hartwell Avenue
Lexington, MA 02421-3136

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QUALITY NARRATIVE STATEMENT

ERG selected reviewers according to selection criteria provided by ATSDR. ATSDR confirmed that the scientific credentials of the reviewers proposed by ERG fulfilled ATSDR's selection criteria. Reviewers conducted the review according to a charge prepared by ATSDR and instructions prepared by ERG. ERG checked the reviewers' written comments to ensure that each reviewer had provided a substantial response to each charge question (or that the reviewer had indicated that any question[s] not responded to was outside the reviewer's area of expertise). Since this is an independent external review, ERG did not edit the reviewers' comments in any way, but rather transmitted them unaltered to ATSDR

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There were no annotated pages from the draft profile document submitted for this review

SECTION I

PEER REVIEWERS' SUMMARY COMMENTS

SUMMARY COMMENTS RECEIVED FROM

John DeSesso, Ph.D.
Senior Fellow, Noblis
Falls Church, VA
703-610-2130
Email: jdesesso@noblis.org

Critique of
Draft Toxicological Profile for Ethylbenzene

John M. DeSesso, PhD
Fellow ATS, DABFM, FACFEI, DABFE, CHS-V

Noblis
3150 Fairview Park Drive
Falls Church, Virginia 22042

Commentary on
Draft Toxicological Profile for Ethylbenzene

This is a well-written, readily understandable, balanced and non-judgmental document that summarizes a great amount of literature for ethylbenzene (EB). For the most part, except as noted in the Specific Comments, the vocabulary choices were appropriate for the intended audience. The authors write in a straightforward, easy to understand style that is not threatening to their audience despite the technical nature of their subject matter. The authors are commended for their effort at distilling complex and sometimes conflicting scientific information into a comprehensible treatise. In the paragraphs that follow, issues will be raised that may criticize certain aspects of the report, but these are brought forward for the purpose of improving the document. Some of the following general comments may be redundant with the items raised in the Specific Comments.

My areas of expertise are toxicology (with emphasis on reproductive and developmental toxicity) and risk assessment. While questions about the details of the exposure assessment are best left to persons with expertise in that area, I want to make two general points about the exposure chapter of the report. First, it seems to me that an important scenario that should be considered is that of a hobbyist (adult and child). Hobbyists use glues, paints and varnishes in workshops that may not have full ventilation; their faces are often quite close to the items on which they are working, which enhances inhalation of vapors. This group is likely to be highly exposed and should be considered more fully. Second, children's exposure should be divided into age segments that reflect their activities. If data were available, the age ranges would include less than three months; three to twelve months; 1-2 years; 3-5 years; 6-8 years; 9-14 years; 15-18+ years. While all of these groups may be beyond the scope of this generalized report, certainly the exposure values for infants and toddlers differ from children in school, who also differ from teenagers. I believe that 9-14 year old hobbyists or 15-18 year old hobbyists might become the most highly exposed groups. While there may be few effects of EB exposure, it would be good to at least consider these age groups to ensure that they are likely to be harmed.

The literature concerning EB has been well-searched and adequately summarized. Nevertheless, the report can be strengthened in five areas. Each of these areas has at least one specific comment in the following section (which also provides page and line locations).

First, papers that report positive but are mediocre should be cited in a way that reminds the readers of the weakness of their findings. The best example in this report is the paper by Ungvary and Tatrai, which attempts to provide developmental toxicity information from dose-response inhalation studies of 8 chemicals in three experimental species. The paper also provides data on body fluid concentrations of the chemicals on test. All of this is presented in a 5 page manuscript that states that EB caused unspecified anomalies of the urogenital tract. This reference is more of an expanded abstract than a manuscript. In contrast, several other guideline-compliant studies (i.e., the Faber et al reports) that have investigated EB's developmental toxicity at comparable doses and have published fully documented reports in the open literature, have not found any anomalies. The apparent impact of the Ungvary and Tatrai (by the number of times it is cited and the amount of space devoted to it) should be reduced.

Second, statements to the effect that it is not known of EB crosses the placenta should be removed. Virtually every molecule under 600 daltons crosses the placenta. Molecules with structures similar to EB (e.g., toluene and the xylenes) readily cross the placenta.

Third, the metabolism of EB is nicely summarized, but there is no (or little) consideration of age-dependent kinetics or metabolism. While there is little in the literature addressing these issues, the report could at least mention that these are areas that contribute to uncertainty with respect to infants and children as well as the aged.

Fourth, the ATSDR text that introduces section 3.7 does a good job of presenting the concepts that children are not merely small versions of the adult and that development continues past birth. However, the statement that development is complete by age 18 is misguided by being too proscriptive. Muscle mass continues to accrete in males until the beginning of the third decade. The function of the brain (as measured by EEG recordings) is not mature until about age 25. Perhaps the wording could be altered to state that most development is complete by the early 20's (it is better to be non-specific about exact dates).

Fifth, the authors must be cautious about including in vitro data. Specifically, the experiments that studied ED effects on synaptosomes suggested that EB's impact could be ameliorated by stabilizing the membranes. This may work well in vitro, but the stabilization itself could have untoward effects in vivo.

With respect to the Regulations and Advisories (Section 8), the authors should point out that the EPA RfC for EB is 1mg/m^3 which is equivalent to a concentration of

0.23 ppm and that that value compares favorably with the ATSDR-derived intermediate-duration inhalation MRL of 0.2 ppm.

In conclusion, the Draft Toxicological Profile for Ethylbenzene needs a few minor alterations, but in the big picture it is a well executed document that is suitable for its intended audience.

Specific Comments

Page 3, Consumer Products, Bullet 4: varnishes and paints

Page 13, line 1. Do the authors mean to say: for 3 weeks ***prior to*** mating? Three weeks is a very long mating period.

Page 13, line 7: By "caudal weight" do the authors mean to say "weight of the caudal epididymis"?

Page 13, line 25: ...irritation and lacrimation ***have*** been...

Page 14, line 4: What is meant by "neoplastic tumors"? Do the authors mean "malignant tumors" or "neoplasms" or some other term?

Page 15, lines 1-16: The authors should mention that, based on human observations, the rat appears to be the more appropriate animal model.

Page 16, line 34 (and throughout): Correct spelling is uropoeitic. Also, the terminology used by Ungvary and Tatrai is uninformative. The actual anomaly should have been described in the original report. Their statement could be anything from a major malformation (e.g., renal agenesis; polycystic kidney; horseshoe kidney) to a non-life threatening anomaly (e.g., duplicated Ureter; partially ascended kidney) to alterations which may be corrected shortly after birth (e.g., dilated renal pelvis – sometime mistaken for hydronephrosis). It is unfortunate that this poorly documented paper gets so much attention.

Page 17, lines 6-7: Statements should be made about the quality of the studies. For instance, the Faber studies were guideline compliant developmental toxicity studies with good reporting and complete examination of fetuses where as the Ungvary and Tatrai study is extremely weak for use in assessing risks.

Page 17, lines 30 ff: I do not understand the logic of the statement being made. First, there were changes in weights of liver and kidney, but no alterations in histopathology. Then how did the organs enlarge? Is there more tissue present? If the weight change is due to fluid retention, this should have shown up in the histopathological sections. If no alterations in histopathology were observed (and this is usually more sensitive than a

change in gross weight) then I have difficulty seeing how the weight change, unsupported by a histological correlate, suggests that these tissues are sensitive targets.

Page 20, line 27: The name of the test performed is a two-generation ***reproductive*** toxicity test.

Page 26, lines 21-22: The first sentence is repeated.

Page 35, line 23: The sentence should read ...observed in animal<u>s</u> ...

Page 37, line 25: Suggest replacing "postimplantation death" with "postimplantation ***loss***".

Page 39, lines5-14. The Ungvary and Tatrai study is again presented. Documentation for this work is quite minimal. The authors performed inhalation studies of 8 chemicals in each of 3 species and allegedly performed a full developmental toxicity evaluation plus chemical analyses of various fluids. The reported their results in a paper that is only 5 pages long. Rather than providing data, for the most part the authors just give us their conclusions. The problem is that there is no way to independently evaluate their claim of "retarded skeletal development" (line7); how was this determined? Were there criteria for this? What were they? Is this merely a general impression of the authors? If so, how did they arrive at the percentages reported in the paper? While it may be important to include this paper for completeness, the paper's value for risk assessment is poor. As mentioned, if the Ungvary and Tatrai paper is the only source for the non-specified "uropoeitic apparatus" anomalies, and there are several well-documented guideline-compliant studies that did not find malformations of the urogenital tract, statements about this condition should be deleted or strongly modified to indicate the weakness of the source.
Page 40, line18: The word 'tests" is missing from the Second sentence – Neurobehavioral ***tests*** conducted...

Page 41: Dropped word - ...significantly grater than in ***the*** control...

Page 50, line 33: Formatting error: cm^2

Page 52, line 4: Formatting error: cm^2 also note that the units for the second value are incorrect.

Page 56, line 8: The authors do not mention the radiolabel; however, they state that they found metabolites in the expired carbon dioxide. Do they mean that EB is metabolized to CO_2 and C^{14} was detected or did they mean to say that they looked for EB in expired *breath*?

Page 65, line 33: Why did the authors pick 18 years as the time when all biological systems have fully developed? Certainly most of the organs are morphologically mature in most people by that age, but the muscular system among males continues to mature past that age and the brain does not reach full maturity until ~25 years. This could be corrected by not making the rather firm statement about all systems being fully developed by 18.

Page 66, line 30: Delete "all" from "in newborns who all have..."

Page 67, lines 13 -16. Once again, it must be noted that Ungvary and Tatrai did not report the specific effects that they claim are anomalies of the urogenital tract. Consequently, the statement about EB causing urinary tract anomalies is unsupported by data.

Page 67, Lines 26-27: The statement that "It is not known if ethylbenzene crosses the placenta" is shocking. First, virtually everything crosses the placenta. Second, the Ungvary and Tatrai paper claims to have found the tested xylene-related chemicals in fetal blood and amniotic fluid. I suggest dropping this sentence. The other sentences remain true. And I do not think it would be appropriate to cite Ungvary and Tatrai for the purpose of demonstrating that EB crosses the placenta.

Page 71, line 10: Dropped word - ...with pig ***skin*** treated...

Page 73, line 7: Obtundation is likely not going to be understood by most of the general population. Consider other terminology like "stupor" or "decreased alertness."

Page 73, lines 10-11: I do not understand the part of the sentence that appears to state that swallowing is a direct route to the lungs.

Page 74, lines 3-6: The in vitro findings relative to synaptosome preparations are interesting, but putting forth in this document the notion of stabilizing the membranes to prevent EB from entering the lipid bilayer as a protective mechanism seems out of place. This would not work in vivo because 1) stabilizing the membrane would likely interfere with the capacity of the synapses to work efficiently and 2) agents that affect the

membranes of neurons at the synapse would also affect other membranes throughout the body. These sentences could be deleted without affecting the flow or quality of the document.

Page 90, line 18: The decimal point is missing from "0.7±02".

Page 118, line 29: In the rest of the document, the spelling used is: absorbent.

Page 125, line 26: Delete *a* from "...dividing the a NOAEL..."

SUMMARY COMMENTS RECEIVED FROM

James McDougal, Ph.D.
Professor and Director of Toxicology Research
Boonshoft School of Medicine
Wright State University
Department of Pharmacology and Toxicology
Dayton, OH 45435
937-775-3697
Email: james.mcdougal@wright.edu

SUMMARY REPORT FOR REVIEW OF TOX PROFILE FOR ETHYLBENZENE

CHAPTER 1. PUBLIC HEALTH STATEMENT

Under the section – How can families reduce the risk of exposure to ethylbenzene?:

The recommendation to limit exposure to tobacco smoke should be eliminated. It is true that ethylbenzene (EB) is a component of tobacco smoke, but the amounts are too small to provide any risk. This recommendation makes ethylbenzene seem like a dangerous component of second-hand smoke when it is very unlikely to cause harm at levels that occur.

CHAPTER 2. RELEVANCE TO PUBLIC HEALTH

2.1 Background and environmental exposures

Exposure conditions are not adequately described. This document reports exposures without describing their importance - leaving the reader to believe that they are much more important that they actually are. This section needs to be put into perspective. Someone from the general public reading this would get the wrong impression about the impact of EB on public health. Examples are:

Page 9, line 13 – "routine human activities, such as driving automobiles, boats, or aircraft, or using gasoline powered tools and equipment, release ethylbenzene to the environment". This is a true statement, but without the proper perspective, an individual might be concerned about this when the actual amounts released are many orders of magnitude below the levels that have been shown to cause health effects.

Page 10, paragraph starting line 4 discussed EB in food and ETS. Without perspective the reader might think these were important sources of exposure. Also, the ETS concentration should be converted to ppm for the reader (approx .0018 ppm).

A paragraph at the beginning or the end of section 2.1 should say something like: Environmental and background exposures to EB are generally very small and therefore have minimal impact on public heath. Trace levels of EB can be found in internal combustion engine exhaust, food, soil, water and second-hand tobacco smoke,but at levels that are well below those that have been shown to have toxic effects in laboratory animals or human exposure studies.

2.2 SUMMARY OF HEALTH EFFECTS

Page 15 – because there is such a big difference in ototoxic effects between guinea pigs and rats, the authors should try to find evidence about how chemical-induced ototoxicity differs between species rather than using rats as representative of humans. It is likely that this level of sensitivity is not found in humans and therefore not of such concern.

2.3 MRLS

Page 19, line 3 – text says partition coefficient (Blood/gas) in animals is greater than in humans. A default value of one is used. The difference between the partition coefficients should be documented and referenced. This default value is a conservative assumption that probably negates the need for the 3-fold safety factor for extrapolation.

CHAPTER 3. HEALTH EFFECTS

Section 3.2 DISCUSSION OF HEALTH EFFECTS BY ROUTE OF EXPOSURE

In general, the discussion of health effects seems to be complete and thorough.

Page 26, line 21 & 22 – sentence about lack of inhalation lethality reports in humans is repeated.

Page 26, line 23 – Lethality to gasoline should not be reported without putting the amount of EB in gasoline in perspective, i.e. about 1%. Not only is it "not possible to determine the extent to which his death was due to exposure to ethylbenzene versus the other components of gasoline", it is extremely unlikely that it was due to EB.

Page 45, line 12 - Probably not fair to characterize that as a dermal exposure without a lot of caveats. First, as pointed out above EB makes up about 1% of gasoline. Second, in a closed environment, the exposure to all the volatile components of gasoline would be much more likely to be by inhalation (inhalation was only mentioned as another route). This study should probably not be listed under dermal effects.

Section 3.3 GENOTOXICITY

Page 47, line 9 – The Holz study was in a styrene plant with reported air levels of benzene, toluene and xylene in addition to EB. This citation should be changed to show the presence of the other VOCs.

Section 3.4 TOXICOKINETICS

In general the toxicokinetic section is complete, if not a little lengthy. If possible a summary covering storage major organs, toxicokinetic differences between humans and animals and their relevance should be included.

Page 48, paragraph starting on line 25 – my Italian is not good enough to be sure, but it doesn't seem that this paragraph represents the paper (at least not the English abstract). Not sure what the point is here. Why is this included? If it remains - the blood and air concentrations should be cited.

Page 50, line 28 – the juxtaposition of the McDougal and Tsuruta studies suggest that they are comparable. That is not the case. McDougal measured the flux from JP-8 and Tsuruta measured the flux of the pure chemical.

Page 50, line 29 – not sure why the 3- 4- & 5-hour duration fluxes are here or where they came from, they are not readily apparent in the paper. Were they calculated by the author? I don't get the same numbers if they were.

Page 51, line 1 – should read 1,200 µg ethylbenzene/mL fuel.

Page 51, line 4 – should read "...diffusivity values of ethylbenzene in pig skin were1.04 µg/cm^2/hour, 0.06 x 10^{-3} cm/hr, and 715 x 10^{-6} cm^2/hour". (The table header says x 1,000 and times 1,000,000 which is an odd way to do it, but the numbers for permeability and diffusivity are very small) The values chosen from the table in the Muhammad paper were the control (not pretreated with JP-8). If the McDougal paper is used (not sure why it is relevant), it should be made clear that EB penetrates from jet fuel.

Section 3.5 MECHANISMS OF ACTION

Mechanisms of action section seems to cover all that is known.

Section 3.9 INTERACTIONS WITH OTHER CHEMICALS

I don't think the first part of the paragraph that starts on page 71 line 1 (discussing the effects of JP-8 vehicle on the penetration of EB from JP-8) is an appropriate interaction with other chemicals for this section. It is well known that the vehicle has important effects on the penetration of chemicals. I think this first part of the paragraph should be deleted. The Muhammad study which is discussed in the last part of the same paragraph is probably not appropriate either, but I feel less strongly about it. If it is left, the authors should explain what it means, rather than just citing the numbers.

Page 71, line 1 – should read "ethylbenzene/mL fuel"

Page 71, line 8,9,10 & 11 – exponents should be negative.

Section 3.12 ADEQUACY OF THE DATABASE

Page 79, line27 – it is important that the need for additional mechanistic studies related to ototoxicity is pointed out. Another need is studies that explore species differences in chemical-induced effects on hearing and ear physiology. Due to the large species differences between rats and guinea pigs in ototoxicity it would be nice to understand which species response is most similar to humans.

CHAPTER 4. CHEMICAL AND PHYSICAL INFORMATION

Looks complete

CHAPTER 5. PRODUCTION, IMPORT/EXPORT, USE, AND DISPOSAL

Looks good.

CHAPTER 6. POTENTIAL FOR HUMAN EXPOSURE

Looks OK. Nice summary of VOC media measurements.

Page 108, lines 9-11 – ppb conversions should be included. Incidentally, "ppbv" and "ppmv" are not listed in appendix C.

CHAPTER 7. ANALYTICAL METHODS

OK

CHAPTER 8. REGULATIONS AND ADVISORIES

OK

CHAPTER 9. REFERENCES

No additional

SUMMARY COMMENTS RECEIVED FROM

Andrew Salmon, Ph.D.
Senior Toxicologist and Chief, Air Toxicology and Risk Assessment Unit
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency
Oakland, CA 94612
510-622-3191
Email: asalmon@oehha.ca.gov

Comments on draft 2 of Toxicological Profile for Ethylbenzene.

Andrew G. Salmon M.A., D.Phil.

General Comments

The general survey of the toxicological and environmental data appears to be thorough up to about the time of the previous version of the profile produced in 1999. However, the extent of updating of the document for this revised draft appears to have been relatively minor, and in some areas less than complete. A few notable findings of recent date relating to developmental toxicity have been included. The coverage of the carcinogenicity findings includes mention of the most recent NTP (1999) studies, and describes the findings of carcinogenicity in mice and rats. Obviously, these findings have given rise to considerable debate as to the mechanism(s) of action of ethylbenzene and the significance of the findings for human health. The reporting of the debate on these questions is incomplete. Initial comments by Hard (2002) are noted, but further debate disputing some of the conclusions of this commentator is not included. Egregiously, the IARC 2000 review is mentioned in passing, but there is no comment or analysis on their finding (2B), which would ordinarily be sufficient to trigger warnings and regulations based on possible human carcinogenicity. It is inappropriate to report this important evaluation so slightly, while at the same time noting without qualification the US EPA evaluation of Category D which predates all the important recent bioassays and evaluations by several years. Recent data on genotoxicity, which although not conclusive may provide some insights into possible mechanisms of action, are also not covered. The non-cancer findings, and derivation of short-term MRLs based on ototoxicity, are in general well described. However, the treatment of available PBPK models of absorption, distribution, metabolism and excretion is curiously limited, dealing only with two published models covering various gasoline components including ethylbenzene, but ignoring a number of other published models.

✓ *Are there any data relevant to child health and developmental effects that have not been discussed in the profile and should be?*

In general this is one of the areas that has been covered and updated reasonably well. Newer results on developmental toxicity have been included.

✓ *Are there any general issues relevant to child health that have not been discussed in the profile and should be?*

The main deficiency is the less than adequate treatment of the most recent findings on carcinogenicity, and supporting issues of genotoxicity, and pharmacokinetics/metabolism. While the potential carcinogenic effects of ethylbenzene are clearly of concern for all exposed individuals, this is certainly a concern for children's health, especially as exposures early in life are expected to give rise to greater cancer risks over the individual's entire lifetime, even in the absence of specific factors enhancing the sensitivity of infants and children (see EPA's 2006 cancer risk assessment guidelines and supplement).

Specific comments

CHAPTER 1. PUBLIC HEALTH STATEMENT

- *The tone of the chapter should be factual rather than judgmental. Does the chapter present the important information in a non-technical style suitable for the average citizen? If not, suggest alternate wording.*

  The style seems generally suitable and understandable.

- *Major headings are stated as a question. In your opinion, do the answers to the questions adequately address the concerns of the lay public? Are these summary statements consistent, and are they supported by the technical discussion in the remainder of the text? Please note sections that are weak and suggest ways to improve them.*

  Page 4 after line 9, subheading "Long-term exposure in air/Cancer". As noted in the comments on the technical section, this statement is completely out of date, and should be replaced with wording noting more recent evaluations, specifically the IARC (2006) evaluation if U.S. EPA has not got around to updating their classification. I suggest replacing the two sentences starting "the EPA

determined... with something like "The International Agency for Research on Cancer (an expert group which is part of the World Health Organization) has determined on the basis of these animal studies that long-term exposure to ethylbenzene may cause cancer in humans.
Similarly, the "Long-term exposure by ingestion" subheading should note that although no studies have examined the risk of cancer by this route, the finding of possible carcinogenicity by inhalation raises the presumption that this effect would be seen by the oral route also.

Page 7, following line 7 (Regulations). It would be desirable for ATSDR to develop and list guidance levels for exposures that would result in *de minimis* cancer risk, i.e. 1 in 10^6, and that these should be listed at least as advisories here.

- *Are scientific terms used that are too technical or that require additional explanation? Please note such terms and suggest alternate wording.*

The level of language used seems well chosen to me.

CHAPTER 2. RELEVANCE TO PUBLIC HEALTH

- *Do you agree with those effects known to occur in humans as reported in the text? If not, provide a copy of additional references you would cite and indicate where (in the text) these references should be included.*

The effects noted in this section are in general noted completely and accurately, given the somewhat limited nature of the human data in some areas.

- *Are the effects only observed in animals likely to be of concern to humans? Why or why not? If you do not agree, please explain.*

The summary describes the non-cancer effects observed in animals accurately, and for the most part makes a good case for their applicability to human risk assessment.. For instance, the ototoxicity which is observed in animals is carefully described, including comparison with the suggestive evidence of similar effects in humans. In contrast,the treatment of the organ weight changes following short-term exposure is a trifle superficial, in that these are largely

dismissed as adaptive without consideration that they may be precursor effects of the frankly pathological changes seen after longer exposures, and even if "adaptive" may have adverse implications if (as is likely) they are also seen in exposed humans. This point is explored in greater detail in some of the line-by-line comments below.

The description of the carcinogenicity data is brief, although accurate as far as it goes. However, it is a deficiency of the document as a whole that no real attempt is made to evaluate the likely human health implications of these findings. The discussion of the human relevance of the more recent NTP findings is limited to reporting some commentary which downplayed the likely relevance of the findings to humans, but fails to report more recent counterarguments. The significant evaluation by IARC (2006) assigning a Group2B classification (possibly carcinogenic to humans) is noted but not in any way responded to. The US EPA classification on IRIS as Group D (not classifiable) is misdescribed by citing the 2007 version of the IRIS database; this evaluation is a very old one which predates all the more recent experimental findings and discussions. Similarly, although ethylbenzene does not appear in the NTP's Report on Carcinogens, this appears to be because they have not considered it recently rather than because they have made a specific negative judgment. There is nothing equivocal about the findings of animal carcinogenicity described in the actual bioassay technical report. Further specific comments on this issue appear below.

- *Have exposure conditions been adequately described? If you do not agree, please explain.*
  Generally, yes, although as noted below this section would benefit from a little more clarity in regard to the source of indoor air contamination by ethylbenzene (which is in fact explained in later sections of the document).

Page 9, line 30. "Ethylbenzene levels in indoor air tend to be higher than corresponding levels monitored in outdoor air." It would be informative to add at this point an observation (based on the description later in the document) that the higher levels in indoor air result from contributions from environmental tobacco smoke (ETS) and

various consumer products, in addition to permeation indoors of ethylbenzene in outside ambient air. The description of the ethylbenzene concentrations measured in ETS (page 10, lines 8 – 13) belongs more logically here than where it currently appears at the end of a paragraph on abundance in foods.

Page 13, lines 21 – 22. It seems unwise to simply dismiss the organ weight increases after acute exposure from further consideration as possible adverse effects, simply on the ground that "some of the weight increases, particularly in the liver" are adaptive. An argument can be made (and evidently was by the Ethylbenzene Producers' Association) that the liver changes are primarily adaptive in nature, but the observation of liver pathology after longer term exposures in some test systems (Wolf et al., 1956; NTP, 1999; Saillenfait et al., 2006) raises the question of whether the changes observed are in fact precursors to later pathological events. Even if what is observed is in fact strictly the induction of P-450 and related xenobiotic metabolizing enzymes and associated proliferation of endoplasmic reticulum, it is not clear that this is a desirable situation for public health since (as noted elsewhere in this document) it carries with it the probability of changes in drug kinetics which could be harmful to individuals on medication, and possibly increased sensitivity to mixed exposures to other toxic chemicals.
In view of the extensive renal pathology reported in other studies it seems unlikely that the kidney weight changes are entirely adaptive, while short-term increases in lung weight are not adaptive but indicative of edema or cellular infiltration (incipient pneumonia?).

Page 14 lines 23-24 and Page 18, lines 1-2. The characterization of ototoxicity as unequivocally "the most sensitive effect following inhalation exposure to ethylbenzene" is true only if the assumption is made that the increases in liver and kidney weight also noted after shorter term exposures are of no toxicological significant. As noted in the previous comment, this conclusion is subject to qualification. The study narratives (pages 16-17) have a tendency to report the exposures used as concentrations but not to specify the exposure durations or schedule, which are important in determining the relative sensitivity of the various endpoints. Admittedly this section is not intended to provide exhaustive detail on the source data, but omission of some key data makes it hard

to understand the significance of the observations or the rationale for the evaluations provided.

A good case can be made that the ototoxicity is the endpoint of concern; it is certainly one of the more sensitive endpoints. It represents a serious and perhaps irreversible effect, and there are data from occupational studies (see the following paragraph, beginning at line 28) suggesting that humans may be sensitive to the effect. Use of this endpoint might result in a lower protective level as the MRL even if other less severe effects are seen at similar or lower levels in animal studies. It is also of concern that developmental effects (skeletal retardation, urinary tract malformations and fetal loss in rats, reduced fetal weight in rabbits) were observed at similar concentration ranges (Ungvary and Tratai, 1985), in spite of acknowledged limitations in the reporting of the study. It would be useful to present this discussion in the narrative, rather than to simply dismiss the short-term organ weight changes as "adaptive" without proper rationalization, or to ignore the concern for developmental effects because the reporting of the study was less than ideal.

Page 22 lines 10-12. The exposure to ethylbenzene of the workers studied by Bardodej and Cirek (1988) was dismissed as unquantified but "negligible". (In fact this description is not entirely accurate – see my fuller analysis in a comment on page 29, below.) Since there were no health-related findings either, this study contributes nothing to the discussion: so why is it even mentioned in this context? If in fact ATSDR's analyst believes that there was some exposure, even though unquantified, this might merit discussion at some other point in the document, but clearly this study has nothing to contribute to the determination of an MRL.

Page 22 lines 26-29. I agree that this endpoint is a reasonable choice on which to base the long-term MRL. It uses the most sensitive endpoint in the most comprehensive and thoroughly reported animal chronic bioassay available. It is interesting that it was not felt necessary to justify this choice for its relevance to humans, given the extensive rebuttals offered by some commentators to the suggestion that the kidney carcinogenicity findings in this study should not be so considered.

Page 23 line 28 to page 24 line 22. It seems to me that in view of the importance of the oral route for exposure to ethylbenzene (e.g. via groundwater contamination leading to its presence in well-derived drinking water and in irrigated crops), it might have been worthwhile to try harder to develop at least some oral MRLs. Possible approaches include using one of the various available PBPK models to extrapolate from the identified inhalation MRLs. The existing data on oral dosing studies may be of insufficient quality to use as the basis of MRLs in isolation, but could be used to confirm (or otherwise) the validity of MRLs obtained by route-to-route extrapolation.

CHAPTER 3. HEALTH EFFECTS

Section 3.1 INTRODUCTION

Section 3.2 DISCUSSION OF HEALTH EFFECTS BY ROUTE OF EXPOSURE

Toxicity - Quality of Human Studies

- *Were adequately designed human studies identified in the text (i.e., good exposure data, sufficiently long period of exposure to account for observed health effects, adequate control for confounding factors)? If not, were the major limitations of the studies sufficiently described in the text without providing detailed discussions. If study limitations were not adequately addressed, please suggest appropriate changes.*

  There are very few data on human effects of ethylbenzene exposure. There are some studies of respiratory irritation, but these are evidently very limited. Two studies of hematological workers at plants where ethylbenzene was manufactured or used are noted, but these are subject to severe limitations. The workers in one study (Angerer and Wulf, 1985) were exposed to a mixture of solvents which included ethylbenzene, and also to lead (another hematologically active toxicant), so there is nothing useful to be concluded about ethylbenzene exposure from this study. The second study (Bardodej and Cirek, 1988), which also looked at cancer incidence, provides very little information either since the exposures the workers received during the study period were low. No adverse health findings were described, and the level of detail in the original report is minimal. In view of the deficiencies of theses studies and the lack of significant findings the summary

descriptions in this section are adequate. The most important human data are those relating to neurotoxicity, especially hearing loss (Sliwinska-Kowalska *et al.*, 2001). This study also is compromised by exposure to multiple solvents, but it is at least suggestive evidence in support of the ototoxicity observed in animals. Again, the summary description is adequate given the nature of the study.

- *Were the conclusions drawn by the authors of the studies appropriate and accurately reflected in the profile? If not, did the text provide adequate justification for including the study (e.g., citing study limitations)? Please suggest appropriate changes.*

  I do have a criticism of the way in which the authors' conclusion about the exposure levels was represented for the study by Bardodej and Cirek (1988). See my detail comment below on misapplication of the term "negligible" in characterizing this parameter. It is certainly justifiable to mention this study as one of the few scraps of evidence available, but the limitations are very clear!

- *Were all appropriate NOAELs and/or LOAELs identified for each study? If not, did the text provide adequate justification for excluding NOAELs/LOAELs including, but not limited to, citing study limitations? Please suggest appropriate changes.*

  Unfortunately it is all too evident that LOAELS or NOAELS cannot be derived from any of the human data available.

- *Were the appropriate statistical tests used in the studies? Would other statistical tests have been more appropriate? Were statistical test results of study data evaluated properly? NOTE: As a rule, statistical values are not reported in the text, but proper statistical analyses contribute to the reliability of the data.*

  Sadly, this question does not, for the most part, arise.

- *Are you aware of other studies which may be important in evaluating the toxicity of the substance?*

  No.

Page 29 line 33 to page 30 line 2. Other references to the study by Bardodej and Cirek (1988) characterize the exposure of the workers to ethylbenzene as "negligible". This

further devalues the study as a source of data on health effects of ethylbenzene exposure. It thus appears misleading to characterize this study as on of "two studies involving long-term monitoring of workers occupationally exposed to ethylbenzene", if in the opinion of the analyst there was no significant exposure even though the workers were employed in a plant where this chemical was manufactured. Actually the description of the study here and elsewhere is somewhat inaccurate when compared to the original paper. The word "negligible" is used by the authors to describe their estimation of the risks of ethylbenzene toxicity (a judgment on their part, rather than a comment on the data), not the concentration. Although not exactly a data-rich source, this paper in fact does report urinary mean post-shift mandelic acid concentrations in the relevant group of workers. The mean value in the period 1975 and later, when no adverse health effects were reported, was 0.2 mmol/l which, according to the authors' report of a chamber study establishing the relationship between urinary mandelic acid and ethylbenzene concentration, is equivalent to 6.4 mg/m^3 of ethylbenzene exposure. Exposure in the earlier period before improvement of the conditions in the works was higher but apparently not dramatically so, at least on average. This negative observation is thus of very low power to detect any effect, as the authors conclude. But it is possible to deduce slightly more real information by studying the paper, than is implied by the very offhand and dismissive account of the study given in this document.

Page 30 lines 20 to 22. See my immediately previous comment.

Toxicity - Quality of Animal Studies

- *Were adequately designed animal studies identified in the text (i.e., adequate number of animals, good animal care, accounting for competing causes of death, sufficient number of dose groups, and sufficient magnitude of dose levels)?*

  Yes. The database of animal toxicity data is much more extensive than that for human effects, and the study quality is for the most part adequate.

- *Were the animal species appropriate for the most significant toxicological endpoint of the study? If not, which animal species would be more appropriate and why?*

  The data are, not unexpectedly, dominated by experiments in rodents (rats and mice), but there are no reasons to think that these are inappropriate in this case.

- *Were the conclusions drawn by the authors of the studies appropriate and accurately reflected in the text? If not, did the text provide adequate justification for including the study (e.g., citing study limitations)?*

  The conclusions of the authors of the studies are appropriately represented.

- *Were all appropriate NOAELs and LOAELs identified for each study? Were all appropriate toxicological effects identified for the studies? If not, please explain.*

  Descriptions of the experimental data are mostly given accurately and with sufficient detail to address the requirements of this section. There is a tendency in a few places to stray into dismissive judgments rather than full description, such as the repeated assertion (Page 31 lines 4 to 6: see my previous comment on page 13) that the acute hepatic effects are purely "adaptive" and thus by implication, deserve less attention than those effects considered adverse. Similarly, the introductory summary for renal toxicity (page 32) is somewhat less than clear in describing the role, if any, of α2u globulin accumulation in the renal toxicity of ethylbenzene (see my line-by-line comments on this section below): perhaps these comments in any case belong better in the later discussion of mechanisms.

- *If appropriate, is there a discussion of the toxicities of the various forms of the substance? If not, please give examples of toxicological effects that might be important for forms of the substance.*

  Other than the distinction between inhalation exposures (the route used in most of the reported studies) and oral exposures to liquid or dissolved ethylbenzene (for which few relevant data are available, as noted previously), this is not an issue for this substance.

- *Were the appropriate statistical tests used in the interpretation of the studies? If not, which statistical tests would have been more appropriate? Were statistical test results of study data evaluated properly? NOTE: As a rule, statistical values are not reported in the text, but proper statistical analyses contribute to the reliability of the data.*

  The human studies of respiratory and neurological effects appear to have been analyzed appropriately by their authors and accurately reported in this document.

Other human studies and case reports are essentially not susceptible to any statistical analysis. The non-cancer animal toxicity data are generally well analyzed, and appropriately presented from this point of view. However, it is notable that no attempt has been made to perform any kind of dose-response analysis on the NTP carcinogenicity data. This is a major and inappropriate omission, since the data are readily susceptible to analysis following the U.S. EPA (2005) guidelines, and such an analysis can be informative even if the analyst has questions as to the relevancy of the effect for humans.

- *Are you aware of other studies that may be important in evaluating the toxicity of the substance? If you are citing a new reference, please provide a copy and indicate where (in the text) it should be included.*

  No major new toxicity studies.

Page 32 line 10 to 15. This sentence is accurate in its intended meaning but is actually a slight mis-statement – the adverse effects (tubular epithelial cell necrosis etc.) are seen in female rats, mice or humans under various circumstances, but not as a result of α2u globulin accumulation. It is the accumulation of that male-rat-specific protein which is the unique event. In fact (and relevant here) the so-called "hyaline droplets" may consist of other proteins: several chemicals, of which ethylbenzene appears to be one, apparently cause accumulation of various proteins which may include α2u globulin in male rats, but also various others which are not sex- or species-specific. Perhaps it would be better to say "Accumulation of in the renal tubular epithelial cells of male rats is associated with tubular epithelial cell necrosis, regenerative proliferation and renal tumors. This accumulation is not observed in female rats, mice or humans (which lack that protein), or in male rats which are genetically lacking α2u globulin. Adverse effects in male rats associated with the renal accumulation exclusively of α2u globulin are therefore not considered relevant to humans (EPA 1991g)."

Page 32 line 18. Better: "... enhance renal accumulation of hyaline droplets in rats of both sexes. In male rats only this accumulation includes α2u globulin, but evidently other proteins are involved in females, and probably also in the males." The data are deficient in their ability to resolve all these issues, but the general consensus at this time

appears to be that the α2u globulin phenomenon is not a major factor in renal toxicity of ethylbenzene.

Levels of Significant Exposure (LSE) Tables and Figures

- *Are the LSE tables and figures complete and self-explanatory? Does the "Users Guide" explain clearly how to use them? Are exposure levels (units, dose) accurately presented for the route of exposure? Please offer suggestions to improve the effectiveness of the LSE tables and figures and the "User's Guide."*

  The tables provided are comprehensive and very "information-dense", and their intended use is explained. However, I am not sure that very many of the intended users of the toxicological profiles actually read them. In my experience short summary tables laying out the actual experimental data, included as part of the study descriptions and linked to the explanatory text are much more accessible than these "mega-tables".

  The results on developmental toxicity (fetal weight reductions) of Saillenfait *et al.* (2007) are noted in the text, but I do not see them in Table 3-1. This isn't a big deal since they are the same as those reported by Saillenfait et al (2006), but they should probably be included for completeness.

- *Do you agree with the categorization of "less serious" or "serious" for the effects cited in the LSE tables?*

  In most cases, yes. But the characterization of the ototoxicity finding (Cappaert *et al.*, 2001; 2003) as "less serious" seems perverse, since this probably represents an irreversible and cumulative loss of some sensory function. That higher doses produce a more severe effect (Gagnaire et al., 2007) does not automatically mean that the initial effects are mild. Similarly, substantial impacts on fetal weight (Saillenfait et al, 2003) are not "less serious", and even minor anatomical terata such as the skeletal abnormalities observed by NIOSH (1981) and Saillenfait (2003, 2006, 2007) could be considered serious, particularly in the context of enhanced concern for children's health.

- *If MRLs have been derived, are the values justifiable? If no MRLs have been derived, do you agree that the data do not support such a derivation?*

  The MRLs derived are justifiable. As noted previously, it should be possible to undertake a route-to route extrapolation to estimate oral MRLs since this is a potentially significant route of exposure for the public and this calculation should be possible with reasonable *confidence* using available PBPK models. The failure to estimate a cancer potency is an unnecessary and undesirable omission. The "cancer effect level" quoted in the LSE tables is a meaningless and confusing concept which provides no information useful in protecting public health. I know this is something that ATSDR has used a lot in the past, but it is misleading in the concept of the current understanding of cancer dose-response characteristics (i.e., for most carcinogens, a non-threshold relationship), and should be abandoned.

Evaluation of Text

- *Have the major limitations of the studies been adequately and accurately discussed? How might discussions be changed to improve or more accurately reflect the proper interpretation of the studies?*

  Study descriptions and discussion of limitations are generally appropriate. This is generally a problem for human studies, and in this case many of the human studies are of so little value that extensive discussion is not warranted, although in one case the analyst was perhaps a little too dismissive of the study by Bardodej and Cirek (1988):

Page 41, Line 12. The original report of this study does in fact provide information which can be used to get a general idea of the exposure levels experienced by the workers. The important point is that the levels are sufficiently small that the study has low power to detect any effect, on cancer or other health endpoints. This should be noted in the study description, here as elsewhere (see my earlier comment relating to page 29). If, as recommended below, the analyst had used the NTP data to estimate a human cancer potency it would have been possible to use these data to determine whether the

observations by Bardodej and Cirek are in fact consistent with this estimate, although negative, based on the expected power of the study.

- *Has the effect, or key endpoint, been critically evaluated for its relevance in both humans and animals?*

  There are several endpoints of concern noted for this compound. Treatment of the non-cancer effects is generally appropriate, particularly for the otoxicity which is a key observation. Other more difficult analyses are deficient. The discussion of renal toxicity (pages 32-33) has some confusing parts as noted previously, and the question of its relevance to humans is not really addressed except to observe the generally accepted non-relevance to humans of the α2u globulin phenomenon (which is probably not an important component of the rodent renal toxicity of ethylbenzene).

  Treatment of the cancer endpoint is inadequate. The animal carcinogenicity findings of NTP are briefly described *(Page 41 line 15 et seq.)* but treated dismissively. Reliance has evidently been placed on the analysis by Hard (2002) who suggested that "chemically induced exacerbation of CPN [chronic progressive nephropathy] was the mode of action underlying the development of renal neoplasia" in the NTP ethylbenzene studies. Whether this would of itself argue that the rat renal tumors are not relevant to humans is itself a matter which could be debated, but more importantly the analyst has failed to take note of more recent rebuttals of that argument. In a retrospective evaluation of NTP chronic studies, Seely *et al.* (2002) found that renal tubule cell neoplasms (RTCNs) "tend to occur in animals with a slightly higher severity of CPN than animals without RTCNs. However, the differential is minimal and clearly there are many male F344 rats with severe CPN without RTCNs." Seely *et al.* (2002) go on to say that "the data from these retrospective reviews suggest that an increased severity of CPN may contribute to the overall tumor response. However, any contribution appears to be marginal, and additional factors are likely involved." The important judgment of IARC (2006) in assigning a Group2B classification (possibly carcinogenic to humans) is not even mentioned in this section, and is only

mentioned in passing and then ignored in other sections of the document. No attempt is made to consider possible relevance to humans of the findings at other sites (rat testis, alveolar/bronchiolar tumors in male mice, hepatocellular tumors in female mice). The analyst appears (page 42, lines 6 and 9) to rely on the observation that the tumor incidences at the latter two sites were within the NTP historical control range. The usefulness of this criterion in establishing significance has been debated, but the most usual conclusion is that the local and concurrent controls are much more important, and the historical range is mostly of interest in identifying experiments where for one reason or another the concurrent controls are outside the historical range – an indication of study problems which does not apply here. NTP's own analysts did not ignore these findings, considering that they in themselves showed "some" evidence of carcinogenicity, and the importance of these data is emphasized by the "clear" finings of renal tumors with ethylbenzene. Consideration of carcinogenicity data and its relevance to humans generally involves consideration of supporting data such as metabolic pathways and genotoxicity, which I do not see in the treatment of the carcinogenicity data in this section (3.2.1.7).

- *Have "bottom-line" statements been made regarding the relevance of the endpoint for human health?*

  These statements do appear appropriately in some cases, but it is a general weakness of this section that such clear statements are often missing or at least hard to find. This may be the result of natural reticence on the part of the analyst to make clear statements on controversial or unresolved issues, but it would be more helpful to highlight this uncertainty rather than to ignore or hide it.

- *Are the conclusions appropriate given the overall database? If not, please discuss your own conclusions based on the data provided and other data provided to you but not presented in the text.*

  The conclusions drawn with regard to non-cancer toxicity are in general reasonable, although I have some concern that some secondary endpoints such as acute liver enlargement and developmental toxicity findings have been ignored or

downplayed in favor of analyzing the "preferred" endpoints. While the analysis of those preferred endpoints is sound, the consideration of the additional effects could be strengthened to provide support and context for the key MRL recommendations.

The cancer analysis is frankly inadequate. My personal view of the overall database on ethylbenzene carcinogenicity follows IARC (2006), who concluded that there was sufficient evidence to justify their Group 2B rating. An IARC 2B evaluation, or its equivalent from U.S. EPA, is generally regarded as a sufficient stimulus to provoke development of a cancer potency ("slope factor"), unit risk of similar quantitative risk measures to support regulatory and precautionary action. That data are sufficient to support such a calculation is shown in an analysis of ethylbenzene carcinogenicity recently presented as public review draft by the State of California's Environmental Protection Agency (OEHHA, 2007)

- *Has adequate attention been paid to dose-response relationships for both human and animal data? Please explain.*

For non-cancer endpoints, yes. For cancer, no – see my immediately previous comment.

- *Has the animal data been used to draw support for any known human effects? If so, critique the validity of the support.*

The report does not really attempt this for any of the endpoints of concern, mainly because of the limited nature of the human data in most cases. Presentation of the data available of itself invites the reader to draw instructive parallels between human and animal responses of the respiratory system, and also in the case of the ototoxicity where the detailed animal data tend to support the conclusion that the human effects seen were in fact related to ethylbenzene exposure, in spite of the inability of the human studies to establish this unequivocally by themselves.

Section 3.3 GENOTOXICITY

The description of findings in this category is mostly complete, but there are two data sets which are not described which may be worth including.

Sram *et al.* (2004) described the effects of benzene and ethylbenzene exposure on chromosomal damage in peripheral blood lymphocytes of exposed workers. Exposure to ethylbenzene resulted in a significant increase in chromosomal aberrations. Reduced ethylbenzene exposures due to improved workplace emissions controls resulted in a reduction in chromosomal damage in exposed workers. However, these workers were also exposed to benzene, so it cannot be proved that the chromosomal damage was due to ethylbenzene; this information may nevertheless be of supportive value if compared with the other reported findings..

A second report,which examined genotoxicity of certain ethylbenzene metabolites, may also be considered useful. Midorikawa *et al* (2004) reported induction of oxidative DNA damage by two metabolites of ethylbenzene, namely ethylhydroquinone and 4-ethylcatechol. (These compounds were shown to be formed from ethylbenzene by rat liver microsomes *in vitro*.) These dihydroxylated metabolites induced DNA damage in ^{32}P-labeled DNA fragments from the human p53 tumor suppressor gene and induced the formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine in calf thymus DNA in the presence of Cu(II). Addition of exogenous NADH enhanced 4-ethylcatechol-induced oxidative DNA damage, but had little effect on ethylhydroquinone action. The authors suggest that Cu(I) and H_2O_2 produced via oxidation of these compounds were involved in oxidative DNA damage. NADH enhancement was attributed to reactive species generated from the redox cycle of EC → 4-ethyl-1, 2-benzoquinone → EC. Similar effects of NADH were observed with benzene metabolites, including catechol (Hirakawa *et al.* 2002).

The overall conclusion of this section, that while most of the "classical" genotoxicity data are negative there appears to be some potential for genotoxic effects in humans, is accurate. It may be of interest to note specifically the analogy with effects of benzene identified by the work of Midorikawa *et al.* (2004)

Section 3.4 TOXICOKINETICS

- *Is there adequate discussion of absorption, distribution, metabolism, and excretion of the substance? If not, suggest ways to improve the text.*

  The description of the available data on absorption, distribution, metabolism *in vivo* and excretion appears to be sufficiently complete for the purpose.
  References to metabolism *in vitro* are confined to a single comment (page 53 lines 31-33) which cites references from 1970 and 1972. While the purpose of this section may be to concentrate on the data *in vivo*, the data from expriments *in vitro* in fact is important in informing both mechanistic analysis of the observed metabolism *in vivo* and the parameterization of PBPK models (see below). This topic deserves more comprehensive coverage. A key reference is Sams *et al.* (2004).

- *Have the major organs, tissues, etc. in which the substance is stored been identified? If not, suggest ways to improve the text.*

  This does not appear to be a major issue for this compound, since it is volatile and relatively rapidly metabolized.

- *Have all applicable metabolic parameters been presented? Have all available pharmacokinetic/pharmacodynamic models and supporting data been presented? If not, please explain.*

  The narrative on PBPK models describes only two models: a systemic model for inhaled gasoline components (including ethylbenzene) reported by Dennison et al. (2004) and a dermal absorption model (Shatkin and Brown , 1991).
  The description of the Dennison model is brief and relatively uninformative: it is pointed out that this model is primarily aimed at dealing with mixed gasoline vapors rather than ethylbenzene. This is certainly a limitation in consideration of risk assessment for pure ethylbenzene, although it may in fact be relevant to some of the exposure scenarios identified elsewhere in this document. This simplified description of PBPK analysis for inhaled ethylbenzene neglects significant published literature on the subject, which should be reviewed and integrated into

the overall narrative on this topic. Key references that should be dealt with in this section include Tardiff *et al.* (1987) and Haddad *et al.* (2001). (Oddly, the paper by Tardiff *et al.* [1987] appears among the references on the CD-ROM, but appears to have been ignored in the text at least in this section.) Treatment of PBPK modeling for ethylbenzene inhalation also appears in an analysis of ethylbenzene carcinogenicity recently presented as public review draft by the State of California's Environmental Protection Agency (OEHHA, 2007)

Description of the Shatkin and Brown (1991) model is more comprehensive and is sufficient for the more limited implications of this model.

- *Is there adequate discussion of the differences in toxicokinetics between humans and animals? What other observations should be made?*

  This topic is not really addressed in the description of the individual studies, other than simply reporting comparable data. The very limited discussion of systemic PBPK models also does not cover this topic at all: this should be addressed as part of a more comprehensive coverage of toxicokinetic modeling. (This topic is addressed in the description of the Shatkin and Brown (1991) dermal absorption model, which used human data for validation.)
  (A general discussion of similarities and differences between animal and human metabolism appears later (Section 3.5.3, page 54) in the mechanism of action section, but this does not address the toxicokinetics to any substantial degree)

- *Is there an adequate discussion of the relevance of animal toxicokinetic information for humans? If not, please explain.*

  No. See my immediately previous comment: this applies here also.

- *If applicable, is there a discussion of the toxicokinetics of different forms of the substance (e.g., inorganic vs. organic mercury)?*

  Not applicable in this case.

Section 3.5 MECHANISMS OF ACTION

Section 3.5.1 (page 62) provides a brief comment on pharmacokinetic mechanisms of action, with the observation that partitioning of somewhat hydrophobic compounds such as ethylbenzene into cell membranes may affect the activity of membrane-bound enzymes. While this is true (and evidently measurable, at least *in vitro*), it is not really clear what this adds to the discussion, or whether it is intended to support the statement at the end of this section (page 62, lines 29-30) that there is no evidence for differences in pharmacokinetic mechanisms between children and adults. This last statement would probably not be supported by more careful examination of PBPK models for ethylbenzene, since although obviously physical processes such as diffusion and partitioning into lipid media would not vary the enzymes responsible for metabolism demonstrably vary, especially in infants, as compared to adults.

Section 3.5.2 (pages 62-63) provides an extremely brief statement of possible mechanisms for CNS toxicity. This appears to relate to the popular hypothesis that alterations in membrane fluidity are responsible for CNS impacts such as narcosis. It is worth noting that although such effects have been regularly observed *in vitro*, more recent work in this area has suggested that actually many effects *in vivo* of anesthetics (and presumably therefore solvents) are mediated by direct interactions with neurotransmitter receptors (there is extensive recent literature on this topic).

Section 3.5.3 (page 64) offers a brief comparison of metabolism in animals and humans. This is accurate as far as it goes, although more recent studies and toxicokinetic issues are not addressed. The observation that overall toxic responses are similar between animals, especially rats, and humans is appropriately, although briefly, expressed.

It is disappointing that this section makes no attempt to address possible mechanisms for the most critical toxic effects, namely the ototoxicity, hepatic and renal damage, and carcinogenesis. While the available literature may not offer

complete answers in this case there are certainly some areas which could be explored, particularly with regard to the observation of reactive and DNA-damaging metabolites (noted in my comments on genotoxicity).

Section 3.6 TOXICITIES MEDIATED THROUGH THE NEUROENDOCRINE AXIS

No data were identified by the analyst suggesting that such effects are important: I am not aware of any myself.

Section 3.7 CHILDREN'S SUSCEPTIBILITY

Data on special sensitivities of infants and children to ethylbenzene toxicity are very limited. This section appropriately notes the developmental toxicity findings described elsewhere: although as noted (page 67, lines 22-23) it is not proven that such effects would be observed in humans it should be stated here that in the interest of protecting public health it is assumed that such effects are relevant.

Possible differences in metabolism between infants, children and adults are appropriately noted, although since the mechanistic significance of metabolism for the major toxic responses is not discussed here or elsewhere in the document these observations are not linked to any particular conclusion as to health impacts.

Since the cancer findings are downplayed and no quantitative analysis is attempted it is not surprising that there is no attempt to address possible increased susceptibility to early-in-life exposures to carcinogens. Nevertheless, this issue should be addressed here in line with U.S. EPA's recent (2005) supplemental guidance on the topic.

Section 3.8 BIOMARKERS OF EXPOSURE AND EFFECT

- *Are the biomarkers of exposure specific for the substance or are they for a class of substances? If they are not specific, how would you change the text?*

The biomarkers of exposure identified (mandelic and phenylglyoxylic acids in urine) are characteristic of exposure to ethylbenzene but also appear following styrene exposure. Unchanged ethylbenzene has been measured in blood, tissues

and expired air and detected in milk. This section describes the issue appropriately.

- *Are there valid tests to measure the biomarker of exposure? Is this consistent with statements made in other sections of the text? If not, please indicate where inconsistencies exist.*

  These biomarkers are in wide use in occupational hygiene studies, and methods have been validated.

- *Are the biomarkers of effect specific for the substance or are they for a class of substances? If they are not specific, how would you change the text?*

  Specific biomarkers for ethylbenzene effects were not identified.

- *Are there valid tests to measure the biomarker of effect? Is this consistent with statements made in other sections of the text? If not, please indicate where inconsistencies exist.*

  Not applicable in this case.

Section 3.9 INTERACTIONS WITH OTHER CHEMICALS

- *Is there adequate discussion of the interactive effects with other substances? Does the discussion concentrate on those effects that might occur at hazardous waste sites? If not, please clarify and add additional references.*

  This section succinctly summarizes various interactions described in the literature. It would be better if it were reorganized to separate the narrative more clearly into interactions observed *in vitro* from those observed *in vivo*, and to highlight those (such as with xylenes or mixed fuels) which would reasonably be expected to occur at hazardous waste sites. These, including both metabolic and skin absorption effects, are much more likely to be important than, for instance, the impact of carbon monoxide. This effect was reported *in vitro* at a CO to oxygen ratio of 2 to 1: any such exposure to CO *in vivo* would be more or less instantly lethal, regardless of any interaction with ethylbenzene! Like the CO effect, interactions with pharmaceutical agents such as phenobarbital and SKF525A are of interest in that they are characteristic of cytochrome P-450 catalyzed reactions,

but are of vanishingly small significance for real-world exposures to toxics. Interaction with ethanol, of course, is an issue worth noting.

- *If interactive effects with other substances are known, does the text discuss the mechanisms of these interactions? If not, please clarify and provide any appropriate references.*

  The text is limited, but adequate in this regard.

Section 3.10 POPULATIONS THAT ARE UNUSUALLY SUSCEPTIBLE

- *Is there a discussion of populations at higher risk because of biological differences which make them more susceptible? Do you agree with the choices of populations? Why or why not? Are you aware of additional studies in this area?*

  A brief but appropriate discussion of generic issues is presented. There do not appear to be any data specific to ethylbenzene toxicity and susceptible populations. Since hearing loss is often observed to be cumulative, those with hearing loss from other causes (rock musicians, sharpshooters? Possibly those with congenital or infection-related hearing loss?) might be considered a susceptible population for the acute ototoxicity.

Section 3.11 METHODS FOR REDUCING TOXIC EFFECTS

- *Is the management and treatment specific for the substance, or is it general for a class of substances?*

  The introductory section cites two standard publications on medical toxicology with sections specific to ethylbenzene.

Subsection 3.11.1 reducing peak absorption

- *Are treatments available to prevent the specific substance from reaching the target organ(s), or are the actions general for a class of substances?*
- *Is there any controversy associated with the treatment?Is it a "well-accepted" treatment? If the discussion concerns an experimental method, do you agree with the conceptual approach of the method?*

  The treatments proposed appear to be generic and widely accepted.

- *Are there any hazards associated with the treatment of populations that are unusually susceptible to the substance (e.g., infants, children)?*

None identified.

Subsection 3.11.2 reducing body burden

- *Are there treatments to prevent adverse effects as the substance is being eliminated from the major organs/tissues where it has been stored (e.g., as a substance is eliminated from adipose tissue, can we prevent adverse effects from occurring in the target organ[s])?*

This does not appear to be a significant issue for ethylbenzene, due to rapid exhalation and metabolism.

Subsection 3.11.3 interfering with toxic effects.

- *Are treatments available to prevent the specific substance from reaching the target organ(s), or are the treatment's actions general for a class of substances?*
- *Is there any controversy associated with the treatment? Is it a "well-accepted" treatment? If the discussion concerns an experimental method, do you agree with the conceptual approach of the method?*
- *Are there any hazards associated with the treatment of populations that are unusually susceptible to the substance (e.g., infants, children)?*

There isn't anything useful to say in this section. The suggestion to use agents that would "stabilize the call membrane" to reduce toxicity is highly speculative and probably would be dangerous if it were possible to identify such agents which were effective *in vivo*. Similarly suggestions to limit changes in neurotransmitter levels are highly speculative, and it is not clear that they provide any useful guidance.

Section 3.12 ADEQUACY OF THE DATABASE

Subsection 3.12.1 Existing Information on Health Effects of Ethylbenzene

- *Do you know of other studies that may fill a data gap? If so, please provide the reference.*

See my previous comments on the sections on metabolism and PBPK modeling, and on genotoxicity.

Subsection 3.12.2 Identification of Data Needs

- *Are the data needs presented in a neutral, non-judgmental fashion? Please note where the text shows bias.*
- *Do you agree with the identified data needs? If not, please explain your response and support your conclusions with appropriate references.*
- *Does the text indicate whether any information on the data need exists?*
- *Does the text adequately justify why further development of the data need would be desirable; or, conversely, justify the "inappropriateness" of developing the data need at present? If not, how can this justification be improved.*

This section deals adequately with the issues raised. I do not have specific comments on this section.

CHAPTER 4. CHEMICAL AND PHYSICAL INFORMATION

- *Are you aware of any information or values that are wrong or missing in the chemical and physical properties tables? Please provide appropriate references for your additions or changes.*

This section deals adequately with the topic. I do not have specific comments on this section.

- *Is information provided on the various forms of the substance? If not, please explain.*

Not applicable in this case.

CHAPTER 5. PRODUCTION, IMPORT/EXPORT, USE, AND DISPOSAL

- *Are you aware of any information that is wrong or missing?*

No.

CHAPTER 6. POTENTIAL FOR HUMAN EXPOSURE

- *Has the text appropriately traced the substance from its point of release to the environment until it reaches the receptor population? Does the text provide sufficient and technically sound information regarding the extent of occurrence at NPL sites? Do you know of other relevant information? Please provide references for added information.*
- *Does the text cover pertinent information relative to transport, partitioning, transformation, and degradation of the substance in all media? Do you know of other relevant information? Please provide references for added information.*
- *Does the text provide information on levels monitored or estimated in the environment, including background levels? Are proper units used for each medium? Does the information include the form of the substance measured? Is there an adequate discussion of the quality of the information? Do you know of other relevant information? Please provide references for added information.*
- *Does the text describe sources and pathways of exposure for the general population and occupations involved in the handling of the substance, as well as populations with potentially high exposures? Do you agree with the selection of these populations? If not, why? Which additional populations should be included in this section?*

This chapter covers the issues raised adequately as far as I can tell. Specific studies and data presented include consideration of exposure to children. They constitute a potentially more heavily exposed sub-population, although available data do not particularly demonstrate this.

There is one issue which does not seem to be addressed here or elsewhere which is that ethylbenzene, like several other aromatics but most notably benzene itself, could be formed as a combustion by-product. This may contribute to the levels in vehicle exhaust noted in Section 6.2.1, page 89, and is also the reason for its appearance as a component of tobacco smoke, as noted in several places in the document. This is of some significance in discussing ethylbenzene as an air pollutant, since it implies an additional source besides evaporation of fuels containing it. But this probably isn't important in the context of hazardous waste site evaluation.

CHAPTER 7. ANALYTICAL METHODS

This chapter covers the issues raised adequately as far as I can tell (although this isn't my particular area of expertise).

CHAPTER 8. REGULATIONS AND ADVISORIES

- *Are you aware of other regulations or guidelines that may be appropriate for the table? If so, please provide a copy of the reference.*

The State of California has some guidelines which may be of interest:

Chronic Reference Exposure level (Air, community exposure: OEHHA, 2005):
400 ppb.
Public Health Goal (Drinking water: OEHHA, 1997):
300 ppb
Cancer risk assessment (DRAFT: public comment version: OEHHA, 2007):
Unit Risk: 2.5 x 10-6 $(\mu g/m^3)^{-1}$
Inhalation Cancer Potency: 0.0087 $(mg/kg\text{-}day)^{-1}$
Oral Cancer Potency: 0.011 $(mg/kg\text{-}day)^{-1}$

Other comments:
In Table 8.1, i t seems unnecessarily confusing to list only the AEGLs and HAP classification under the heading "National regulations and guidelines/a. Air/EPA", but to later all the IRIS entries, including the RfC, which is obviously an air standard, under "d. Other". It is similarly confusing to find the RfD under "d. Other" but not to find at least a mention of it under either food or water.

As noted previously, it is disingenuous to provide only the reference "IRIS 2007" for the very old and outdated US EPA carcinogenicity classification which in fact predates most of the important new data and evaluations.

The statement that the NTP has "no data" is accurate as far as the Report on Carcinogens goes, but is misleading in that it fails to mention that the most recent bioassay Technical Report (NTP, 1999) listed a finding of clear evidence at one site and suggestive evidence at others (as noted elsewhere in this document)..

CHAPTER 9. REFERENCES

- *Are there additional references that provide new data or are there better studies than those already in the text? If so, please provide a copy of each additional reference.*

  Here are some additional references cited in my comments which I did not find in the refernce list or on the CD-ROM (I will endeavor to provide copies of these papers):

Haddad S, Beliveau M, Tardif R and Krishnan K, 2001, A PBPK modeling-based approach to account for interactions in the health risk assessment of chemical mixtures. Toxicol Sci 63:125-131.

Hirakawa K, Oikawa S, Hiraku Y, Hirosawa I, Kawanishi S, 2002. Catechol and hydroquinone have different redox properties responsible for their differential DNA-damaging ability. Chem Res Toxicol 15:76-82.

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OEHHA (1997). Public Health Goal for Ethylbenzene in Drinking Water. State of California, Office of Environmental Health Hazard Assessment, Sacramento, CA. http://www.oehha.ca.gov/water/phg/pdf/etbx2_c.pdf

OEHHA (2005). Chronic Toxicity Summary for Ethylbenzene. http://www.oehha.ca.gov/air/chronic_rels/pdf/100414.pdf

OEHHA (2007). Proposal for the adoption of a unit risk factor for ethylbenzene. State of California, Office of Environmental Health Hazard Assessment, Sacramento, CA. http://www.oehha.ca.gov/air/toxic_contaminants/pdf_zip/Ethylbenzene_2007_Public%20Review%20Draft.pdf

Sams C, Loizou GD, Cocker J and Lennard MS, 2004. Metabolism of ethylbenzene by human liver microsomes and recombinant human cytochrome P450s (CYP). Toxicol Lett 147:253-260.

Seely JC, Haseman JK, Nyska A, Wolf DC, Everitt JI and Hailey JR, 2002. The effect of chronic progressive nephropathy on the incidence of renal tubule cell neoplasms in control male F344 rats. Toxicol Pathol 30(6):681-686.

Sram RJ, Beskid O, Binkova B, Rossner P and Smerhovsky Z, 2004. Cytogenetic analysis using fluorescence in situ hybridization (FISH) to evaluate occupational exposure to carcinogens. Toxicol Lett 149:335-344.

US. EPA (2005). Guidelines for Carcinogen Risk Assessment (EPA/630/P-03/001B), and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens (EPA/630/R-03/003F). www.epa.gov/cancerguidelines.

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SECTION II

ADDITIONAL REFERENCES AND DATA SUBMITTED BY THE PEER REVIEWERS

ADDITIONAL REFERENCES AND DATA SUBMITTED BY

Andrew Salmon, Ph.D.
Senior Toxicologist and Chief, Air Toxicology and Risk Assessment Unit
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency
Oakland, CA 94612
510-622-3191
Email: asalmon@oehha.ca.gov

Long-term Health Effects of Exposure to Ethylbenzene

Background and Status of Ethylbenzene as a Toxic Air Contaminant and its Potential Carcinogenicity

Ethylbenzene (CAS Registry Number: 100-41-4) is a natural constituent of crude petroleum and is found in gasoline and diesel fuels (HSDB, 2003). It is used as a chemical intermediate, primarily in the production of styrene (ATSDR, 1999). Ethylbenzene is included on a list of "inert" or "other ingredients" found in registered pesticide products (U.S. EPA, 2004a).

Ethylbenzene enters the atmosphere both from emissions from industrial facilities and other localized sources, and from mobile sources. Vehicle exhaust contains ethylbenzene due to its presence in fuel and possibly due to formation during the combustion process. Ethylbenzene is a component of environmental tobacco smoke (CARB, 1997) and a number of consumer products (ATSDR, 1999), resulting in its presence as a contaminant of indoor air.

The statewide annual emissions of ethylbenzene in California were estimated to be 116 tons (232,000 lb) from stationary point sources and 9,892 tons (19.7 million lb) from area sources, including on and off-road mobile sources (CARB, 2004). U.S. EPA's Toxics Release Inventory reported 7,463,252 pounds total on- and off-site releases of ethylbenzene for the year 2002 in the U.S., of which 6,441,052 pounds were fugitive or point source air emissions (U.S. EPA, 2004b).

The average statewide ambient air concentration of ethylbenzene in 2003 was 0.22 ppb (0.96 $\mu g/m^3$) with a range of 0.1 to 2.0 ppb (503 observations, CARB, 2005).

The primary route of atmospheric transformation for ethylbenzene is reaction with the OH radical. For a 24-hr average OH radical concentration of 1.0×10^6 molecule cm^{-3}, the calculated lifetime of ethylbenzene is 1.7 days (Arey and Atkinson, 2003). Observed products of ethylbenzene reaction with the OH radical include acetophenone and benzaldehyde (Hoshino *et al.*, 1978).

Ethylbenzene is identified under the section 112(b)(1) of the U.S. Clean Air Act amendment of 1990 as a Hazardous Air Pollutant (HAP). This followed the U.S. EPA's determination that ethylbenzene is known to have, or may have, adverse effects on human health or the environment. On April 8, 1993, the California Air Resources Board (ARB) identified, by regulation, all 189 of the then listed HAPs as Toxic Air Contaminants (TACs). This was in response to the requirement of Health and Safety Code Section 39657(b).

Non-cancer health effects of ethylbenzene have been recognized for some time, and these were the basis for a Chronic Inhalation Reference Exposure Level (cREL) developed by OEHHA (2000) for use in the Air Toxics Hot Spots (AB2588) program. The cREL

adopted was 2000 $\mu g/m^3$ (400 ppb), based on effects in the alimentary system (liver), kidney and endocrine system.

Summary of Carcinogenic Health Effects of Ethylbenzene

Maltoni *et al.* (originally reported in 1985; additional information published in 1997) studied the carcinogenicity of ethylbenzene in male and female Sprague-Dawley rats exposed via gavage. The authors reported increases in the percentage of animals with malignant tumors and with tumors of the nasal and oral cavities associated with exposure to ethylbenzene. Reports of these studies lacked detailed information on the incidence of specific tumors, statistical analysis, survival data, and information on historical controls. Results of the Maltoni *et al.* studies were considered inconclusive by IARC (2000) and NTP (1999).

Because of the potential for significant human exposure to ethylbenzene, NTP (1999) carried out inhalation studies in $B6C3F_1$ mice and F344/N rats. NTP found clear evidence of carcinogenic activity in male rats and some evidence in female rats, based on increased incidences of renal tubule adenoma or carcinoma in male rats and renal tubule adenoma in females. NTP (1999) also noted increases in the incidence of testicular adenoma in male rats. Increased incidences of lung alveolar/bronchiolar adenoma or carcinoma were observed in male mice and liver hepatocellular adenoma or carcinoma in female mice, which provided some evidence of carcinogenic activity in male and female mice (NTP, 1999).

IARC (2000) classified ethylbenzene as Group 2B, possibly carcinogenic to humans, based on the NTP studies. The State of California's Proposition 65 program listed ethylbenzene as a substance known to the state to cause cancer on June 11, 2004. In view of the NTP data and the identification of ethylbenzene as known to the state to cause cancer, it is appropriate to provide a cancer risk estimate for ethylbenzene for use in the Toxic Air Contaminants program. The following summary (to be included as an addendum to the *Air Toxics Hot Spots Program Risk Assessment Guidelines: Part II, Technical Support Document for describing available Cancer Potency Factors)* provides an analysis of the carcinogenicity data for ethylbenzene, and derives a cancer potency factor $(mg/kg\text{-}d)^{-1}$ and unit risk factor $(\mu g/m^3)^{-1}$ for use in risk assessments of environmental exposures to ethylbenzene.

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Arey J and Atkinson R. 2003. Photochemical reactions of PAHs in the atmosphere. In: PAHs: An Ecotoxicological Perspective, Ed. P.E.T. Douben, John Wiley & Sons Ltd., pp. 47-63.

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National Toxicology Program (NTP). 1999. Toxicology and Carcinogenesis Studies of Ethylbenzene (CAS No. 100-41-4) in F344/N Rats and in B6C3F$_1$ Mice (Inhalation Studies). Technical Report Series No. 466. NIH Publication No. 99-3956. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health. NTP, Research Triangle Park, NC.

U.S. Environmental Protection Agency (U.S. EPA). 2004a. Complete List of all "Inert" or "Other Ingredients" Found in Pesticide Products Registered by EPA. Available at: http://www.epa.gov/opprd001/inerts/completelist_inerts.pdf. Accessed 7/19/04; last revision date not available.

ETHYLBENZENE

CAS No: 100-41-4

I. PHYSICAL AND CHEMICAL PROPERTIES
(From HSDB, 2003)

Molecular weight	106.2
Boiling point	136.2°C
Melting point	-94.9°C
Vapor pressure	9.6 mm Hg @ 25°C
Air concentration conversion	1 ppm = 4.35 mg/m^3 @ 25°C

II. HEALTH ASSESSMENT VALUES

Unit Risk:	2.5 x 10-6 (µg/m3)-1
Inhalation Cancer Potency:	0.0087 (mg/kg-day)-1
Oral Cancer Potency:	0.011 (mg/kg-day)-1

The unit risk and cancer potency values for ethylbenzene were derived from the National Toxicology Program (NTP, 1999) male rat renal tumor data, using the linearized multistage (LMS) methodology with lifetime weighted average (LTWA) doses. Methods are described in detail below. The use of a physiologically-based pharmacokinetic (PBPK) model to derive internal doses for the rodent bioassays was explored. Unit risk and cancer potency values based on the PBPK internal doses were not markedly different than those based on the LTWA doses, and involved a number of assumptions. Because the PBPK modeling is uncertain and the results were relatively insensitive to the approach used, the LMS results based on the LTWA doses were selected as most appropriate.

III. METABOLISM and CARCINOGENIC EFFECTS

Metabolism

Ethylbenzene is rapidly and efficiently absorbed in humans via the inhalation route (ATSDR, 1999). Human volunteers exposed for 8 hours to 23-85 ppm retained 64% of inspired ethylbenzene vapor (Bardodej and Bardodejova, 1970). Gromiec and Piotrowski (1984) observed a lower mean uptake value of 49% with similar ethylbenzene exposures. There are no quantitative oral absorption data for ethylbenzene or benzene in humans but studies with [^{14}C]-benzene in rats and mice indicate gastrointestinal absorption in these species was greater than 97% over a wide range of doses (Sabourin *et al.*, 1987).

Most of the metabolism of ethylbenzene is governed by the oxidation of the side chain (Fishbein, 1985). Engstrom (1984) studied the disposition of ethylbenzene in rats exposed to 300 or 600 ppm (1305 or 2610 mg/m^3) for six hours. Engstrom assumed 60 percent absorption of inhaled ethylbenzene and calculated that 83% of the 300 ppm dose

was excreted in the urine within four hours of exposure. At the higher exposure of 600 ppm only 59 percent of the dose was recovered in the urine within 48 hr of exposure. Fourteen putative ethylbenzene metabolites were identified in the urine of exposed rats. The principal metabolites were 1-phenylethanol, mandelic acid, and benzoic acid. Metabolism proceeded mainly through oxidation of the ethyl moiety with ring oxidation appearing to play a minor role. Other metabolites included acetophenone, ω-hydroxyacetophenone, phenylglyoxal, and 1-phenyl-1, 2-ethandiol. Ring oxidation products include p-hydroxy- and m-hydroxyacetophenone, 2-ethyl- and 4-ethylphenol. With the exception of 4-hydroxyacetophenone all these other metabolites were seen only in trace amounts.

The metabolism of ethylbenzene was studied in humans (number unstated) exposed at 23 to 85 ppm (100 to 370 mg/m^3) in inhalation chambers for eight hours (Bardodej and Bardodejova, 1970). About 64 percent of the vapor was retained in the respiratory tract and only traces of ethylbenzene were found in expired air after termination of exposure. In 18 experiments with ethylbenzene, the principal metabolites observed in the urine were: mandelic acid, 64%; phenylglyoxylic acid, 25%; and 1-phenylethanol, 5%.

Engstrom *et al.* (1984) exposed four human male volunteers to 150 ppm ethylbenzene (653 mg/m^3) for four hours. Urine samples were obtained at two-hr intervals during exposure and periodically during the next day. Metabolites identified in the 24-hr urine included: mandelic acid, 71.5 ± 1.5%; phenylglyoxylic acid, 19.1 ± 2.0%; 1-phenylethanol, 4.0 ± 0.5%; 1-phenyl-1, 2-ethanediol, 0.53 ± 0.09%; acetophenone, 0.14 ± 0.04%; ω- hydroxyacetophenone, 0.15 ± 0.05%; m-hydroxyacetophenone, 1.6 ± 0.3%; and 4-ethylphenol, 0.28 ± 0.06%. A number of the hydroxy and keto metabolites were subject to conjugation. Differences were observed between the concentrations obtained with enzymatic and acid hydrolysis. For example, 50% of maximal yield of 4-ethylphenol was obtained with glucuronidase or acid hydrolysis and 100% with sulfatase indicating the presence of glucuronide and sulfate conjugates of this metabolite. Alternatively, acetophenone gave only 30-36% yield with enzymatic treatment but 100% with acid hydrolysis indicating the presence of other conjugates not susceptible to glucuronidase or sulfatase. The metabolic scheme proposed by Engstrom *et al.* (1984) is shown in Figure 1.

Gromiec and Piotrowski (1984) measured ethylbenzene uptake and excretion in six human volunteers exposed at concentrations of 18 to 200 mg/m^3 for eight hours. Average retention of ethylbenzene in the lungs was 49 ± 5% and total excreted mandelic acid accounted for 55 ± 2% of retained ethylbenzene.

Tardif *et al.* (1997) studied physiologically-based pharmacokinetic (PBPK) modeling of ternary mixtures of alkyl benzenes including ethylbenzene in rats and humans. As part of this investigation they determined Vmax and Km kinetic parameters for the rat by best fit of model simulations to the time-course data on the venous blood concentrations of ethylbenzene following single exposures. The maximal velocity (Vmax) was 7.3 mg/hr-kg body weight and the Michaelis-Menten affinity constant (Km) was 1.39 mg/L. For the

human PBPK model the Vmax value from the rat was scaled on the basis of (body weight)$^{0.75}$. All other chemical and metabolic parameters were unchanged.

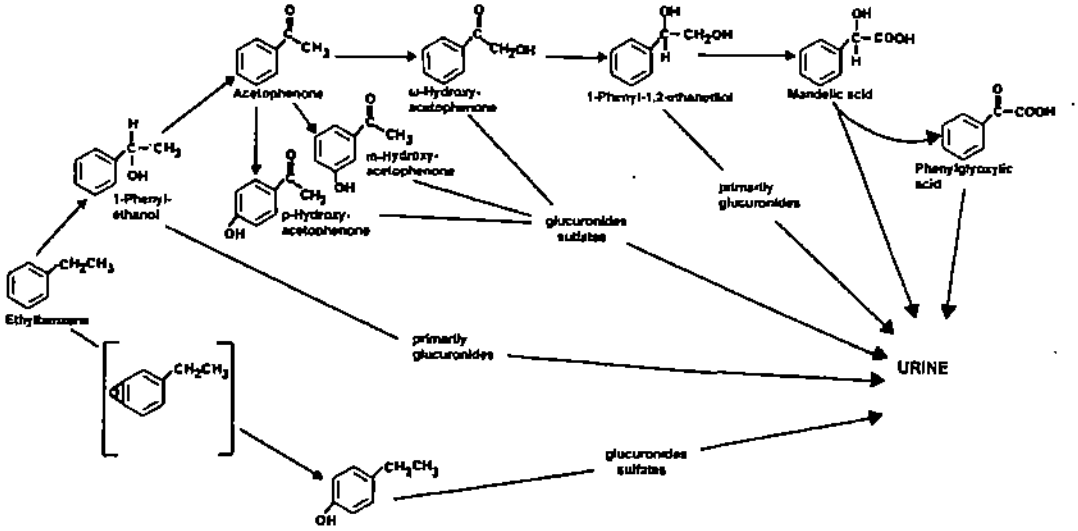

Figure 1. Human Ethylbenzene Metabolism (adapted from Engstrom et al., 1984).

The scaling of rodent metabolism of alkylbenzenes to humans was evaluated using kinetic data in an exposure study with human volunteers. Four adult male subjects (age, 22-47; body weight, 79-90 kg) were exposed to 33 ppm ethylbenzene for 7 hr/d in an exposure chamber. Urine samples were collected during (0-3 hr) and at the end (3-7 hr) of exposure and following exposure (7-24 hr). For the 0-24 hr collections mandelic acid amounted to 927 ± 281 μmol and phenylglyoxylic acid 472 ± 169 μmol. Venous blood (5.5 to 8 hr) and expired air (0.5 to 8 hr) were also measured in the subjects and exhibited good correspondence with PBPK model predictions. It is interesting that the metabolism of ethylbenzene in these human subjects was not significantly affected by simultaneous exposure to the other alkyl benzenes (toluene and xylene) studied. The metabolic parameters for ethylbenzene used by Haddad *et al.* (2001) and in the internal dosimetry modeling presented below were based on this study.

The oxidation of ethylbenzene to 1-phenylethanol by human liver microsomes and recombinant human cytochrome P450s was investigated by Sams *et al.* (2004). Human liver microsomes from seven subjects (four male, three female, age 37-74) and microsomes expressing recombinant human CYP1A2, 2A6, 2B6, 2C9*1(Arg144), 2C19, 2D6, 2E1, and 3A4 co-expressed with cytochrome P450 reductase/cytochrome b5 were both obtained from commercial sources. Kinetic experiments were conducted with microsomes and ethylbenzene over a 10-5000 μ*M* substrate concentration range. For chemical inhibition experiments, selective inhibitors of specific CYP isoforms were used to obtain maximum inhibition of the target CYP with minimum effect on other CYPs. Eadie-Hofstee plots (V vs. V/S) indicated that the reaction of ethylbenzene to 1-

phenylethanol with human liver microsomes was biphasic with low and high affinity components. The Michaelis-Menten equation was fit to the data and kinetic constants obtained by regression analysis. One microsome preparation was found to give a noticeably less curved Eadie-Hofstee plot and metabolized ethylbenzene at a much higher rate than the other preparations (Vmax = 2922 pmol/min/mg). It was excluded from the statistical analysis. For the high affinity reaction the mean Vmax was 689 ± 278 pmol/min/mg microsomal protein and the Km = 8.0 ± 2.9 μM (n = 6). For the low affinity reaction the Vmax was 3039 ± 825 pmol/min/mg and Km = 391 ± 117 μM (n = 6). The intrinsic clearance values of Vmax/Km were 85.4 ± 15.1 and 8.3 ± 3.0 for the high and low affinity reactions, respectively. The high affinity component of pooled human liver microsomes was inhibited 79%-95% by diethyldithiocarbamate, and recombinant CYP2E1 metabolized ethylbenzene with a low Km of 35 μM and low Vmax of 7 pmol/min/pmol P450, indicating that the CYP2E1 isoform catalyzed this component. Recombinant CYP1A2 and CYP2B6 exhibited high Vmaxs (88 and 71 pmol/min/pmol P450, respectively) and Km's (502 and 219 μM, respectively), indicating their role in the low affinity component. The mean Vmax and Km values above were used by OEHHA in addition to those from Haddad *et al.* (2001) in our human PBPK modeling of ethylbenzene.

Charest-Tardif *et al.* (2006) characterized the inhalation pharmacokinetics of ethylbenzene in male and female B6C3F1 mice. Initially groups of animals were exposed for four hr to 75, 200, 500 or 1000 ppm ethylbenzene. Subsequently groups of animals were exposed for six hr to 75 and 750 ppm for one or seven consecutive days. The maximum blood concentration (Cmax, mean (± SD), n = 4) observed after four hr exposure to 75, 200, 500 and 1000 ppm was 0.53 (0.18), 2.26 (0.38), 19.17 (2.74), and 82.36 (16.66) mg/L, respectively. The blood AUCs were 88.5, 414.0, 3612.2, and 19,104.1 (mg/L)-min, respectively, in female mice, and 116.7, 425.7, 3148.3, 16039.3 (mg/L)-min, respectively in male mice. The comparison of Cmax and kinetics of ethylbenzene in mice exposed to 75 ppm indicated similarity between 1 and 7-day exposures. However, at 750 ppm elimination of ethylbenzene appeared to be greater after repeated exposures. Overall, the single and repeated exposure PK data indicate that ethylbenzene kinetics is saturable at exposure concentrations above 500 ppm but is linear at lower concentrations.

Backes *et al.* (1993) demonstrated that alkylbenzenes with larger substituents (e.g., ethylbenzene, m-, p-xylene, n-propylbenzene) were effective inducers of microsomal enzymes compared to those with no or smaller substituents (benzene, toluene). Cytochrome P450 2B1 and 2B2 levels were induced with the magnitude of induction increasing with hydrocarbon size. P450 1A1 was also induced but less than 2B. A single intraperitoneal (i.p.) dose of 10 mmol/kg in rats was selected for optimum induction response with no overt toxic effects.

Bergeron *et al.* (1999) using the same daily dose of ethylbenzene for up to ten days observed changes in expression of CYP 2B1, 2B2, 2E1, and 2C11. While CYP 2C11 and 2E1 were attenuated by repeated dosing of ethylbenzene, CYP 2Bs were elevated after initial dosing despite the absence of detectable 2B1 or 2B2 mRNA. The authors

interpreted this observation as the initial ethylbenzene dose leading to an increase in ethylbenzene clearance and an overall decrease in tissue ethylbenzene levels with repeated dosing and decreased induction effectiveness.

Serron *et al.* (2000) observed that treatment of rats with ethylbenzene (i.p., 10 mmol/kg) led to increased free radical production by liver microsomes compared to corn oil controls. Oxygen free radical generation was measured *in vitro* by conversion of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to its fluorescent product 2', 7'-dichlorofluorescein (DCF). A significant elevation (40%) of DCF was seen despite lack of effect on overall P450 levels. The DCF product formation was inhibited by catalase but not by superoxide dismutase suggesting a H_2O_2 intermediate. Anti-CYP2B antibodies inhibited DCF production indicating involvement of CYP2B. As noted above ethylbenzene treatment induces increased production of CYP2B.

While the doses in these studies were quite high at over 1000 mg/kg-d by the intraperitoneal route, earlier studies by Elovaara *et al.* (1985) showed P450 induction in livers of rats exposed to 50, 300 and 600 ppm (218, 1305 and 2610 mg/m^3) for 6 hours/day, 5 days/week for up to 16 weeks. So it is possible that the types of effects discussed above, notably the production of reactive oxygen species via induced CYP 2B, may have occurred during the cancer bioassays.

Genotoxicity

***In vitro* and *in vivo* animal studies**

Ethylbenzene has been tested for genotoxicity in a variety of *in vitro* and *in vivo* genotoxicity assays. Those studies have been reviewed by ATSDR (1999). Ethylbenzene has not demonstrated genotoxicity in *Salmonella* reverse mutation assays. Those studies are listed in Table 1. All studies were performed in the presence and absence of metabolic activation (rat liver S9), and were negative. It has not been tested in *Salmonella* strains sensitive to oxidative DNA damage.

Table 1. Ethylbenzene *Salmonella* reverse mutation studies

Test strains	Reference
TA98, TA100, TA1535, TA1537	Florin *et al.*, 1980
TA98, TA100, TA1535, TA1537, TA1538	Nestmann *et al.*, 1980
TA98, TA100, TA1535, TA1537, TA1538	Dean *et al.*, 1985
TA97, TA98, TA100, TA1535	NTP, 1986
TA97, TA98, TA100, TA1535	NTP, 1999
TA98, TA100	Kubo *et al.*, 2002

Ethylbenzene also did not induce mutations in the WP2 and WP2uvrA strains of *Escherichia coli* in the presence and absence of metabolic activation (Dean *et al.*, 1985), or in *Saccharomyces cerevisiae* strains JD1 (Dean *et al.*, 1985), XV185-14C, and D7 as measured by gene conversion assays (Nestmann and Lee, 1983).

Ethylbenzene has been observed to induce mutations in L5178Y mouse lymphoma cells at the highest nonlethal dose tested (80 µg/mL) (McGregor *et al.*, 1988; NTP, 1999). However, NTP noted significant cytotoxicity at this dose level (relative total growth was reduced to 34% and 13% of the control level in each of two trials).

Data on the ability of ethylbenzene to induce chromosomal damage in non-human mammalian cells are negative. Ethylbenzene did not cause chromosomal damage in rat liver epithelial-like (RL4) cells (Dean *et al.*, 1985). Additionally, ethylbenzene did not induce an increase in either sister chromatid exchanges (SCE) or chromosomal aberrations in Chinese hamster ovary (CHO) cells in the presence or absence of metabolic activation (NTP 1986, 1999).

The frequency of micronucleated erythrocytes in bone marrow from male NMRI mice exposed to ethylbenzene by intraperitoneal injection was not significantly increased compared to controls (Mohtashamipur *et al.*, 1985). Additionally, ethylbenzene did not increase the frequency of micronucleated erythrocytes in peripheral blood from male and female $B6C3F_1$ mice treated for 13 weeks with ethylbenzene (NTP, 1999).

Midorikawa *et al* (2004) reported oxidative DNA damage induced by the metabolites of ethylbenzene, namely ethylhydroquinone and 4-ethylcatechol. Ethylbenzene was metabolized to 1-phenylethanol, acetophenone, 2-ethylphenol, and 4-ethylphenol by rat liver microsomes *in vitro*. 2-Ethylphenol and 4-ethylphenol were ring-dihydroxylated to ethylhydroquinone (EHQ) and 4-ethylcatechol (EC). These dihydroxylated metabolites induced DNA damage in ^{32}P-labeled DNA fragments from the human p53 tumor suppressor gene and induced the formation of 8-oxo-7, 8-dihydro-2'-deoxyguanosine in calf thymus DNA in the presence of Cu(II). Addition of exogenous NADH enhanced EC-induced oxidative DNA damage but had little effect on EHQ action. The authors suggest that Cu(I) and H_2O_2 produced via oxidation of EHQ and EC were involved in oxidative DNA damage. NADH enhancement was attributed to reactive species generated from the redox cycle of EC → 4-ethyl-1, 2-benzoquinone → EC. Similar effects of NADH were observed with benzene metabolites and catechol (Hirakawa *et al.* 2002).

***In vitro* and *in vivo* human studies**

Norppa and Vainio (1983) exposed human peripheral blood lymphocytes to ethylbenzene in the absence of metabolic activation. The authors reported that ethylbenzene induced a marginal increase in SCEs at the highest dose tested, and that the increase demonstrated a dose-response.

Holz *et al.* (1995) studied genotoxic effects in workers exposed to volatile aromatic hydrocarbons (styrene, benzene, ethylbenzene, toluene and xylenes) in a styrene production plant. Peripheral blood monocytes were assayed for DNA adducts using a nuclease P1-enhanced ^{32}P-postlabeling assay, and DNA single strand breaks, SCEs and micronuclei frequencies in peripheral blood lymphocytes were determined in workers and

controls. No significant increases in DNA adducts, DNA single strand breaks, SCEs or total micronuclei were noted in exposed workers. Significantly increased kinetochore-positive micronuclei (suggestive of aneuploidy-induction) were noted in total exposed workers, exposed smokers, and exposed non-smokers. However, the mixed exposures made it impossible to ascribe the kinetochore-positive micronuclei increase in exposed workers solely to ethylbenzene or other chemical exposure.

The effects of benzene and ethylbenzene exposure on chromosomal damage in exposed workers were examined by Sram *et al.* (2004). Peripheral blood lymphocytes from exposed workers and controls were analyzed for chromosomal aberrations. Exposure to ethylbenzene resulted in a significant increase in chromosomal aberrations. A reduction in ethylbenzene concentration due to improved workplace emissions controls resulted in a reduction in chromosomal damage in exposed workers. However, these workers were also exposed to benzene, making it impossible to determine if the chromosomal damage was due to ethylbenzene.

Ethylbenzene sunlight-irradiation products

Toda *et al.* (2003) found that sunlight irradiation of ethylbenzene resulted in the formation of ethylbenzene hydroperoxide (EBH). EBH induced oxidative DNA damage in the presence of Cu^{2+} as measured by the formation of 8-hydroxy-deoxyguanosine (8-OH-dG) adducts in calf thymus DNA. The Cu^{2+}-specific chelator bathocuproine strongly inhibited EBH-induced oxidative DNA damage. Superoxide dismutase (catalyzes superoxide decomposition) partly inhibited 8-OH-dG adduct formation, and catalase (catalyzes hydrogen peroxide decomposition) slightly inhibited 8-OH-dG adduct formation.

Summary of ethylbenzene genotoxicity

The above data indicate that ethylbenzene generally has not been demonstrated to induce gene mutations or chromosomal damage in bacteria, yeast or non-human mammalian cells, with the exception of positive results in the L5178Y mouse lymphoma cell mutation assay at concentrations producing significant cytotoxicity (McGregor *et al.*, 1988; NTP, 1999). Data on the genotoxicity of ethylbenzene in humans is mixed (Norppa and Vainio, 1983; Holz *et al.*, 1995; Sram *et al.*, 2004), and interpretation of the epidemiological studies is made difficult because of confounding due to coexposures to other chemicals, including benzene. Ethylbenzene has been demonstrated to generate reactive oxygen species in liver microsomes from exposed rats (Serron *et al.*, 2000), and ethylbenzene hydroperoxide (a sunlight-irradiation product) has been demonstrated to induce oxidative DNA damage in calf thymus DNA *in vitro* (Toda *et al.*, 2003). The ethylbenzene metabolites EHQ and EC have demonstrated the ability to induce oxidative DNA damage in human DNA *in vitro* (Midorikawa *et al.*, 2004).

Animal Cancer Bioassays

Maltoni *et al.* (originally reported in 1985; additional information published in 1997) studied the carcinogenicity of ethylbenzene in male and female Sprague-Dawley rats exposed via gavage. The authors reported an increase in the percentage of animals with malignant tumors associated with exposure to ethylbenzene. In animals exposed to 800 mg/kg bw ethylbenzene, Maltoni *et al.* (1997) reported an increase in nasal cavity tumors, type not specified (2% in exposed females versus 0% in controls), neuroesthesioepitheliomas (2% in exposed females versus 0% in controls; 6% in exposed males versus 0% in controls), and oral cavity tumors (6% in exposed females versus 2% in controls; 2% in exposed males versus 0% in controls). These studies were limited by inadequate reporting and were considered inconclusive by NTP (1999) and IARC (2000).

The National Toxicology Program (NTP, 1999; Chan *et al.*, 1998) conducted inhalation cancer studies of ethylbenzene using male and female F344/N rats and B6C3F$_1$ mice. Groups of 50 animals were exposed via inhalation to 0, 75, 250 or 750 ppm ethylbenzene for 6.25 hours per day, 5 days per week for 104 (rats) or 103 (mice) weeks.

Survival probabilities were calculated by NTP (1999) using the Kaplan-Meier product-limit procedure. For male rats in the 75 ppm and 250 ppm exposure groups, survival probabilities at the end of the study were comparable to that of controls but significantly less for male rats in the 750 ppm exposure group (30% for controls and 28%, 26% and 4% for the 75 ppm, 250 ppm and 750 ppm exposure groups, respectively). NTP (1999) stated that the mean body weights of the two highest exposure groups (250 and 750 ppm) were "generally less than those of the chamber controls from week 20 until the end of the study." Expressed as percent of controls, the mean body weights for male rats ranged from 97 to 101% for the 75 ppm group, 90 to 98% for the 250 ppm group, and 81 to 98% for the 750 ppm group.

In female rats, survival probabilities were comparable in all groups (62% for controls and 62%, 68% and 72% for the 75 ppm, 250 ppm and 750 ppm exposure groups, respectively). NTP (1999) reported that the mean body weights of exposed female rats were "generally less than those of chamber controls during the second year of the study." Expressed as percent of controls, the mean body weights for female rats ranged from 92 to 99% for the 75 ppm group, 93 to 100% for the 250 ppm group, and 92 to 99% for the 750 ppm group.

The incidences of renal tumors (adenoma and carcinoma in males; adenoma only in females) were significantly increased among rats of both sexes in the high-dose group (males: 3/50, 5/50, 8/50, 21/50; females: 0/50, 0/50, 1/50, 8/49 in control, 75 ppm, 250 ppm and 750 ppm groups respectively [standard and extended evaluations of kidneys combined]). The incidence of testicular adenomas (interstitial and bilateral) was significantly elevated among high-dose male rats (36/50, 33/50, 40/50, 44/50 in control, 75 ppm, 250 ppm and 750 ppm groups respectively). NTP noted that this is a common neoplasm, which is likely to develop in all male F344/N rats that complete a natural life span; exposure to ethylbenzene "appeared to enhance its development." NTP concluded that there was clear evidence of carcinogenicity in male rats and some evidence in female rats, based on the renal tumorigenicity findings.

The survival probabilities at the end of the study for exposed male mice were comparable to that of controls (57% for controls and 72%, 64% and 61% for the 75 ppm, 250 ppm and 750 ppm exposure groups, respectively). The same was true for exposed female mice (survival probabilities at end of study: 71% for controls and 76%, 82% and 74% for the 75 ppm, 250 ppm and 750 ppm exposure groups, respectively). Mean body weights in exposed male mice were comparable to those of controls. NTP (1999) reported that the mean body weights in exposed female mice were greater in the 75 ppm group compared to controls after week 72, and generally lower in the 750 ppm group compared to controls from week 24 through week 68. Expressed as percent of controls, the ranges of mean body weights in exposed female mice were 96 to 110% in the 75 ppm group, 93 to 108% in the 250 ppm group, and 92 to 101% in the 750 ppm group.

Increased incidences of alveolar/bronchiolar adenoma and adenoma or carcinoma (combined) were observed in male mice in the high-dose group (7/50, 10/50, 15/50, 19/50 in control, 75 ppm, 250 ppm and 750 ppm groups respectively). Among female mice in the high-dose group, the incidences of combined hepatocellular adenoma or carcinoma and hepatocellular adenoma alone were significantly increased over control animals (for adenomas and carcinomas the tumor incidences were 13/50, 12/50, 15/50, 25/50 in control, 75 ppm, 250 ppm and 750 ppm groups, respectively). NTP (1999) concluded that these findings provided some evidence of carcinogenicity in male and female mice.

Human Studies of Carcinogenic Effects

Studies on the effects of workplace exposures to ethylbenzene have been complicated by concurrent exposures to other chemicals, such as xylenes and benzene. IARC (2000) concluded that there was inadequate evidence in humans for the carcinogenicity of ethylbenzene.

IV. DERIVATION OF CANCER POTENCY

Mechanism of Action and Basis for Cancer Potency

The derivation of a cancer potency value is based on either the demonstration of a mode of action (MOA) supporting a low dose linear dose-response or insufficient evidence supporting an alternative nonlinear low dose response leading to a NOAEL or margin of exposure for the observed tumor response. Thus, when no MOA can be convincingly established, a low dose linear dose-response is assumed by default in cancer risk assessment. The U.S. EPA (2005) has provided a detailed framework for evaluating the evidence supporting potential MOAs. In this analysis we evaluated data relevant to the MOA for ethylbenzene carcinogenicity .

Hard (2002) suggested that "chemically induced exacerbation of CPN [chronic progressive nephropathy] was the mode of action underlying the development of renal neoplasia" in the NTP ethylbenzene studies. In a retrospective evaluation of NTP chronic

studies, Seely *et al.* (2002) found that renal tubule cell neoplasms (RTCNs) "tend to occur in animals with a slightly higher severity of CPN than animals without RTCNs. However, the differential is minimal and clearly there are many male F344 rats with severe CPN without RTCNs." Seely *et al.* (2002) go on to say that "the data from these retrospective reviews suggest that an increased severity of CPN may contribute to the overall tumor response. However, any contribution appears to be marginal, and additional factors are likely involved."

Stott *et al.* (2003) reported accumulation of the male rat specific protein α2u-globulin in 1-week and 4-week inhalation studies of ethylbenzene in groups of six (1-week study) or eight (4-week study) male rats; the accumulation measured as an increase in hyaline droplets in proximal convoluted tubules was statistically significant only in the 1-week study. In the 13-week and 2-year inhalation studies of ethylbenzene, NTP (1992; 1999) found no evidence of an increase in hyaline droplets in treated rats. NTP (1999) therefore dismissed any involvement of α2u-globulin accumulation in renal tumor development in rats. The fact that the lesion appears in both male and female rats further argues against the involvement of α2u-globulin in the development of kidney toxicity. This mechanism was discounted by Hard (2002) as well. Stott *et al.* (2003) also postulated mechanisms of tumorigenic action involving cell proliferation and/or altered cell population dynamics in female mouse liver and male mouse lung. Stott et al. (2003) propose various hypothetical mechanisms which might involve nonlinear dose responses but the metabolism data clearly show the formation of epoxides and related oxidative metabolites, which could potentially be involved in a genotoxic mechanism of carcinogenic action possibly similar to benzene. Midorikawa *et al.* (2004) reported that the oxidative metabolism of ethylbenzene metabolites ethylhydroquinone and 4-ethylcatechol resulted in oxidative DNA damage *in vitro*. In view of the variety of metabolites and possible modes of action a low-dose linearity assumption is considered appropriate when extrapolating from the point of departure to obtain an estimate of the cancer risk at low doses with the BMD methodology as is use of the LMS approach.

Unit risk values for ethylbenzene were calculated based on data in male and female rats and mice from the studies of NTP (1999) utilizing both linearized multistage and benchmark dose methods. The incidence data used to calculate unit risk values are listed below in Tables 2 thru 6. The methodologies for calculating average concentration, lifetime weighted average (LTWA) dose and PBPK adjusted internal dose are discussed below. An internal dose metric representing the amount of ethylbenzene metabolized per kg body weight per day (metabolized dose) was used in the dose response analysis with published PBPK modeling parameters. In addition, for the mouse, recent pharmacokinetic data simulating mouse bioassay conditions were used to improve PBPK model predictions (Tables 5 and 6).

The metabolized dose metric is considered the most appropriate metric for assessment of carcinogenic risks when the parent compound undergoes systemic metabolism to a variety of oxidative metabolites which may participate in one or more mechanisms of carcinogenic action, and the parent compound is considered unlikely to be active. In this case the dose response relation is likely to be more closely related to the internal dose of

metabolites than of the parent compound. Other metrics commonly investigated using PBPK methods are the area under the concentration-time curve (AUC), and the maximum concentration (Cmax) for parent or metabolites in blood and target tissues. The PBPK metabolized dose metric was used in the ethylbenzene dose-response analysis.

Table 2. Incidence of renal tubule adenoma or carcinoma in male rats exposed to ethylbenzene via inhalation and relevant dose metrics (from NTP, 1999).

Chamber concentration (ppm)	Average concentration[a] (mg/m^3)	LTWA dose[b] (mg/kg-day)	PBPK metabolized dose[c] (mg/kg-d)	Tumor incidence[d]	Statistical significance[e]
0	0	0	0	3/42	$p < 0.001$[f]
75	60.7	35.6	21.15	5/42	$p = 0.356$
250	202	119	56.87	8/42	$p = 0.0972$
750	607	356	105.47	21/36	$p < 0.001$

a. Average concentration during exposure period calculated by multiplying chamber concentration by 6.25 hours/24 hours, 5 days/7 days, and 4.35 mg/m^3/ppm.

b. Lifetime weighted average doses determined by multiplying the lifetime average concentrations during the dosing period by the male rat breathing rate (0.264 m^3/day) divided by the male rat body weight (0.450 kg). The duration of exposure was 104 weeks, so no correction for less than lifetime exposure was required.

c. Rodent PBPK models were used to estimate internal doses under bioassay conditions; methods are described in detail below.

d. Effective rate. Animals that died before the first occurrence of tumor (day 572) were removed from the denominator.

e. The *p*-value listed next to dose groups is the result of pair wise comparison with controls using the Fisher exact test.

f. The *p*-value listed next to the control group is the result of trend tests conducted by NTP (1999) using the life table, logistic regression and Cochran-Armitage methods, with all methods producing the same result.

Table 3. Incidence of testicular adenoma in male rats exposed to ethylbenzene via inhalation and relevant dose metrics (from NTP, 1999).

Chamber concentration (ppm)	Average concentration[a] (mg/m^3)	LTWA dose[b] (mg/kg-day)	PBPK metabolized dose[c] (mg/kg-d)	Tumor incidence[d]	Statistical significance[e]
0	0	0	0	36/48	$p < 0.001$[f]
					$p = 0.010$[g]
75	60.7	35.6	21.15	33/46	p = 0.450N
250	202	119	56.87	40/49	p = 0.293
750	607	356	105.47	44/47	p < 0.05

a. Average concentration during exposure period calculated by multiplying chamber concentration by 6.25 hours/24 hours, 5 days/7 days, and 4.35 mg/m^3/ppm.
b. Lifetime weighted average doses determined by multiplying the lifetime average concentrations during the dosing period by the male rat breathing rate (0.264 m^3/day) divided by the male rat body weight (0.450 kg). The duration of exposure was 104 weeks, so no correction for less than lifetime exposure was required.
c. Rodent PBPK models were used to estimate internal doses under bioassay conditions; methods are described in detail below.
d. Effective rate. Animals that died before the first occurrence of tumor (day 420) were removed from the denominator.
e. The *p*-value listed next to dose groups is the result of pair wise comparison with controls using the Fisher exact test. An "N" after the *p*-value signifies that the incidence in the dose group is lower than that in the control group. The *p*-values listed next to the control group are the result of trend tests conducted by NTP (1999) using the methods specified in the following footnotes.
f. Results of trend tests conducted by NTP (1999) using the life table and logistic regression tests.
g. Result of Cochran-Armitage trend test conducted by NTP (1999).

Table 4. Incidence of renal tubule adenoma in female rats exposed to ethylbenzene via inhalation and relevant dose metrics (from NTP, 1999).

Chamber concentration (ppm)	Average concentration[a] (mg/m^3)	LTWA dose[b] (mg/kg-day)	PBPK metabolized dose[c] (mg/kg-d)	Tumor incidence[d]	Statistical significance[e]
0	0	0	0	0/32	$p < 0.001$[f]
75	60.7	41.6	24.22	0/35	--
250	202	139	63.72	1/34	$p = 0.515$
750	607	416	115.3	8/37	$p < 0.01$

a. Average concentration during exposure period calculated by multiplying chamber concentration by 6.25 hours/24 hours, 5 days/7 days, and 4.35 mg/m3/ppm.
b. Lifetime weighted average doses were determined by multiplying the lifetime average concentrations during the dosing period by the female rat-breathing rate (0.193 m3/day) divided by the female rat body weight (0.282 kg). The duration of exposure was 104 weeks, so no correction for less than lifetime exposure was required.
c. Rodent PBPK models were used to estimate internal doses under bioassay conditions; methods are described in detail below.
d. Effective rate. Animals that died before the first occurrence of tumor (day 722) were removed from the denominator.
e. The p-value listed next to dose groups is the result of pair wise comparison with controls using the Fisher exact test.
f. The p-value listed next to the control group is the result of trend tests conducted by NTP (1999) using the life table, logistic regression and Cochran-Armitage methods, with all methods producing the same result.

Table 5. Incidence of lung alveolar/bronchiolar carcinoma or adenoma in male mice exposed to ethylbenzene via inhalation and relevant dose metrics (from NTP, 1999).

Chamber concentration (ppm)	Average concentration[a] (mg/m^3)	LTWA dose[b] (mg/kg-day)	PBPK metabolized dose[c] (mg/kg-d)	PBPK metabolized dose - Charest-Tardif[d] (mg/kg-d)	Tumor incidence[e]	Statistical significance[f]
0	0	0	0	0	7/46	$p = 0.004$[g]
75	60.7	69.3	40.40	46.60	10/48	$p = 0.331$
250	202	231	89.38	152.8	15/50	$p = 0.0688$
750	607	693	134.77	340.2	19/48	$p < 0.01$

a. Average concentration during exposure period calculated by multiplying chamber concentration by 6.25 hours/24 hours, 5 days/7 days, and 4.35 mg/m^3/ppm.
b. Lifetime weighted average doses were determined by multiplying the average concentrations during the dosing period by the male mouse breathing rate (0.0494 m^3/day) divided by the male mouse body weight (0.0429 kg) and by 103 weeks/104 weeks to correct for less than lifetime exposure.
c. Rodent PBPK models were used to estimate internal doses under bioassay conditions; methods are described in detail below.
d. PBPK metabolized dose based on published parameters from Charest-Tardif *et al.* (2006).
e. Effective rate. Animals that died before the first occurrence of tumor (day 418) were removed from the denominator.
f. The *p*-value listed next to dose groups is the result of pair wise comparison with controls using the Fisher exact test.
g. The *p*-value listed next to the control group is the result of trend tests conducted by NTP (1999) using the life table, logistic regression and Cochran-Armitage methods, with all methods producing the same result.

Table 6. Incidence of liver hepatocellular carcinoma or adenoma in female mice exposed to ethylbenzene via inhalation and relevant dose metrics (from NTP, 1999).

Chamber concentration (ppm)	Average concentration[a] (mg/m^3)	LTWA dose[b] (mg/kg-day)	PBPK metabolized dose[c] (mg/kg-d)	PBPK metabolized dose - Charest-Tardif[d] (mg/kg-d)	Tumor incidence[e]	Statistical significance[f]
0	0	0	0	0	13/47	p = 0.004[g] p = 0.002[h]
75	60.7	71.6	41.53	47.98	12/48	p = 0.479N
250	202	239	91.22	157.3	15/47	p = 0.411
750	607	716	136.68	348.1	25/48	p < 0.05

a. Average concentration during exposure period calculated by multiplying chamber concentration by 6.25 hours/24 hours, 5 days/7 days, and 4.35 mg/m^3/ppm.
b. Lifetime weighted average doses were determined by multiplying the average concentrations during the dosing period by the female mouse breathing rate (0.0463 m^3/day) divided by the female mouse body weight (0.0389 kg) and by 103 weeks/104 weeks to correct for less than lifetime exposure.
c. Rodent PBPK models were used to estimate internal doses under bioassay conditions; methods are described in detail below.
d. PBPK metabolized dose based on published parameters from Charest-Tardif *et al.* (2006).
e. Effective rate. Animals that died before the first occurrence of tumor (day 562) were removed from the denominator.
f. The *p*-value listed next to dose groups is the result of pair wise comparison with controls using the Fisher exact test. An "N" after the *p*-value signifies that the incidence in the dose group is lower than that in the control group. The *p*-value listed next to the control group is the result of trend tests conducted by NTP (1999) using the methods specified in the footnotes.
g. Result of trend test conducted by NTP (1999) using the life table method.
h. Results of trend tests conducted by NTP (1999) using the logistic regression and Cochran-Armitage trend tests.

Linearized Multistage Approach

The default approach, as originally delineated by CDHS (1985), is based on a linearized form of the multistage model of carcinogenesis (Armitage and Doll, 1954). Cancer potency is estimated from the upper 95% confidence limit, q_1^*, on the linear coefficient q_1 in a model relating lifetime probability of cancer (p) to dose (d):

$$p = 1 - \exp[-(q_0 + q_1 d + q_2 d^2 + \cdots + q_j d^j)] \quad (1)$$

with constraints, $q_i \geq 0$ for all i. The default number of parameters used in the model is n, where n is the number of dose groups in the experiment, with a corresponding polynomial degree of n-1.

The parameter q_1^* is estimated by fitting the above model to dose response data using MSTAGE (Crouch, 1992). For a given chemical, the model is fit to one or more data sets. The default approach is to select the data for the most sensitive species and sex.

To estimate animal potency, q_{animal}, when the experimental exposure is less than lifetime the parameter q_1^* is adjusted by assuming that the lifetime incidence of cancer increases with the third power of age. The durations of the NTP experiments were at least as long as the standard assumed lifetime for rodents of 104 weeks, so no correction for short duration was required.

Benchmark Dose Methodology

U.S. EPA (2003) and others (*e.g.* Gaylor *et al.*, 1994) have more recently advocated a benchmark dose method for estimating cancer risk. This involves fitting a mathematical model to the dose-response data. A linear or multistage procedure is often used, although others may be chosen in particular cases, especially where mechanistic information is available which indicates that some other type of dose-response relationship is expected, or where another mathematical model form provides a better fit to the data. A point of departure on the fitted curve is defined: for animal carcinogenesis bioassays this is usually chosen as the lower 95% confidence limit on the dose predicted to cause a 10% increase in tumor incidence (LED_{10}). Linear extrapolation from the point of departure to zero dose is used to estimate risk at low doses either when mutagenicity or other data imply that this is appropriate, or in the default case where no data on mechanism are available. The slope factor thus determined from the experimental data is corrected for experimental duration in the same way as the q_1^* adjustments described for the linearized multistage procedure. In the exceptional cases where data suggesting that some other form of low-dose extrapolation is appropriate, a reference dose method with uncertainty factors as required may be used instead.

The quantal tumor incidence data sets were analyzed using the BMDS software (version 1.3.2) of U.S.EPA (2000). In general the program models were fit to the data with the X^2 fit criterion ≥ 0.1. In those cases when more than one model gave adequate fit the model that gave the best fit in the low dose region (visually and by X^2 residual) was chosen for the LED_{10} estimation.

Implementation of LMS and BMD Methodology

The linearized multistage approach and the benchmark dose methodology were both applied to the tumor incidence data for ethylbenzene in the NTP (1999) studies.

Calculation of Lifetime Weighted Average Dose

Male and female rats (NTP, 1999) were exposed to ethylbenzene for 6.25 hours/day, five days/week for 104 weeks. Male and female mice (NTP, 1999) were exposed to ethylbenzene for 6.25 hours/day, five days/week for 103 weeks. Average concentrations,

expressed in mg/m^3, during the exposure period were calculated by multiplying the reported chamber concentrations by 6.25 hours/24 hours, five days/seven days and 4.35 mg/m^3/ppm.

The average body weights of male and female rats were calculated to be 0.450 kg and 0.282 kg, respectively, based on data for controls reported by NTP (1999). The average body weights of male and female mice were estimated to be approximately 0.0429 kg and 0.0389 kg, respectively, based on data for controls reported by NTP (1999). Inhalation rates (I) in m^3/day for rats and mice were calculated based on Anderson *et al.* (1983):

$$I_{rats} = 0.105 \times (bw_{rats}/0.113)^{2/3} \quad (3)$$

$$I_{mice} = 0.0345 \times (bw_{mice}/0.025)^{2/3} \quad (4)$$

Breathing rates were calculated to be 0.264 m^3/day for male rats, 0.193 m^3/day for female rats, 0.0494 m^3/day for male mice, and 0.0463 m^3/day for female mice. Lifetime weighted average (LTWA) doses were determined by multiplying the average concentrations during the dosing period by the appropriate animal breathing rate divided by the corresponding animal body weight. For mice, the exposure period (103 weeks) was less than the standard rodent lifespan (104 weeks), so an additional factor of 103 weeks/104 weeks was applied to determine lifetime average doses.

Physiologically Based Pharmacokinetic (PBPK) Modeling

The carcinogenic potency of ethylbenzene was calculated using rodent PBPK models to estimate internal doses under bioassay conditions. Extrapolations to human potencies were done using interspecies scaling. For comparison, a human PBPK model was used to estimate risk-specific doses for occupational and ambient environmental exposure scenarios. The PBPK models were comprised of compartments for liver, fat, vessel poor tissues (e.g., muscle), vessel rich tissues, and lung. Typical model parameters are given in Table 7 for flow-limited PBPK models and a model diagram is shown in Figure 2. Chemical and metabolic parameters were taken from Haddad *et al.* (2001) for all species studied and additionally from Sams *et al.* (2004) for human metabolism. Simulations were conducted using Berkeley Madonna (v.8.0.1) software (e.g., 6.25 hr exposure/day x 5 days/wk for one week simulations of bioassay exposure levels, see sample model equations in the appendix). The chemical partition coefficients used in the model were the same for all species: blood:air, 28.0; fat:blood, 55.57; liver:blood, 2.99; muscle:blood, 0.93; and lung:blood, 2.15 (Haddad *et al.*, 2001). The metabolic parameters were also from Haddad *et al.* (2001): VmaxC = 6.39 mg/hr/kg body weight scaled to the ¾ power of body weight; Km = 1.04 mg/L for all species. A second set of human metabolic parameters from Sams *et al.* (2004) was also used. In this case constants for low and high affinity saturable pathways were incorporated into the models: high affinity Vmax = 689 pmol/min/mg microsomal protein, Km = 8.0 μM; low affinity Vmax = 3039 pmol/min/mg protein, Km = 391 μM. A value of 28 mg/mL liver for microsomal protein concentration was assumed. Published values we reviewed ranged from 11 to 35 mg/g tissue. The value we used was similar to that of Kohn and Melnick (2000) (30 mg/g

liver) and Medinsky *et al.* (1994) (35 mg/g liver). All model units were converted to moles, liters, or hours for simulation. A molecular weight of 106.16 g/mol for ethylbenzene was used throughout. In addition to PBPK modeling based on published parameters the recent pharmacokinetic data of Charest-Tardif *et al.* (2006) was used in the mouse PBPK modeling for comparison purposes.

Johansen and Filser (1992) studied a series of volatile organic chemicals including ethylbenzene and developed theoretical values for clearance of uptake (CLupt) defined as the product of the rate constant for transfer of chemical from air to body and the volume of air in a closed chamber. The CLupt values were based on alveolar ventilation (Qalv), cardiac output (Qtot), and blood:air partition coefficients (Pbi). For most chemicals the experimentally determined values for inhalation uptake in rats and mice were about 60% of the theoretical values. The values determined for ethylbenzene in the rat of 70 mL/min for CLupt and 73 mL/min for alveolar ventilation are about 50% of the value given in Table 7 (i.e., 4.38 L/hr vs. 8.58 L/hr). In the work described below selected simulations were run with lower alveolar ventilation rates for comparison with the main analysis.

The primary model prediction was the amount of ethylbenzene metabolized over the course of the simulation. The AUCs, the areas under the concentration x time curves for mixed venous concentration and liver concentration of ethylbenzene, were also recorded. The values for one week simulations of the amount metabolized (mmoles) were divided by 7d/week and body weight in kg to give daily values and multiplied by the molecular weight to give the PBPK metabolized dose in mg/kg-d. These values were then used in the dose response assessment of individual tumor site incidences using the benchmark dose software of U.S. EPA (BMDS v. 1.3.2) to obtain the dose at which tumor incidence was predicted to be 10% (ED_{10}), LED_{10} (lower 95% confidence limit of ED_{10}) and curve fit statistics for each experiment.

Table 7. Parameters for Ethylbenzene PBPK Models.

Parameter	Mouse	Rat	Human
Alveolar ventilation rate Qalv, L/hr	$15*BW^{0.7}$	$15*BW^{0.7}$	$36*BW^{0.7}$ occ $15*BW^{0.7}$ env
Cardiac output Qtot, L/hr	$15*BW^{0.7}$	$15*BW^{0.7}$	$18*BW^{0.7}$ occ $15*BW^{0.7}$ env
Blood flows (fraction of cardiac output)			
Fat, Qf	0.09	0.09	0.05
Liver, Ql	0.25	0.25	0.26
Muscle, Qm	0.15	0.15	0.25
Vessel Rich Group, Qvrg	0.51	0.51	0.44
Tissue volumes, L (fraction of body weight)			
Fat, Vf	0.06	0.09	0.20, 0.40
Liver, Vl	0.04	0.049	0.026
Muscle, Vm	0.76	0.72	0.61, 0.41
Vessel Rich Group, Vvrg	0.05	0.036	0.036
Lung, Vlu	0.014	0.014	0.014
Body weight, BW kg	0.043 male 0.039 female	0.45 male 0.28 female	70
Metabolism VmaxC (Haddad *et al.*, 2001) mg/hr/$kg^{3/4}$ BW	6.39 25.56*	6.39	6.39
Km mg/L (Haddad *et al.* 2001)	1.04	1.04	1.04
Metabolism (Sams *et al.* 2004) High/Low Affinity Vmax mg/hr/L_{liver} High/Low Affinity Km mg/L			122.8/542.0 0.85/40.4

Note: occ = occupational scenario values; env = environmental exposure scenario; * this value provided better fit to the data of Charest-Tardif *et al.* (2006).

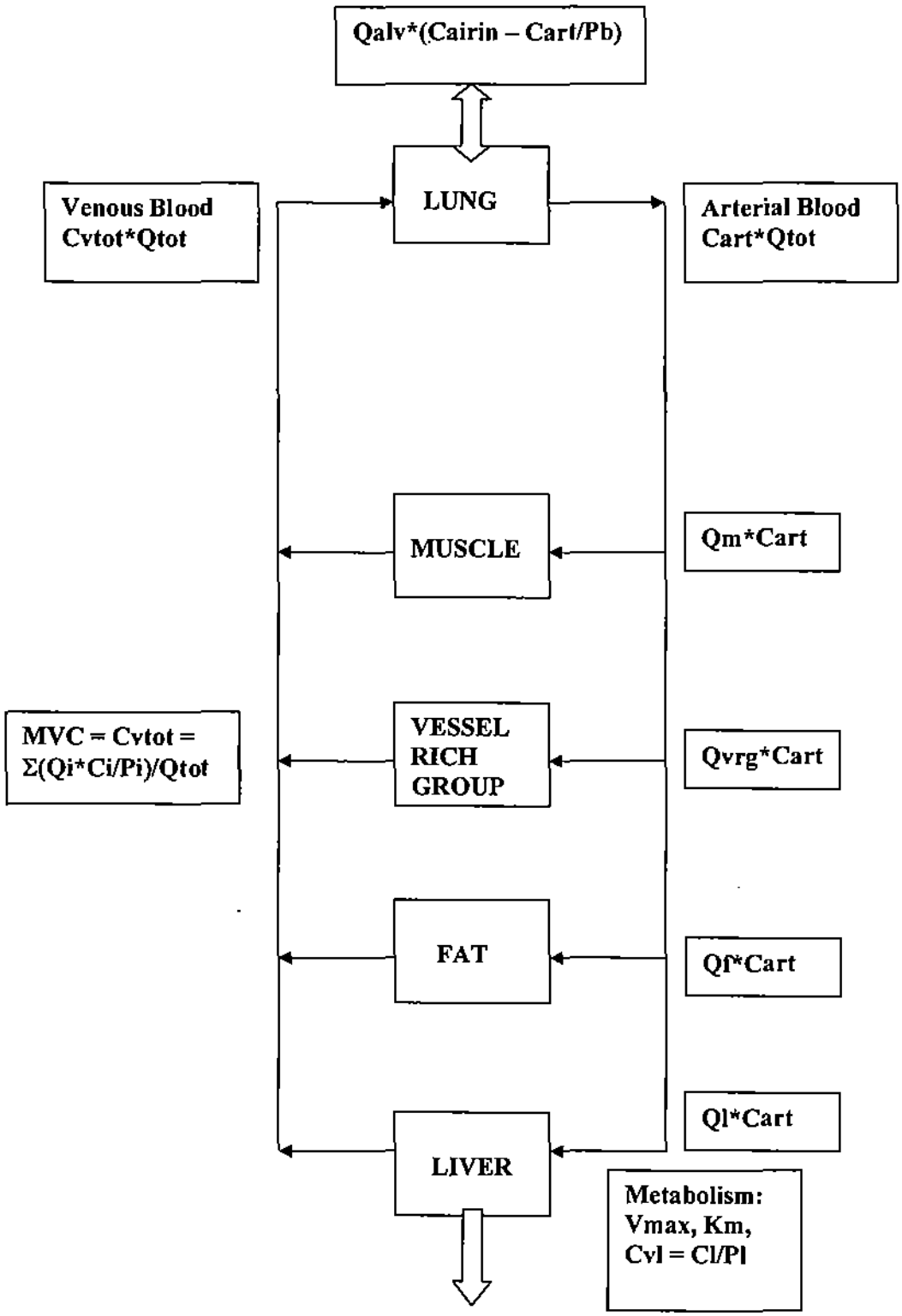

Figure 2. General Scheme for Ethylbenzene PBPK Model: Qtot = Cardiac Output; Qalv = Alveolar Ventilation Rate ; Pb = Blood/Air Partition Coefficient; Pi = Tissue/Blood Partition Coefficients; Qi = Tissue Fractional Blood Flows ;Cart = Arterial Blood Concentration; Cvtot = Mixed Venous Blood Concentration; Cairin = Inhaled Concentration (e.g. ppm Ethylbenzene); Cexhaled = Cart/Pb(Concentration of Ethylbenzene Exhaled); Ci = Ai/Vi = Mass/Volume.

Internal to External Dose Conversion

In order to estimate external equivalent air concentrations associated with internal doses, the PBPK models were used. Simulation of 10 ppb ethylbenzene for 8 hours in the human PBPK model with the Haddad *et al.* (2001) parameters resulted in the predicted uptake of 3.04 µmoles in tissues and blood compared to 3.96 µmoles inhaled, or an uptake of 77%. Practically all of the 3.04 µmoles represents metabolized ethylbenzene. Based on these results, OEHHA assumed that all absorbed ethylbenzene is metabolized at low dose. Thus, for the inhalation route, the internal metabolized dose is converted to an external dose by applying an uptake factor of 77%. As noted above, uptake values of 49 to 65% have been observed in studies with human subjects exposed via inhalation to ethylbenzene. OEHHA has occasionally used a default value of 50% for inhalation uptake of similar volatile organic compounds.

For the oral route at low dose, OEHHA assumed that ethylbenzene is 100% metabolized (based on the model predictions noted above) and that uptake of ethylbenzene is also 100% (a conventional assumption made for lack of more specific data at low doses in humans). Thus, at low dose, the internal metabolized dose of ethylbenzene would be equivalent to an external applied dose by the oral route. No conversion factor for internal to external dose is necessary in this case.

Interspecies Extrapolation

Interspecies extrapolation from experimental animals to humans is normally based on the following relationship, where bw_h and bw_a are human and animal body weights, respectively, and potency (*e.g.*, q_{animal}) is expressed on a per dose per body weight basis (e.g., $(mg/kg\text{-}d)^{-1}$ see Watanabe *et al.* (1992):

$$q_{human} = q_{animal} \times \left(\frac{bw_h}{bw_a}\right)^{1/4} \quad (2)$$

Alternatively, when performing calculations based on applied dose in terms of air concentrations, the assumption has sometimes been made that air concentration values are equivalent between species (CDHS, 1985). However, using the interspecies scaling factor shown above is preferred because it is assumed to account not only for pharmacokinetic differences (*e.g.*, breathing rate, metabolism), but also for pharmacodynamic considerations.

When extrapolating from an animal potency in terms of PBPK adjusted internal dose, only a pharmacodynamic scaling factor is required. Since an equal contribution of pharmacokinetic and pharmacodynamic considerations is assumed, animal potency values already adjusted for pharmacokinetic considerations require a scaling factor of only $(bw_h/bw_a)^{1/8}$:

$$q_{human} = q_{animal} \times \left(\frac{bw_h}{bw_a}\right)^{1/8} \quad (3)$$

Derivation of the Human Inhalation Unit Risk Value

To derive the human inhalation unit risk value, the human internal potency value based on PBPK metabolized dose is multiplied by the human breathing rate (assumed to be 20 m^3/day), divided by the human body weight (assumed to be 70 kg) and multiplied by the estimated inhalation uptake factor in humans (0.77 for ethylbenzene). This yields a human inhalation unit risk value in terms of external air concentration.

For the case of LTWA doses, the human inhalation unit risk value is derived by multiplying the human inhalation cancer potency value by the human breathing rate (assumed to be 20 m^3/day), divided by the human body weight (assumed to be 70 kg). Because the LTWA doses represent external applied dose from an inhalation study, no uptake factor is necessary in deriving the unit risk value.

Inhalation and Oral Cancer Potency Values

The cancer potency derived based on internal doses (i.e., PBPK metabolized dose) is equivalent to the oral cancer potency, because of the assumption of 100% oral uptake and 100% metabolism of ethylbenzene at low doses. To derive the inhalation cancer potency, the human inhalation unit risk value is multiplied by the human body weight (assumed to be 70 kg) and divided by the human breathing rate (assumed to be 20 m^3/day).

For the case of LTWA doses, the human cancer potency derived based on these external applied doses from the inhalation study is equivalent to the inhalation cancer potency. To determine the oral cancer potency, the inhalation cancer potency is multiplied by the ratio of the oral to inhalation uptake factors (i.e., 1/0.77).

Example Calculations – BMD Approach

In this section, example calculations of the human cancer potency values (oral and inhalation) and the human unit risk value based on the LED_{10} for the male rat kidney tumor data and either the PBPK metabolized doses or the LTWA doses are provided. The same logic would apply to the derivation using the LMS methodology, with the only difference being that the animal potency is taken directly from the MSTAGE program under the LMS approach instead of being calculated from the LED_{10} in the BMD approach. To distinguish the results obtained under the two approaches, the terms P_{animal}, P_{human}, and U_{human} were used for the values derived using the BMD methodology.

Calculations based on BMD methodology and PBPK metabolized doses

Under the BMD methodology, the ED_{10}s and LED_{10}s are obtained from the BMDS program, with the animal potency value being simply $0.1/LED_{10}$ (i.e., 10% risk (0.1) divided by the 95% lower confidence limit on the dose that induced 10% risk or LED_{10}; this is the definition of a slope). To obtain the animal potency based on internal dose

($P_{animal\_internal}$), 0.1 is divided by the LED_{10} derived for the male rat kidney tumor data and the PBPK metabolized doses:

$$P_{animal\_internal} = 0.1/LED_{10} = 0.1/22.96 = 0.004355 \text{ (mg/kg-d)}^{-1}$$

The human potency value based on internal dose ($P_{human\_internal}$) is calculated from the animal potency as follows:

$$\begin{aligned} P_{human\_internal} &= 0.004355 \text{ (mg/kg-day)}^{-1} \times (70 \text{ kg}/0.450 \text{ kg})^{1/8} \\ &= 0.0082 \text{ (mg/kg-day)}^{-1} \end{aligned}$$

$P_{human\_internal}$ is equivalent to the oral human potency, because of the assumptions of 100% oral uptake and 100% metabolism of ethylbenzene at low dose.

The human unit risk value (U_{human}) is derived from the internal human cancer potency as follows:

$$\begin{aligned} U_{human} &= 0.0082 \text{ (mg/kg-day)}^{-1} \times (20 \text{ m}^3/\text{day}/70 \text{ kg}) \times 0.77 \\ &= 1.8 \times 10^{-3} \text{ (mg/m}^3)^{-1} \\ &= 1.8 \times 10^{-6} \text{ (}\mu\text{g/m}^3)^{-1} \end{aligned}$$

As noted above the value of 0.77 for the proportion of inhaled dose metabolized was based on the prediction of the human ethylbenzene PBPK model, assuming exposure to low levels of ethylbenzene, and is similar to values obtained in studies with human subjects (Bardodej and Bardodejova, 1970; Engstrom et al., 1984; Gromiec and Piotrowski,1984). By applying this uptake factor and assuming that the metabolism of ethylbenzene is 100% at low dose, the resulting unit risk value is expressed in terms of external concentration.

The inhalation cancer potency is derived from the unit risk value as follows:

$$\begin{aligned} P_{human\_inhalation} &= 1.8 \times 10^{-3} \text{ (mg/m}^3)^{-1} \times (70 \text{ kg}/20 \text{ m}^3/\text{day}) \\ &= 0.0063 \text{ (mg/kg-day)}^{-1} \end{aligned}$$

Calculations based on BMD methodology and LTWA doses

The LED_{10} based on the male rat kidney data (Table 4) and the LTWA doses (Table 3) is determined using the BMDS software. The animal potency, which in this case is the inhalation animal potency (P_{animal_inh}), is determined by dividing the LED_{10} into 0.1:

$$P_{animal\_inh} = 0.1/LED_{10} = 0.1/42.62 = 0.002346 \text{ (mg/kg-d)}^{-1}$$

The human inhalation cancer potency (P_{human_inh}) is derived from the animal potency using the interspecies scaling factor:

$$P_{human\_inh} = 0.002346 \text{ (mg/kg-day)}^{-1} \times (70 \text{ kg}/0.450 \text{ kg})^{1/4}$$

$$= 0.0083 \text{ (mg/kg-day)}^{-1}$$

The unit risk factor is derived from the human inhalation cancer potency as follows:

$$\begin{aligned} U_{human} &= 0.0083 \text{ (mg/kg-day)}^{-1} \times (20 \text{ m}^3\text{/day}/70 \text{ kg}) \\ &= 2.4 \times 10^{-3} \text{ (mg/m}^3\text{)}^{-1} \\ &= 2.4 \times 10^{-6} \text{ (}\mu\text{g/m}^3\text{)}^{-1} \end{aligned}$$

For the calculation based on LTWA doses, the oral cancer potency is derived from the inhalation cancer potency by multiplying by the ratio of uptake factors (1/0.77):

$$\begin{aligned} P_{human\_oral} &= 0.0083 \text{ (mg/kg-day)}^{-1} \times (1/0.77) \\ &= 0.011 \text{ (mg/kg-day)}^{-1} \end{aligned}$$

Results and Discussion

Linearized multistage approach

Tables 8a and 8c list the q_{animal}, q_{human} and unit risk values based on the linearized multistage approach. The cancer potencies and unit risk values were derived using the applied LTWA doses and PBPK adjusted internal doses, as described above. The most sensitive tumor sites are the male rat testicular interstitial cell adenoma and the male rat kidney adenoma and carcinoma, when the LTWA doses are used. If PBPK doses are used, the most sensitive sites are the male rat testicular interstitial cell adenoma and the male mouse lung. Regardless of whether LTWA or PBPK doses are used, the results based on the male mouse lung tumor data, the female mouse liver tumor data, and the male rat renal tumor data are comparable, producing unit risk values of approximately 0.002 $(\text{mg/m}^3)^{-1}$. Further, the results using either the LTWA doses or the PBPK metabolized doses are quite similar indicating that the PBPK modeling does not markedly improve the estimates. Some of the inherent uncertainty associated with PBPK modeling is demonstrated by the fact that the results based on the PBPK modeling using the Charest-Tardif parameters differ by roughly a factor of two for the mice compared to the results derived based on the other equally valid PBPK modeling approach.

The testicular interstitial cell adenoma site gives the highest values. However, the very high background incidences of this tumor make it less reliable and suitable for dose-response analysis than the male rat kidney site.

Thus, the unit risk value of 0.0025 $(\text{mg/m}^3)^{-1}$ derived based on the LMS approach from the male rat kidney tumor data using the LTWA doses is selected as the representative value for ethylbenzene. It is very similar to the estimate derived using the PBPK approach (0.0026 $(\text{mg/m}^3)^{-1}$), and does not require the many assumptions made in applying the more complex PBPK approach.

Table 8a. Cancer potency and unit risk values for ethylbenzene derived using the linearized multistage procedure (LMS) with applied LTWA doses based on data from NTP (1999).

Sex, species	Site, tumor type	q_{animal_inh} $(mg/kg\text{-}day)^{-1}$	q_{human_inh}[a] $(mg/kg\text{-}day)^{-1}$	Human unit risk value[b] $(mg/m^3)^{-1}$	Goodness-of-fit test[c]
Male rats	Renal tubule carcinoma or adenoma	0.002472	0.0087	0.0025	p = 0.81
	Testicular interstitial cell adenoma	0.006547	0.023	0.0066	p = 0.52
Female rats	Renal tubule adenoma	0.0005528	0.0022	0.00063	p = 0.95
Male mice	Lung alveolar/ bronchiolar carcinoma or adenoma	0.0008494	0.0054	0.0015	p = 0.75
Female mice	Liver hepatocellular carcinoma or adenoma	0.0009421	0.0061	0.0017	p = 0.68

a. The interspecies extrapolation was applied to q_{animal_inh} in $(mg/kg\text{-}d)^{-1}$ to determine q_{human_inh} $(mg/kg\text{-}day)^{-1}$, as described above.

b Unit risk was determined by multiplying the human cancer potency in $(mg/kg\text{-}day)^{-1}$ by the human breathing rate (20 m^3/day) divided by human body weight (70 kg), as described above.

c. A *p*-value of greater than 0.05 for the chi-square goodness-of-fit test indicates an adequate fit.

Table 8b. Cancer potency and unit risk values for ethylbenzene derived using the BMD procedure with applied LTWA doses based on data from NTP (1999).

Sex, species	Site, tumor type	P_{animal_inh} $(mg/kg\text{-}day)^{-1}$	P_{human_inh}[a] $(mg/kg\text{-}day)^{-1}$	Human unit risk value[b] $(mg/m^3)^{-1}$	Model Goodness-of-fit test[c]
Male rats	Renal tubule carcinoma or adenoma	0.002589	0.0091	0.0026	Quantal Linear p = 0.49
	Testicular interstitial cell adenoma	0.006333	0.022	0.0063	Quantal Linear p = 0.73
Female rats	Renal tubule adenoma	0.0004704	0.0019	0.00054	Quantal Quadratic p = 0.99
Male mice	Lung alveolar/ bronchiolar carcinoma or adenoma	0.0008062	0.0051	0.0015	Quantal Linear p = 0.75
Female mice	Liver hepatocellular carcinoma or adenoma	0.0009256	0.0060	0.0017	Quantal Linear p = 0.74

a. The interspecies extrapolation of $(BW_h/BW_a)^{1/4}$ was applied to P_{animal_inh} in $(mg/kg\text{-}d)^{-1}$ to determine P_{human_inh} $(mg/kg\text{-}day)^{-1}$, as described above.

b Unit risk was determined by multiplying the human cancer potency in $(mg/kg\text{-}day)^{-1}$ by the human breathing rate (20 m^3/day) divided by human body weight (70 kg).

c. A *p*-value ≥ 0.1 for the chi-square goodness-of-fit test indicates an adequate fit with the BMD procedure.

Table 8c. Cancer potency and unit risk values for ethylbenzene derived using the linearized multistage procedure with PBPK metabolized doses and bioassay data from NTP (1999).

Sex, species	Site, tumor type	$q_{animal\_internal}$ $(mg/kg\text{-}day)^{-1}$	$q_{human\_internal}$[a] $(mg/kg\text{-}day)^{-1}$	Human unit risk value[b] $(mg/m^3)^{-1}$	Goodness-of-fit test[c]
Male rats	Renal tubule carcinoma or adenoma	0.004465	0.0084	0.0018	p = 0.57
	Testicular interstitial cell adenoma	0.01586	0.030	0.0066	p = 0.62
Female rats	Renal tubule adenoma	0.0009037	0.0018	0.00040	p = 0.98
Male mice	Lung alveolar/ bronchiolar carcinoma or adenoma	0.003747 0.001680[d]	0.0094 0.0042[d]	0.0021 0.00092[d]	p = 0.99 p = 0.93[d]
Female mice	Liver hepatocellular carcinoma or adenoma	0.002702 0.001705[d]	0.0069 0.0044[d]	0.0015 0.00097[d]	p = 0.86 p = 0.73[d]

a. The interspecies extrapolation of $(bw_h/bw_a)^{1/3}$ was applied to $q_{animal\_internal}$ in $(mg/kg\text{-}d)^{-1}$ to determine $q_{human\_internal}$ in $(mg/kg\text{-}day)^{-1}$, as described above.

b. Unit risk was determined by multiplying the human internal cancer potency in $(mg/kg\text{-}day)^{-1}$ by the human breathing rate (20 m^3/day) divided by human body weight (70 kg) and by an uptake factor of 0.77, as described above.

c. A *p*-value of greater than 0.05 for the chi-square goodness-of-fit test indicates an adequate fit.

d. These values obtained with PBPK model adjusted to approximate the PK data of Charest-Tardif *et al.* (2006).

Table 8d. Cancer potency and unit risk values for ethylbenzene derived using the BMD procedure with PBPK metabolized doses and bioassay data from NTP (1999).

Sex, species	Site, tumor type	$P_{animal\_internal}$ $(mg/kg\text{-}day)^{-1}$	$P_{human\_internal}$[a] $(mg/kg\text{-}day)^{-1}$	Human unit risk value[b] $(mg/m^3)^{-1}$	Model Goodness-of-fit test[c]
Male rats	Renal tubule carcinoma or adenoma	0.004355	0.0082	0.0018	Multistage (order = 3) p = 0.57
	Testicular interstitial cell adenoma	0.004570	0.0086	0.0019	Quantal Quadratic p = 0.87
Female rats	Renal tubule adenoma	0.001443	0.0029	0.00064	Multistage (order = 3) p = 0.98
Male mice	Lung alveolar/ bronchiolar carcinoma or adenoma	0.003557	0.0090	0.0020	Multistage (order = 3) p = 0.99
		0.001595[d]	0.0040[d]	0.00088[d]	Quantal Linear p = 0.93
Female mice	Liver hepatocellular carcinoma or adenoma	0.002604	0.0066	0.0015	Multistage (order = 3) p = 0.86
		0.0007523[d]	0.0019[d]	0.00042[d]	Quantal Quadratic p = 0.94[d]

a. The interspecies extrapolation of $(BWh/BWa)^{1/8}$ was applied to $P_{animal\_internal}$ in $(mg/kg\text{-}d)^{-1}$ to determine $P_{human\_internal}$ $(mg/kg\text{-}day)^{-1}$, as described above.

b. Unit risk was determined by multiplying the human internal cancer potency in $(mg/kg\text{-}day)^{-1}$ by the human breathing rate (20 m^3/day) divided by human body weight (70 kg) and by an uptake factor of 0.77, as described above.

c. A *p*-value of 0.1 or greater for the chi-square goodness-of-fit test indicates an adequate fit with the BMD procedure.

d. These values obtained with PBPK model adjusted to approximate the mouse pharmacokinetic data of Charest-Tardif *et al.* (2006).

Benchmark dose approach

Tables 8b and 8d list the P_{animal}, P_{human}, and human unit risk values based on the BMD approach. The cancer potencies and unit risk values were derived using the applied LTWA doses and PBPK adjusted internal doses, as described above. As expected the results from the BMD approach are quite similar to those just described using the LMS approach. Unit risk values ranged from 0.00042 to 0.0063 $(mg/m^3)^{-1}$. When LTWA doses are used, the most sensitive sites are the male rat testicular interstitial cell adenoma and the male rat kidney adenoma and carcinoma. When PBPK doses are used, the most sensitive sites are the male rat testicular interstitial cell adenomas and the male mice lung tumors. Regardless of whether LTWA or PBPK doses are used, the unit risk values based on male rat kidney, male mouse lung, and female mouse liver are comparable at approximately 0.002 $(mg/m^3)^{-1}$ The results based on the Charest-Tardif PBPK parameters are about a factor of two to four less than those based on the PBPK parameters from Haddad, again indicating some of the uncertainty in the PBPK approach.

As discussed above, the male rat testicular tumors are not considered appropriate for unit risk and potency estimation because of the high background rate. The preferred unit risk value of 0.0025 $(mg/m^3)^{-1}$, is derived from the male rat kidney data based on LTWA doses with the LMS method. The value derived using the BMD approach based on LTWA doses is not significantly different (0.0026 $(mg/m^3)^{-1}$).

Human PBPK Models

Initial predictions of risk-specific exposure concentrations from a human PBPK model used metabolic parameters from Haddad *et al.* (2001), two exposure scenarios, and two methods of risk estimation. The exposure scenarios utilized were an occupational-like time of exposure (8.0 hr exposure/day x 5 d/week; 7 days simulation) and a continuous environmental time of exposure (24 hr/d x 7d/week; 10 days simulation). Two methods of risk estimation were used. In method I a human potency value, P_{human}, was used to estimate an internal dose equivalent to 1 x 10^{-6} lifetime theoretical risk (e.g., 10^{-6} risk/0.0087 $(mg/kg`-d)^{-1}$ = 1.15 x 10^{-4} mg/kg-d.) The human PBPK model with differing exposure scenarios was then used to estimate the external ethylbenzene concentrations resulting in that internal dose. In method II the animal LED_{10} was divided by 10^5 to obtain the 10^{-6} risk specific dose and the equivalent external concentration was adjusted for possible pharmacodynamic (PD) differences between rats and humans (i.e., $(70/0.45)^{1/8}$). For the tumor site of male rat kidney the 1 x 10^{-6} values from the human models vary by 2-fold (0.48 to 0.79 ppb; Table 9). The same analysis was repeated with the human metabolic parameters from Sams *et al.* (2004) and the range was similar (0.33 to 0.74 ppb). PBPK models with higher body weight of 90 kg and 40% body fat gave only slightly higher ppb predictions. According to the discussion above, the preferred value for the unit risk of ethylbenzene is 2.5 x 10^{-6} $(\mu g/m^3)^{-1}$, based on the data for male rat kidney tumors. With the human model, unit risk estimates ranged from 1.27 x 10^{-6} to 3.06 x 10^{-6} ppb^{-1} (2.9 x 10^{-7} to 7.0 x 10^{-7} $[\mu g/m^3]^{-1}$ at 4.35 $\mu g/m^3$/ppb) or somewhat lower than the animal PBPK based values. These unit risk estimates from the human PBPK

models were not used as final values due to issues of tumor site concordance and human variability and parameter uncertainty.

Table 9. Estimates of Exposure Levels (ppb) for 10^{-6} Theoretical Lifetime Cancer Risk, based on Human PBPK Modeling[a]

Method/Model	Occupational Scenario	Environmental Scenario
I . Human Potency based		
70 kg human 20% fat Haddad	0.70	0.50
20% fat Sams	0.66	0.33
90 kg human 40% fat Haddad	0.79	0.56
40% fat Sams	0.74	0.34
II. Animal LED_{10} based		
70 kg human 20% fat Haddad	0.68	0.48
20% fat Sams	0.64	0.32
90 kg human 40% fat Haddad	0.74	0.53
40% fat Sams	0.69	0.34

[a]Note: Values are calculated for 1×10^{-6} theoretical lifetime cancer risk. Occupational scenario was 8.0 hr/d x 5 days/week, for one-week simulations; environmental scenario was continuous exposure for one week. Method I used the human potency (Ph) in $(mg/kg\text{-}d)^{-1}$ to calculate a 10^{-6} risk internal dose in metrics of ethylbenzene metabolized by the liver (AMET, µmol/d). Method II uses the animal LED_{10} to calculate a 10^{-6} risk dose. The human models were the 70 kg default with 20% fat and a 90 kg variant with 40% fat (and comparatively less muscle). The Ph was based on the male rat kidney tumors of 0.0087 $(mg/kg\text{-}d)^{-1}$. Inhalation was 20 m^3/d. The models were run with metabolic parameters from Haddad *et al.* (2001) and Sams *et al.* (2004).

Conclusion

The male rat was the most sensitive sex and species tested by NTP (1999) in the inhalation carcinogenesis studies of ethylbenzene. While the highest potency and unit risk values were obtained for rat testicular adenomas, the high background rate of this common tumor made interpretation difficult. NTP considered the increased incidences of renal tubule carcinoma or adenoma to provide clear evidence of the carcinogenic activity of ethylbenzene, and this site was considered to be the more reliable basis for estimating human cancer potency.

Using either the LMS or BMD methodology with different dose metrics, the 95% upper confidence bound on the unit risk value for purposes of calculating cancer risks associated with exposure to ethylbenzene is in the range 4.0×10^{-4} to 6.6×10^{-3} $(mg/m^3)^{-1}$, based on the incidence data from the NTP (1999) studies (Table 10). The unit risk value of 2.5×10^{-3} $(mg/m^3)^{-1}$, or 2.5×10^{-6} $(\mu g/m^3)^{-1}$, based on the renal tubule carcinoma or adenoma incidence data in male rats and using the LMS methodology applied to LTWA doses, is considered the most appropriate for purposes of calculating cancer risks associated with exposure to low levels of ethylbenzene. As noted above and summarized in Table 10 below, unit risks based on the PBPK internal doses were not markedly different than those based on the LTWA doses, and involved a number of assumptions. Because the PBPK modeling is uncertain and the results were relatively insensitive to the approach used, the LMS results based on the LTWA doses were selected as most appropriate. The inhalation cancer potency, from which the unit risk value was derived, is 0.0087 $(mg/kg\text{-}d)^{-1}$. The oral cancer potency value of 0.011 $(mg/kg\text{-}d)^{-1}$ is derived from the inhalation potency value by multiplying by the ratio of the uptake values (i.e., 1/0.77). The inhalation and oral cancer potency values are considered applicable to low dose ethylbenzene exposures.

Table 10. Comparison of unit risk values for ethylbenzene

Species/sex/tumor site	Unit Risk value $(mg/m^3)^{-1}$			
	LTWA doses, LMS approach	LTWA doses, BMD approach	PBPK doses, LMS approach	PBPK doses, BMD approach
Male rat kidney	0.0025	0.0026	0.0018	0.0018
Male rat testicular	0.0066	0.0063	0.0066	0.0019
Female rat kidney	0.00063	0.00054	0.00040	0.00064
Male mouse lung	0.0015	0.0015	0.0021	0.0020
Female mouse liver	0.0017	0.0017	0.0015	0.0015

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Appendix

Berkeley Madonna Model Code Example (Male Rat 75 ppm x 6.25 hr/d x 5days/week, 1 week simulation. If cut and pasted into BM demo program available online this model will run)

```
METHOD Stiff

STARTTIME = 0
STOPTIME= 168
DT = 0.001

{ethylbenzene moles}
init Af = 0
Limit Af >= 0
init Al = 0
Limit Al >= 0
init Am = 0
Limit Am >= 0
init Avrg = 0
Limit Avrg >= 0
init Alu = 0
Limit Alu >= 0

{moles  metabolized}
init Ametl = 0
init Ametlg = 0

{tissue flows L/hr}
Qtot = 15*BW^0.7
Qalv = 15*BW^0.7
Qf = 0.09*Qtot
Qvrg = 0.51*Qtot
Ql = 0.25*Qtot
Qm = 0.15*Qtot
Qlu = Qtot

{tissue volumes L}
Vf = 0.09*BW
Vl = 0.049*BW
Vm = 0.72*BW
Vvrg = 0.036*BW
Vlu = 0.014*BW
BW = 0.45

{blood/air and tissue/blood partition coefficients, unitless}
Pb = 28.0
Pl = 2.99
Pf = 55.57
Pm = 0.93
Pvrg = 1.41
Plu = 2.15
```

```
{ethylbenzene metabolic parameters, CLh, Vmax mol/hr, Km, M}
VmaxC = 6.39
Vmax = VmaxC*BW^0.75/(1000*106.16)
Km = 1.04/(1000*106.16)
{exposure in ppm converted to moles/L}
Cair = IF TIME <= 6.25 THEN 75*(1E-6/25.45) ELSE IF (24<TIME) AND (TIME <= 30.25) THEN
75*(1E-6/25.45) ELSE IF (48<TIME) AND (TIME <= 54.25) THEN 75*(1E-6/25.45) ELSE IF
(72<TIME) AND (TIME <= 78.25) THEN 75*(1E-6/25.45) ELSE IF (96<TIME) AND (TIME <=
102.25) THEN 75*(1E-6/25.45) ELSE 0

{calculated concentrations of ethylbenzene}
Cart = Pb*(Qalv*Cair + Qtot*Cvtot)/(Pb*Qtot + Qalv)
Cvf = Af/(Vf*Pf)
Cvl = Al/(Vl*Pl)
Cvvrg = Avrg/(Vvrg*Pvrg)
Cvm = Am/(Vm*Pm)
Cvlu = Alu/(Vlu*Plu)
Cvtot = (Ql*Cvl + Qf*Cvf + Qm*Cvm + Qvrg*Cvvrg)/Qtot
Cexh = Cart/Pb
Tmass = Ametl + Alu + Al + Af + Am + Avrg

{differential equations for ethylbenzene uptake and metabolism}
d/dt(Alu) = Qtot*(Cvtot - Cvlu)
d/dt(Al) = Ql*(Cart - Cvl)  - Vmax*Cvl/(Km + Cvl)
d/dt(Af) = Qf*(Cart - Cvf)
d/dt(Avrg) = Qvrg*(Cart - Cvvrg)
d/dt(Am) = Qm*(Cart - Cvm)

{amount of ethylbenzene metabolized}
d/dt(Ametl)  = Vmax*Cvl/(Km + Cvl)
d/dt(Ametlg) = (Vmax*Cvl/(Km + Cvl))/BW
init AUCvtot = 0
init AUCvl = 0
d/dt(AUCvtot) = Cvtot
d/dt(AUCvl) =  Cvl
```

CHRONIC TOXICITY SUMMARY

ETHYLBENZENE

(Phenylethane; NCI-C56393)

CAS Registry Number: 100-41-4

I. Chronic Toxicity Summary

Inhalation reference exposure level	**2000 µg/m^3 (400 ppb)**
Critical effect(s)	Liver, kidney, pituitary gland in mice and rats
Hazard index target(s)	Alimentary system (liver); kidney; endocrine system

II. Physical and Chemical Properties (HSDB, 1994)

Description	colorless liquid
Molecular formula	C_8H_{10}
Molecular weight	106.16 g/mol
Boiling point	136.2°C
Melting point	-95°C
Vapor pressure	10 torr @ 25.9°C
Density	0.867 g/cm^3 @ 20°C
Solubility	Soluble in ethanol and ether, low solubility in water (0.014 g/100 ml at 15°C)
Conversion factor	1 ppm = 4.35 mg/m^3

III. Major Uses or Sources

Ethylbenzene is used as a precursor in the manufacture of styrene (HSDB, 1994). It is also used in the production of synthetic rubber, and is present in automobile and aviation fuels. It is found in commercial xylene (Reprotext, 1994). In 1996, the latest year tabulated, the statewide mean outdoor monitored concentration of ethylbenzene was approximately 0.4 ppb (CARB, 1999a). The latest annual statewide emissions from facilities reporting under the Air Toxics Hot Spots Act in California, based on the most recent inventory, were estimated to be 161,846 pounds of ethylbenzene (CARB, 1999b).

IV. Effects of Human Exposure

Studies on the effects of workplace exposures to ethylbenzene have been complicated by concurrent exposures to other chemicals, such as xylenes (Angerer and Wulf, 1985). Bardodej

and Cirek (1988) reported no significant hematological or liver function changes in 200 ethylbenzene production workers over a 20-year period.

V. Effects of Animal Exposure

Rats and mice (10/sex/group) were exposed to 0, 100, 250, 500, 750, and 1000 ppm (0, 434, 1086, 2171, 3257, and 4343 mg/m^3) ethylbenzene 6 hours/day, 5 days/week for 90 days (NTP, 1988; 1989; 1990). Rats displayed significantly lower serum alkaline phosphatase in groups exposed to 500 ppm or higher. Dose-dependent increases in liver weights were observed in male rats beginning at 250 ppm, while this effect was not seen until 500 ppm in the females. An increase in relative kidney weights was seen in the 3 highest concentrations in both sexes. Minimal lung inflammation was observed in several of the treatment groups, but this phenomenon was attributed to the presence of an infectious agent rather than to ethylbenzene exposure. The mice in this study did not show any treatment-related effects except for elevated liver and kidney weights at 750 and 1000 ppm, respectively.

Rats and mice were exposed to ethylbenzene (greater than 99% pure) by inhalation for 2 years (NTP, 1999; Chan *et al.*, 1998). Groups of 50 male and 50 female F344/N rats were exposed to 0, 75, 250, or 750 ppm, 6 hours per day, 5 days per week, for 104 weeks. Survival of male rats in the 750 ppm group was significantly less than that of the chamber controls. Mean body weights of 250 and 750 ppm males were generally less than those of the chamber controls beginning at week 20. Mean body weights of exposed groups of females were generally less than those of chamber controls during the second year of the study. In addition to renal tumors, the incidence of renal tubule hyperplasia in 750 ppm males was significantly greater than that in the chamber controls. The severity of nephropathy in 750 ppm male rats was significantly increased relative to the chamber controls. Some increases in incidence and severity of nephropathy were noted in all exposed female rats, but these were statistically significant only at 750 ppm.

Groups of 50 male and 50 female B6C3F1 mice were exposed to 0, 75, 250, or 750 ppm ethylbenzene by inhalation, 6 hours per day, 5 days per week, for 103 weeks. Survival of exposed mice was similar to controls. Mean body weights of females exposed to 75 ppm were greater than those of the chamber controls from week 72 until the end of the study. In addition to lung and liver tumors, the incidence of eosinophilic liver foci in 750 ppm females was significantly increased compared to that in the chamber controls. There was a spectrum of nonneoplastic liver changes related to ethylbenzene exposure in male mice, including syncytial alteration of hepatocytes, hepatocellular hypertrophy, and hepatocyte necrosis. The incidences of hyperplasia of the pituitary gland pars distalis in 250 and 750 ppm females and the incidences of thyroid gland follicular cell hyperplasia in 750 ppm males and females were significantly increased compared to those in the chamber control groups. Based on an evaluation of all the non-cancer data in mice and rats OEHHA staff selected 75 ppm as the NOAEL for the NTP (1999) study.

Rats (17-20 per group) were exposed to 0, 600, 1200, or 2400 mg/m^3 for 24 hours/day on days 7 to 15 of gestation (Ungvary and Tatrai, 1985). Developmental malformations in the form of "anomalies of the uropoietic apparatus" were observed at the 2400 mg/m^3 concentration.

Skeletal retardation was observed in all exposed groups compared with controls. The incidence of skeletal abnormalities increased with higher concentrations of ethylbenzene.

Rabbits exposed by these investigators to the same concentrations as the rats on days 7 to 15 of gestation, exhibited maternal weight loss with exposure to 1000 mg/m^3 ethylbenzene. There were no live fetuses in this group for which abnormalities could be evaluated. No developmental defects were observed in the lower exposure groups.

Rats (78-107 per group) and rabbits (29-30 per group) were exposed for 6 or 7 hours/day, 7 days/week, during days 1-19 and 1-24 of gestation, respectively, to 0, 100, or 1000 ppm (0, 434, or 4342 mg/m^3) ethylbenzene (Andrew *et al.*, 1981; Hardin *et al.*, 1981). No effects were observed in the rabbits for maternal toxicity during exposure or at time of necropsy. Similarly, no effects were seen in the fetuses of the rabbits. The only significant effect of ethylbenzene exposure in the rabbits was a reduced number of live kits in the 1000 ppm group. A greater number and severity of effects were seen in rats exposed to 1000 ppm ethylbenzene. Maternal rats exposed to 1000 ppm exhibited significantly increased liver, kidney, and spleen weights compared with controls. Fetal rats showed an increase in skeletal variations at the 1000 ppm concentration, but the results of the 100 ppm exposure were not conclusive.

Clark (1983) found no significant effects on body weight, food intake, hematology, urinalysis, organ weights or histopathology in rats (18 per group) exposed to 100 ppm (434 mg/m^3) ethylbenzene for 6 hours/day, 5 days/week, for 12 weeks.

Degeneration of the testicular epithelium was noted in guinea pigs and a rhesus monkey exposed to 600 ppm (2604 mg/m^3) for 6 months (Wolf *et al.*, 1956). No effects were reported for female monkeys exposed to the same conditions.

Cragg *et al.* (1989) exposed mice and rats (5/sex/group) to 0, 99, 382, and 782 ppm (0, 430, 1659, and 3396 mg/m^3) 6 hours/day, 5 days/week for 4 weeks. Some evidence of increased salivation and lacrimation was seen in the rats exposed to 382 ppm. No other gross signs of toxicity were observed. Both male and female rats had significantly enlarged livers following exposure to 782 ppm. Female mice also showed a significant increase in liver weight at this concentration. No histopathological lesions were seen in the livers of these mice.

Dose-dependent induction of liver cytochrome P450 enzymes in rats by ethylbenzene was observed by Elovaara *et al.* (1985). Rats (5 per group) were exposed to 0, 50, 300, or 600 ppm (0, 217, 1302, or 2604 mg/m^3) ethylbenzene for 6 hours/day, 5 days/week for 2, 5, 9, or 16 weeks. Cytochrome P450 enzyme induction, and microscopic changes in endoplasmic reticulum and cellular ultrastructure were evident at all ethylbenzene concentrations by week 2, and persisted throughout the exposure. Liver weights were not elevated in these studies.

VI. Derivation of the Chronic Reference Exposure Level

Study	NTP, 1999; Chan *et al.*, 1998
Study population	Male and female rats and mice (50 per group)
Exposure method	Discontinuous inhalation
Critical effects	Nephrotoxicity, body weight reduction (rats) hyperplasia of the pituitary gland; liver cellular alterations and necrosis (mice)
LOAEL	250 ppm
NOAEL	75 ppm
Exposure continuity	6 hours/day, 5 days/week
Exposure duration	103 weeks.
Average experimental exposure	13 ppm for NOAEL group
Human equivalent concentration	13 ppm for NOAEL group (gas with systemic effects, based on RGDR = 1.0 using default assumption that lambda (a) = lambda (h))
LOAEL uncertainty factor	1
Subchronic uncertainty factor	1
Interspecies uncertainty factor	3
Intraspecies uncertainty factor	10
Cumulative uncertainty factor	30
Inhalation reference exposure level	0.4 ppm (400 ppb; 2 mg/m^3; 2,000 µg/m^3)

The REL is based on a lifetime toxicity/carcinogenesis study. The NOAEL for non-neoplastic effects in the study was 75 ppm, and the LOAEL was 250 ppm. Some shorter duration studies discussed above (e.g. NTP, 1988, 1989, 1990) identify higher concentrations as NOAELs, but the study used (NTP 1999) is the most recent available and is considered the most reliable for assessing chronic effects.

U.S. EPA based its RfC on developmental toxicity studies in rats and rabbits (Andrew *et al.*, 1981; Hardin *et al.*, 1981; U.S. EPA, 1994). The NOAEL in the studies was 100 ppm, and the LOAEL was 1000 ppm. In accordance with its methodology, U.S. EPA did not use a time-weighted average concentration for the discontinuous exposure experiment since the key effect was developmental toxicity. If OEHHA methodology is followed (which includes the time-weighted averaging of the exposure concentrations, and uncertainty factors of 3 (interspecies, with RGDR = 1) and 10 (intraspecies), this study would indicate a REL of 0.6 ppm (3 mg/m^3). The study by Ungvary and Tatrai (1985) reported a NOAEL of 600 mg/m^3 for developmental and maternal effects in several species. However, the reporting and general quality of this paper create less confidence in its results.

For comparison to the proposed REL of 0.4 ppm, Clark (1983) found no significant effects in rats exposed to 100 ppm ethylbenzene 6 h/day, 5 d/week, for 12 weeks. This NOAEL can be time-adjusted to 18 ppm, then divided by a subchronic UF of 3, an interspecies UF of 3, and an intraspecies UF of 10 which results in a REL of 0.2 ppm. (The default value of 1 for RGDR was used). It appears that the proposed REL provides a sufficient margin of safety to provide

protection against the reported developmental effects (Andrew *et al.*, 1981; Hardin *et al.*, 1981; Ungvary and Tatrai, 1985)

VII. Data Strengths and Limitations for Development of the REL

The strengths of the inhalation REL for ethylbenzene include the availability of controlled exposure inhalation studies in multiple species at multiple exposure concentrations and with adequate histopathogical analysis, and the observation of a NOAEL in lifetime chronic inhalation exposure studies. The major area of uncertainty is the lack of adequate human exposure data.

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Public Health Goal for ETHYLBENZENE in Drinking Water

Prepared by
Pesticide and Environmental Toxicology Section
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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LIST OF CONTRIBUTORS

PHG PROJECT MANAGEMENT	REPORT PREPARATION	SUPPORT
Project Officer Anna Fan, Ph.D. ***Chemical Prioritization Report Outline*** Joseph Brown, Ph.D. Coordinator David Morry, Ph.D. Yi Wang, Ph.D. ***Document Development*** Michael DiBartolomeis, Ph.D. Coordinator George Alexeeff, Ph.D. Hanafi Russell, M.S. Yi Wang, Ph.D. ***Public Workshop*** Michael DiBartolomeis, Ph.D. Coordinator Judy Polakoff, M.S. Organizer ***Methodology/Approaches/Review Comments*** Joseph Brown, Ph.D. Robert Howd, Ph.D. Coordinators Lubow Jowa, Ph.D. David Morry, Ph.D. Rajpal Tomar, Ph.D. Yi Wang, Ph.D.	***Author*** John Faust, Ph.D. ***Primary Reviewer*** Robert Howd, Ph.D. ***Secondary Reviewer*** Michael DiBartolomeis, Ph.D. ***Final Reviewers*** Anna Fan, Ph.D. William Vance, Ph.D. ***Editor*** Michael DiBartolomeis, Ph.D.	***Administrative Support*** Edna Hernandez Coordinator Laurie Bliss Sharon Davis Kathy Elliott Vickie Grayson Michelle Johnson Juliet Rafol Genevieve Shafer Tonya Turner ***Library Support*** Mary Ann Mahoney Valerie Walter ***Website Posting*** Robert Brodberg, Ph.D. Edna Hernandez Laurie Monserrat, M.S. Judy Polakoff, M.S. Hanafi Russell, M.S.

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PREFACE

Drinking Water Public Health Goal of the
Office of Environmental Health Hazard Assessment

This Public Health Goal (PHG) technical support document provides information on health effects from contaminants in drinking water. The PHG describes concentrations of contaminants at which adverse health effects would not be expected to occur, even over a lifetime of exposure. PHGs are developed for chemical contaminants based on the best available toxicological data in the scientific literature. These documents and the analyses contained in them provide estimates of the levels of contaminants in drinking water that would pose no significant health risk to individuals consuming the water on a daily basis over a lifetime.

The California Safe Drinking Water Act of 1996 (amended Health and Safety Code, Section 116365) requires the Office of Environmental Health Hazard Assessment (OEHHA) to adopt PHGs for contaminants in drinking water based exclusively on public health considerations. The Act requires OEHHA to adopt PHGs that meet the following criteria:

1. PHGs for acutely toxic substances shall be set at levels at which scientific evidence indicates that no known or anticipated adverse effects on health will occur, plus an adequate margin-of-safety.
2. PHGs for carcinogens or other substances which can cause chronic disease shall be based solely on health effects without regard to cost impacts and shall be set at levels which OEHHA has determined do not pose any significant risk to health.
3. To the extent the information is available, OEHHA shall consider possible synergistic effects resulting from exposure to two or more contaminants.
4. OEHHA shall consider the existence of groups in the population that are more susceptible to adverse effects of the contaminants than a normal healthy adult.
5. OEHHA shall consider the contaminant exposure and body burden levels that alter physiological function or structure in a manner that may significantly increase the risk of illness.
6. In cases of scientific ambiguity, OEHHA shall use criteria most protective of public health and shall incorporate uncertainty factors of noncarcinogenic substances for which scientific research indicates a safe dose-response threshold.
7. In cases where scientific evidence demonstrates that a safe dose-response threshold for a contaminant exists, then the PHG should be set at that threshold.
8. The PHG may be set at zero if necessary to satisfy the requirements listed above.
9. OEHHA shall consider exposure to contaminants in media other than drinking water, including food and air and the resulting body burden.
10. PHGs adopted by OEHHA shall be reviewed periodically and revised as necessary based on the availability of new scientific data.

PHGs adopted by OEHHA are for use by the California Department of Health Services (DHS) in establishing primary drinking water standards (State Maximum Contaminant Levels, or MCLs). Whereas PHGs are to be based solely on scientific and public health considerations without regard to economic cost considerations, drinking water standards adopted by DHS are to consider economic factors and technical feasibility. For this reason PHGs are only one part of the information used by DHS for establishing drinking water standards. PHGs established by OEHHA exert no regulatory burden and represent only non-mandatory goals. By federal law, MCLs established by DHS must be at least as stringent as the federal MCL if one exists.

PHG documents are developed for technical assistance to DHS, but may also benefit federal, state and local public health officials. While the PHGs are calculated for single chemicals only, they may, if the information is available, address hazards associated with the interactions of contaminants in mixtures. Further, PHGs are derived for drinking water only and are not to be utilized as target levels for the contamination of environmental waters where additional concerns of bioaccumulation in fish and shellfish may pertain. Often environmental water contaminant criteria are more stringent than drinking water PHGs, to account for human exposures to a single chemical in multiple environmental media and from bioconcentration by plants and animals in the food chain.

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SUMMARY

A Public Health Goal (PHG) of 0.3 mg/L (300 ppb) is developed for ethylbenzene in drinking water. U.S. EPA determined that ethylbenzene is not classifiable as to human carcinogenicity (Group D). Therefore, the PHG calculation is based on noncarcinogenic effects observed in experimental animals. The National Toxicology Program (NTP) study cited in the development of the PHG provides evidence of hepatotoxicity in mice exposed to 250 ppm ethylbenzene in air for two years. This type of effect is consistent with other reports on the toxicity of ethylbenzene. A no-observed-adverse-effect-level (NOAEL) for hepatotoxicity was determined to be 75 ppm from the NTP study, corresponding to a daily dose of 49 mg/kg. For the calculation of the PHG, factors accounting for uncertainty in inter-species extrapolation, potentially sensitive human subpopulations and the potential for a severe effect (cancer) were incorporated, for a cumulative uncertainty factor of 1,000. Based on these considerations, OEHHA calculates a PHG for ethylbenzene of 0.3 mg/L (300 ppb).

INTRODUCTION

The purpose of this document is to develop a PHG for ethylbenzene in drinking water. In an evaluation of the available literature as of 1991, the U.S. Environmental Protection Agency (U.S. EPA) determined that ethylbenzene is not classifiable as to human carcinogenicity (Group D; U.S. EPA, 1991a). The International Agency for Research on Cancer (IARC) has not evaluated the carcinogenicity of ethylbenzene.

In this document, we evaluate the available data on the toxicity of ethylbenzene, with the primary focus on the literature related to oral exposures which may be most appropriate for the establishment of a PHG for drinking water. To determine a public health-protective level for ethylbenzene in drinking water, an effort was made to identify more sensitive subgroups in the general population (and if there is inadequate information to identify such groups, appropriate uncertainty factors were incorporated into the PHG). The studies which can be used to identify public health-protective levels are reviewed and evaluated.

CHEMICAL PROFILE

Ethylbenzene (phenylethane; CAS No. 100-41-4) is a colorless liquid at room temperature with the molecular formula C_8H_{10} and a molecular weight of 106.16 g/mole (4.42 mg/m^3 per ppm in air at 20°C) (chemical data from HSDB, 1997, except as noted). It has a melting point of -95.0°C, a boiling point of 136.2°C and a vapor pressure of 10 mm Hg at 25.9°C. It is minimally soluble in water (140 mg/L at 15°C), but is miscible with many organic solvents, including ethanol and ethyl ether.

Ethylbenzene has an odor which has been described as aromatic, pungent or sweet and gasoline-like (HSDB, 1997). The odor threshold has been approximated at 2.3 ppm (Amoore and Hautala, 1983), although lower values have been reported (0.09 to 0.6 ppm) (AIHA, 1989). A taste threshold in water has been estimated at 0.1 mg/L (Fazzalari, 1978).

PRODUCTION AND USE

The primary use of ethylbenzene is as a chemical intermediate in the production of styrene monomer, which accounts for more than 99% of its use (HSDB, 1997). It has been used in the manufacture of synthetic rubber, acetophenone and cellulose acetate. As an organic liquid, it also has use as an industrial solvent for insecticides and acetophenone and as a diluent in the paint industry (a replacement for benzene). Ethylbenzene is a component of gasoline (added to 2% by weight as an anti-knocking agent) and is also present in preparations of naphtha, asphalt and xylene.

Most ethylbenzene is produced by the Friedel-Crafts alkylation reaction with benzene, ethylene and an aluminum chloride catalyst and promoter (Fishbein, 1985). Production of ethylbenzene in the United States (U.S.) has been estimated at 11.76 billion pounds (1993), while 1983 estimates of ethylbenzene imports were 87 million pounds (HSDB, 1997). For the years 1982 and 1983, ethylbenzene ranked among the top 20 chemical products (Fishbein, 1985).

ENVIRONMENTAL OCCURRENCE AND HUMAN EXPOSURE

The high level of production and use of ethylbenzene in industry results in the potential for contamination of air, soil and water. As a component of crude petroleum and a product of combustion, ethylbenzene is also a naturally occurring compound (Fishbein, 1985).

Air

The presence of ethylbenzene in gasoline as well as its common use as a solvent results in a significant potential for release to air. Urban air has been shown to have higher levels of ethylbenzene than rural air. Vehicle emissions have been proposed to be the major contributor to air contamination (ATSDR, 1990). Estimates of ethylbenzene in urban air have included levels up to 23.1 ppb and a range of 3 to 15 ppb ethylbenzene (Fishbein, 1985; ATSDR, 1990, citing Jonsson *et al.*, 1985). Other surveys have reported remote or rural air levels of ethylbenzene of less than 0.2 ppb and suburban and urban median concentrations of 0.6 ppb; higher estimates near roads have been reported (10 to 16 ppb) (ATSDR, 1990).

The use of consumer products containing xylene and ethylbenzene such as degreasers, insecticides, lacquers and paint removers results in the potential for exposure of the general population. Ethylbenzene has also been identified as a component of tobacco smoke. Because of the enclosed environment, indoor air estimates of ethylbenzene frequently are higher than those outdoor.

Toxic Release Inventory (TRI) data for California indicate that for the years 1987 to 1994, air releases ranged from 89,836 to 211,362 pounds (U.S. EPA, 1997). Nationwide air emissions for 1988 were estimated at 47 billion pounds (ATSDR, 1990).

Soil

Soil contamination by ethylbenzene may potentially occur through fuel spillage, solvent disposal or storage tank leakage. Of the hazardous waste sites tested for ethylbenzene contamination, 25% showed detectable levels with a geometric mean soil concentration of 67 ppb (ATSDR, 1990).

Water

Water has the potential to become contaminated by ethylbenzene from its use in industry (discharges), as a fuel component and by storage tank leakage. Drinking water supplies taken near leaking gasoline storage tanks or from surface waters have the highest potential for contamination.

Among the approximately 4% of surface water samples in which ethylbenzene has been detected, the geometric mean concentration was approximately 340 ppb (ATSDR, 1990; citing U.S. EPA, 1989). Median concentrations for surface water samples, however, were reported to be less than 5 ppb. Among the approximately 11% of ground water samples in which ethylbenzene has been detected, the mean concentration was approximately 70 ppb.

Public drinking water samples in Rhode Island were reported to have ethylbenzene levels ranging from 1 to 3 ppb (ATSDR, 1990). Likewise, water supplies in New Orleans (1974) were reported to contain 1.6 to 2.3 ppb ethylbenzene. When detected, well water sampling has generally shown higher ethylbenzene concentrations.

Food

There are some reports of measurable quantities of ethylbenzene in food products (ATSDR, 1990; citing Lovegren *et al.*, 1979). Concentrations of 0.005 to 0.013 ppm have been measured for food products such as split peas, lentils and beans.

The chemical and pharmacokinetic properties (low bioconcentration factor, rapid metabolism - see below) of ethylbenzene suggest little potential for significant bioaccumulation in aquatic organisms.

METABOLISM AND PHARMACOKINETICS

Absorption

Both oral and inhalation exposure of human subjects to ethylbenzene results in rapid absorption (NTP, 1996; citing Bardodej and Bardodejova, 1970; Climie *et al.*, 1983). Inhalation exposure of 18 human male volunteers to 100, 187, 200 or 370 mg/m^3 ethylbenzene resulted in an absorption estimate of 64% (Bardodej and Bardodejova, 1970). Inhalation exposure of rats to 1 mg/L for six hours resulted in an absorption estimate of 44%, although the possibility of dermal absorption (due to whole-body exposure) was not considered (Chin *et al.*, 1980). Six human volunteers exposed to 18, 34, 80 and 200 mg/m^3 ethylbenzene demonstrated a lung retention of 49% of the ethylbenzene vapors (Gromiec and Piotrowski, 1984).

The dermal absorption of ethylbenzene has been studied in 14 human volunteers exposed to aqueous solutions of 112 and 156 mg/L (Dutkiewicz and Tyras, 1967). The skin absorption rate was determined to be 0.12 and 0.21 mg/cm^2/hour which was described as rapid relative to other organic compounds such as benzene and styrene. Dermal absorption of liquid ethylbenzene was also estimated at 22 to 33 mg/cm^2-hour (Dutkiewicz and Tyras, 1967). Percutaneous absorption of ethylbenzene in rat skin (*in vitro*) was estimated at 105 ng/cm^2-minute (approximately 0.064 mg/cm^2-hour) (Tsuruta, 1982). Total percutaneous absorption of 3.4% of the applied dose (occluded) was estimated for ethylbenzene applied to hairless mice (Susten *et al.*, 1990).

Distribution

The low solubility of ethylbenzene in blood and moderate lipophilicity will result in rapid distribution to all tissues, including the brain. Ethylbenzene does not highly concentrate in any tissue, but reaches equilibrium within a few minutes in rapidly perfused tissues and more slowly, to a higher concentration, in fat.

Exposure of rats to radiolabeled ethylbenzene by inhalation has demonstrated distribution to the liver, gastrointestinal tract and adipose tissue (Chin *et al.*, 1980). Although the experimental design measured ethylbenzene levels within two days, distribution to these sites would be expected to be very rapid. Similarly, oral administration of radiolabeled ethylbenzene to rats resulted in distribution to the liver, kidney, intestine and adipose tissue (Climie *et al.*, 1983). Humans exposed to ethylbenzene orally or by inhalation have exhibited low levels in subcutaneous and body fat (Engstrom and Bjurstrom, 1978; Wolf *et al.*, 1977). Transplacental transport appears to occur as evidenced by the appearance of ethylbenzene in cord blood (HSDB, 1997)

Metabolism and Excretion

1-Phenylethanol (α-methylbenzyl alcohol), mandelic acid and phenylglyoxylic acid have been identified as metabolites in the urine of human subjects exposed to ethylbenzene (Bardodej and Bardodejova, 1970; Engström *et al.*, 1984). Oxidation of the side chain appeared to be the primary metabolic conversion for excretion into urine among human subjects exposed to ethylbenzene by inhalation (150 ppm), while ring oxidation accounted for only 4% of the metabolic products (Engström *et al.*, 1984). Minor metabolites identified in human urine included methylphenyl carbinol and 2-ethylphenol (Bardodej and Bardodejova, 1970).

In rats exposed to ethylbenzene by inhalation, the primary metabolites were 1-phenylethanol, mandelic acid and benzoic acid, although 11 other probable metabolites were identified. Minor metabolites identified were ω-hydroxyacetophenone, 1-phenyl-1,2-ethanediol, acetophenone, p-hydroxyacetophenone and phenylglyoxal (Engström, 1984). Metabolic products were found to be conjugated with glucuronide, sulfate and glycine. Mandelic acid and phenylglyoxylic acid were identified as minor metabolites in another study in rats and rabbits (Kiese and Lenk, 1974). As in the case of human metabolites, side chain oxidation products predominated. Differences in the metabolic products of ethylbenzene in experimental animals and humans have been reported to be minor (NTP, 1996; citing Chin *et al.*, 1980, Climie *et al.*, 1983).

In rats, urinary elimination of total identified metabolites after 48 hours accounted for 59 and 83% of absorbed doses resulting from six hour inhalation exposure to 300 and 600 ppm ethylbenzene, respectively (Engström, 1984). A minor level of respiratory elimination of unchanged ethylbenzene is also likely (HSDB, 1997). Urinary elimination of the metabolite mandelic acid from human volunteers exposed by inhalation was reported to be biphasic, with elimination half-lives of 3.1 and 24.5 hours (Gromiec and Piotrowski, 1984).

TOXICOLOGY

Toxicological Effects in Animals

Acute Effects

Estimates of the LD_{50} from oral exposure to ethylbenzene have included 5.5 g/kg (rat; Smyth *et al.*, 1962) and 3.5 g/kg (rat; Wolf *et al.*, 1956). An LD_{50} estimate from intraperitoneal exposure was 2.3 g/kg (mouse; Lewis, 1992).

Inhalation LC_{50} estimates for ethylbenzene include 4,000 ppm (four-hour, rat) (Smyth *et al.*, 1962), 8,000 ppm (one-hour, rat) (Smyth *et al.*, 1962) and approximately 8,000 and approximately 13,000 ppm (two-hour, mice and rats, respectively) (Ivanov, 1962). Symptoms among affected animals included sleepiness, leukocytosis, pulmonary congestion and hyperemia of the viscera (Yant *et al.*, 1930). Eye and nose irritation of guinea pigs has resulted from short-term exposure to 1,000 to 2,000 ppm ethylbenzene (Lewis, 1992). Higher concentrations (10,000 ppm) have resulted in tremor, ataxia and loss of consciousness and ultimately death to the guinea pigs (Lewis, 1992; ACGIH, 1991). Pulmonary irritation, decreased respiration and anesthesia were observed following 30 minute exposure of mice to ethylbenzene concentrations ranging from 410 to 9,640 ppm ethylbenzene (Nielsen and Alarie, 1982).

An LD_{50} of 15.4 g/kg was estimated in rabbits exposed dermally to ethylbenzene (Smyth *et al.*, 1962).

Three-day exposure of rats to 2,000 ppm ethylbenzene (six hours/day) resulted in significant increases in kidney and liver weight as well as the induction of hepatic cytochrome P_{450} and microsomal enzymes (Toftgård and Nilsen, 1981; Toftgård and Nilsen, 1982). Male rats exposed for three days (six hours/day) to 2,000 ppm ethylbenzene exhibited several biochemical changes, including an increased turnover of dopamine and noradrenaline in the hypothalamus and median eminence, and a 30% decrease in serum prolactin concentrations (Andersson *et al.*, 1981).

Subchronic Effects

F344 rats, B6C3F1 mice and New Zealand white rabbits (five/sex/group) were exposed to 0, 99, 382 or 782 ppm (rats and mice) or 0, 382, 782 or 1,610 ppm (rabbits) ethylbenzene for six hours/day, five days/week for four weeks (Cragg *et al.*, 1989). Among male rats, liver weight was significantly increased in the mid-dose group, while among male and female rats in the high-dose group, liver weight, liver-to-body weight ratio and liver-to-brain weight ratio were increased. Significantly increased liver weight (female mice) and liver-to-brain weight ratios (male and female mice) were observed among animals in the high-dose group. Platelet count and leukocyte count were increased among male and female rats, respectively, in the high-dose group. Neither gross nor microscopic changes in over 30 tissues collected from the animals were observed. Body weight gain was decreased among female rabbits in the high-dose group. Transient decrease in body weight gain was observed among male rabbits in the high-dose group. No clinical chemistry effects were observed in rats or rabbits for a variety of tests. From this study a lowest-observed adverse-effect-level (LOAEL) of 382 ppm and a NOAEL of 99 ppm for rats for changes in liver

weight were identified. For mice an LOAEL of 782 ppm and an NOAEL of 382 ppm for organ weight changes were identified. For rabbits, an LOAEL of 1,610 ppm and an NOAEL of 782 ppm for body weight changes were determined.

F344/N rats and B6C3F1 mice (10/sex/group) were exposed to 0, 100, 250, 500, 750 or 1,000 ppm ethylbenzene for six hours/day, five days/week for 13 weeks (NTP, 1992). Among exposed rats, absolute and relative liver, lung and kidney weights were increased, with the increase in absolute and relative liver weights observed among male rats in the 250 ppm dose group and higher, and among female rats in the 500 ppm dose group and higher. Absolute and relative kidney weights were significantly increased among male and female rats in the 500, 750 and 1,000 ppm dose groups (with the exception of male rats in the 500 ppm dose group where this effect was not significant). Regeneration of the kidney tubules was observed in male rats in all dose groups with increased severity with dose. Serum alkaline phosphatase was significantly increased among male and female rats at doses of 500 ppm and higher. Enlarged lymph nodes (bronchial and mediastinal) and lung inflammation observed in exposed groups was determined by the investigators to be an infection rather than an exposure-related effect, although further evaluation of this observation was recommended. Among male and female mice, absolute and relative liver weights were increased in the two highest dose groups. Among female mice in the high-dose group, relative kidney weights were significantly increased. NTP concluded that there was only minimal evidence for the toxicity of ethylbenzene in rats and mice at the doses tested. In this study, the LOAEL was considered to be 250 ppm ethylbenzene for liver weight changes and increased renal tubular regeneration in rats; the study NOAEL was 100 ppm.

Several species were repeatedly exposed to ethylbenzene by inhalation (Wolf *et al.*, 1956). Among rats (10 to 25/group) exposed to 400, 600, 1,250 or 2,200 ppm ethylbenzene for seven hours/day, five days/week for 186 to 214 days, all groups exhibited slightly increased liver and kidney weights. Rats in the two highest dose groups also exhibited growth depression as well as liver and kidney histopathology characterized as cloudy swelling. Among guinea pigs (5 to 10 per group) similarly exposed to 400, 600 or 1,250 ppm ethylbenzene, animals in the highest dose group exhibited growth depression and those in the mid-dose group exhibited a slight increase in liver weight. Among rabbits (one to two/group) similarly exposed to 400, 600 or 1,250 ppm ethylbenzene, testicular histopathology (degeneration of the germinal epithelium) was observed in the mid-dose group. Among Rhesus monkeys exposed to 400 ppm (two females) or 600 ppm (one male) ethylbenzene, the male exhibited testicular histopathology as well as slightly increased liver weight. An LOAEL of 400 ppm ethylbenzene was established in rats for changes in liver and kidney weights. In guinea pigs an LOAEL of 1,250 ppm was established, with an NOAEL of 600 ppm. The utility of this study is somewhat limited by scant reporting of the experimental findings and, with rabbits and monkeys, a limited number of experimental animals. The nature of the control group for each of the experiments was also unclear.

Female rats (10/group) were also administered ethylbenzene 0, 13.6, 136, 408 or 608 mg/kg-day orally by intubation for six months, five days/week (Wolf *et al.*, 1956). Effects observed in the two highest dose groups included cloudy swelling of liver cells and the renal tubular epithelium with increased liver and kidney weight. The LOAEL for this study is 408 mg/kg-day and the NOAEL is 136 mg/kg-day.

Wistar rats (18/sex/group) were exposed by inhalation to 0 or 100 ppm ethylbenzene for six hours/day, five days/week for 12 weeks (Clark, 1983). No statistically significant adverse effects

were observed among the exposed animals. An NOAEL of 100 ppm ethylbenzene was identified from this study.

In a study of liver effects, male Wistar rats (five/group) were exposed by inhalation to 0, 50, 300 or 600 ppm ethylbenzene for six hours/day, five days/week for 2, 5, 9 or 16 weeks (Elovaara *et al.*, 1985). Proliferation of the smooth endoplasmic reticulum and degranulation of the rough endoplasmic reticulum was evident at two to nine weeks. A number of serum enzyme activities were increased after 16 weeks including NADPH-cytochrome reductase and UDPG-transferase (300 and 600 ppm), and aminopyrine N-demethylase and 7-ethoxycoumarin-O-deethylase (all dose groups).

Six-month exposure of rabbits to 400 mg/kg ethylbenzene (presumably oral) was reported to produce segmentation of the nuclei of blood leukocytes (Pokkrovskii and Volchkova, 1968). Seven-months exposure of rabbits to 100 or 1,000 mg ethylbenzene/m^3 was reported to lead to hematological effects (white blood cell count changes), dystrophia of the liver and kidney and muscle chronaxia (Ivanov, 1962; Ivanov, 1964).

Noncarcinogenic Chronic Effects

Fisher 344/N rats and B6C3F1 mice (50/sex/group) were exposed by inhalation to 0, 75, 250 or 750 ppm ethylbenzene for two years (six hours/day, five days/week) (NTP, 1996). Survival was significantly decreased among high-dose male rats. Among male and female rats in the high-dose group the severity of nephropathy was increased over control animals. It was speculated by the investigators that the reduced survival rate observed among male rats in the high-dose group was caused in part by the exacerbation of nephrotoxicity, which is frequently observed among aging male rats. Cystic degeneration of the liver was significantly increased in the high-dose group. Increased incidences of edema, congestion and hemorrhage of the lungs and hemorrhage of the renal lymph nodes were slightly but significantly increased among animals in the high-dose group, although it was speculated that these were indirect effects among moribund animals. Prostate gland inflammation, characterized as infiltration of mononuclear cells into the glandular acini and interstitium, were also increased in all groups of male rats relative to controls. Hypercellularity of the bone marrow (increased erythroid and myeloid precursors) was increased in animals in both high- and low-dose groups. A clear dose-response was not evident for either the prostate or bone marrow effects. An LOAEL for renal and liver effects was established to be 750 ppm ethylbenzene for rats, with an NOAEL of 250 ppm.

Among male mice, hepatotoxicity was evident and included significantly increased observations of liver hypertrophy (high-dose), necrosis (high-dose) and alterations of hepatic syncytia (mid- and high-dose). Eosinophilic liver foci were significantly increased among female mice in the high-dose group. For B6C3F1 mice, an LOAEL of 250 ppm ethylbenzene is established for hepatotoxicity in males, with a corresponding NOAEL of 75 ppm.

Developmental and Reproductive Toxicity

Female CFY rats (17 to 20/group) were exposed to 0, 600, 1,200 or 2,400 mg/m^3 ethylbenzene (0, 136, 271 and 543 ppm, respectively) continuously from days 7 to 15 of pregnancy (Ungváry and Tátrai, 1985). The authors reported "moderate and dose-dependent" maternal toxicity in rats, although the nature of the toxicity was not presented. Skeletal retardation was also reported among the exposed rats, and the incidences of extra ribs, anomalies of the uropoietic apparatus and

skeletal malformations were increased in the high-dose group. Post-implantation loss was also increased among exposed rats. The LOAEL for rats in this study is 543 ppm ethylbenzene, with an NOAEL of 271 ppm. Female CFLP mice and New Zealand rabbits were exposed to 0, 500 or 1,000 mg/m^3 ethylbenzene (0, 113 and 226 ppm, respectively) continuously from days 6 to 20 of pregnancy. Among mice, an increase in the incidence of skeletal retardation and weight retarded fetuses was observed. Mice also showed an increase in the incidence of anomalies to the uropoietic apparatus. Among rabbits, mild maternal toxicity (decreased weight gain) and increased loss to abortion were observed in the high-dose group. Weight retardation was observed among fetuses in the low-dose group. No teratogenic effects were observed.

Female Wistar rats (78 to 107/group) and New Zealand white rabbits (29 to 30/group) were exposed by inhalation for six to seven hours/day to 0, 100 or 1,000 ppm ethylbenzene during gestational days 1 to 19 (rats) or 1 to 24 (rabbits) (Andrew *et al.*, 1981; also reported in Hardin *et al.*, 1981). There was no evidence of embryotoxicity, fetotoxicity or teratogenicity among rabbits, nor was there evidence of maternal toxicity. A significant decrease in the number of live rabbit kits/litter was observed in both exposed groups, although there was some question regarding the reporting of the data in the low-dose group. Among rat dams in the high-dose group, evidence of toxicity included increases in the absolute and relative weight of the liver, kidney and the spleen. Increased incidences of fetuses with supernumerary and rudimentary ribs (high-dose) and extra ribs (high- and low-dose) were also observed. In this study, the LOAEL was considered to be 1,000 ppm ethylbenzene for developmental effects in rabbits and rats and maternal toxicity in rat dams. The corresponding NOAEL is 100 ppm.

In a supplemental experiment, female rats were exposed to 0, 100 or 1,000 ppm ethylbenzene, six to seven hours/day, for three weeks prior to mating with exposure continuing into pregnancy (Andrew *et al.*, 1981). Among rat dams in the high-dose group, absolute and relative liver and spleen weights were increased and relative kidney weight was increased significantly. Among the fetuses in the high-dose group, the incidence of extra ribs was significantly increased. The LOAEL and NOAEL for this study are 1,000 and 100 ppm ethylbenzene, respectively.

Genetic Toxicity

Five strains of *Salmonella* showed no evidence of mutagenicity from exposure to ethylbenzene either with or without metabolic activation, nor was there evidence in two *Escherichia coli* strains or in a *Saccharomyces cerevisiae* gene conversion assay (Nestmann *et al.*, 1980; Dean *et al.*, 1985; Zeiger *et al.*, 1992; Florin *et al.*, 1980). The lack of mutagenicity of ethylbenzene to *Salmonella* has been confirmed in testing by NTP; additionally no indications of increased sister-chromatid exchange or chromosomal aberrations were observed in Chinese hamster ovary cells (NTP, 1996). Ethylbenzene induced a mutagenic response in a mouse lymphoma assay without metabolic activation, but only at a dose which resulted in cytotoxicity (McGregor *et al.*, 1988; NTP, 1996). In addition, a 13-week exposure of mice by inhalation to ethylbenzene concentrations of 500, 750 or 1,000 ppm did not result in an increase in the frequency of micronucleated erythrocytes (MacGregor *et al.*, 1990).

Carcinogenicity

Fisher 344/N rats and B6C3F1 mice (50/sex/group) were exposed by inhalation to 0, 75, 250 or 750 ppm ethylbenzene for two years (six hours/day, five days/week) (NTP, 1996). Survival rate and mean body weight were lower among male rats in the high-dose group relative to control animals. The incidences of renal tumors among male rats are summarized in Tables 1 and 2. In addition, the incidences of interstitial cell adenoma and renal tubule hyperplasia were significantly increased among male rats in the high-dose group.

Table 1. Kidney Tumors in Male Rats Exposed to Ethylbenzene (Single Sections) (NTP, 1996)

Tumor Type	**Exposure Concentration (ppm)**			
	0	**75**	**250**	**750**
Tubular cell adenoma	0/50	3/50	2/50	4/50*
Tubular cell carcinoma	0/50	0/50	1/50	3/50
Tubular cell tumors (combined)	0/50	3/50	3/50	7/50*

*Significantly increased incidence.

Table 2 presents the results of a further evaluation of renal tumors in male and female rats using the results of the single sections combined with those of step sections. In addition to these observations, the incidences of renal tubule hyperplasia were also increased significantly among both male and female rats in the high-dose group. NTP reported no evidence of hyaline droplet formation in the kidneys in this study (or in the earlier 13-week study), indicating that nephropathy due to the accumulation of $\alpha_{2\mu}$-globulin is unlikely to be the mechanism of kidney toxicity with ethylbenzene.

Table 2. Kidney Tumors in Rats Exposed to Ethylbenzene (Single and Step sections) (NTP, 1996)

Tumor Type	**Exposure Concentration (ppm)**							
	0		**75**		**250**		**750**	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Tubular cell adenoma	3/50	0/50	5/50	0/50	7/50	1/50	20/50*	8/49*
Tubular cell carcinoma	0/50		0/50		1/50		3/50	
Tubular cell tumors (combined)	3/50		5/50		8/50		21/50*	

*Significantly increased incidence.

The incidence of testicular adenomas (interstitial and bilateral) was also increased among high-dose male rats (36/50, control; 33/50, low-dose; 40/50, mid-dose; 44/50, high-dose; $p < 0.05$ by Fisher's Exact Test).

The incidences of several tumor types were increased significantly among the B6C3F1 mice (Table 3). Among male mice in the high-dose group, the incidences of alveolar/bronchiolar adenoma and adenoma or carcinoma (combined) were increased over controls. The incidences among exposed groups, however, was within the range of historical controls (10 to 42% for combined tumors). Among female mice in the high-dose group, the incidences of combined hepatocellular adenoma or carcinoma and hepatocellular adenoma alone were significantly increased over control animals, although again the incidence among exposed animals was within the range of historical controls (3 to 54% for combined tumors).

Table 3. Tumors in B6C3F1 Mice Exposed to Ethylbenzene (NTP, 1996)

Tumor Type	Exposure Concentration (ppm)							
	0		75		250		750	
	Male	Female	Male	Female	Male	Female	Male	Female
Alveolar or bronchiolar adenoma	5/50		9/50		10/50		16/50*	
Alveolar or bronchiolar adenoma + carcinoma	7/50		10/50		15/50		19/50*	
Hepatocellular adenoma		6/50		9/50		12/50		16/50*
Hepatocellular adenoma + carcinoma		13/50		12/50		15/50		25/50*

*Significantly increased incidence ($p < 0.05$).

Thyroid gland follicular cell hyperplasia incidences were increased among male and female mice in the high dose group. Among female mice in the high- and mid-dose groups, the incidences of hyperplasia of the pituitary gland *pars distalis* was significantly increased (10/48, control; 12/49, low-dose; 23/47, mid-dose; 22/49, high-dose; $p < 0.05$ by Fisher's Exact Test).

In another study of the carcinogenicity of ethylbenzene, Sprague-Dawley rats were administered 500 mg/kg ethylbenzene by oral gavage for four or five days/week for 104 weeks (Maltoni *et al.*, 1985). An increase in the incidence of total malignant neoplasms was reported for both male and female rats. Tumor types were not specified in the study.

Weight-of-Evidence for Carcinogenicity

Only two studies have been conducted examining the carcinogenicity of ethylbenzene in experimental animals (NTP, 1996; Maltoni *et al.*, 1985). The study by Maltoni *et al.* (1985) was conducted with only a single dose of ethylbenzene and details of the results were not presented (total tumors). Therefore, the usefulness of the study is limited for the evaluation of carcinogenicity, although a significant increase in total neoplasms was reported.

The chronic bioassay conducted by NTP demonstrated the induction of several tumor types in rats and mice exposed to ethylbenzene by inhalation (NTP, 1996). The study appears to be well-conducted and appropriately designed for the evaluation of the carcinogenicity of the test compound in experimental animals, given the available information on the toxicity of ethylbenzene. Dose selection was made based upon the results of previous subchronic studies and proved to be adequately close to the maximum tolerated dose (MTD) as demonstrated by the limited increase in mortality observed at the end of the two-year study. Significantly increased incidences of tumors included combined renal tubule adenomas and carcinomas in male rats, testicular adenomas in male rats, renal tubule adenomas in female rats, combined alveolar and bronchiolar adenomas and carcinomas in male mice and combined hepatocellular adenomas and carcinomas in female mice. In the case of lung tumors in male mice and liver tumors in female mice, the tumor incidences were within the range of incidences for historical controls.

The most clear evidence of carcinogenicity was demonstrated by the development of renal tubule tumors in male rats. The appearance of renal tubule tumors in male rats raises the possibility that the tumors were induced by a mechanism involving the hyperplastic response mediated by the binding of the test compound to $\alpha_{2\mu}$-globulin leading to accumulation which results in nephrotoxicity and a hyperplastic response, a mechanism which has been hypothesized for certain strains of male rats (including Fisher 344/N) but determined not to be relevant to humans for the purposes of risk assessment because of the absence of significant amounts of $\alpha_{2\mu}$-globulin in humans (U.S. EPA, 1991d). With regard to the involvement of this mechanism in the evaluation of the carcinogenicity of ethylbenzene, there are several observations to consider:

1) The current NTP study as well as the 13-week study which preceded it (NTP, 1992) demonstrated no evidence of the formation of hyaline droplets in the kidneys, a hallmark of the accumulation of $\alpha_{2\mu}$-globulin and a requirement for the induction of nephropathy by this proposed mechanism.

2) There was evidence of renal effects in female rats including a significantly increased incidence of renal tubule adenomas and hyperplasia in the high-dose group as well as an increased severity of nephropathy with increasing dose.

3) Since the $\alpha_{2\mu}$-globulin-mediated effect is specific to male rats, this observation provides evidence that for rats exposed to ethylbenzene, another mechanism leading to renal lesions is likely to be mediating toxicity.

For these reasons, the renal lesions observed in the study were considered relevant to human health risk assessment and the calculation of a PHG for ethylbenzene in drinking water.

While the NTP (1996) study overall provides some evidence for the carcinogenicity of ethylbenzene in experimental animals, there are several issues which need to be considered before a complete appraisal of the carcinogenic effect can be made and its relevance to humans established. These concerns include the contribution of chronic injury or cytotoxicity to tumor development, the appropriateness of using historical controls in decreasing the weight-of-evidence for significantly elevated tumor incidences, and the biological relevance of increased hepatocellular tumors in female B6C3F1 mice.

Toxicological Effects in Humans

Acute Effects

An early report on the toxicity of ethylbenzene in air demonstrated intolerable irritation of the eyes and nose at 5,000 ppm, tearing, dizziness and nose irritation at 2,000 ppm and eye irritation at 1,000 ppm ethylbenzene (Yant *et al.*, 1930). CNS depression occurs at 2,000 ppm ethylbenzene. A later report showed a threshold of 200 ppm ethylbenzene for irritation of the eyes and mucous membranes (Gerarde, 1959). Increasing the exposure level to 2,000 ppm ethylbenzene (six minutes) resulted in dizziness and more severe irritation of the eyes and nose. Eighteen human subjects (male) exposed to 100 ppm ethylbenzene for up to eight hours reported mild irritation of the eyes and respiratory system plus tiredness, insomnia and headache (Bardodej and Bardodejova, 1970). Skin contact may result in erythema and inflammation (Lewis, 1992).

Subchronic Effects

Prolonged inhalation exposure to levels as low as 23 to 230 ppm ethylbenzene may result in leukopenia, lymphocytosis, neurofunctional disorder and hepatitis, while lower levels of exposure (2.3 ppm) may result in inflammation of the mucosa of the upper respiratory tract (HSDB, 1997; citing ILO, 1983).

In an epidemiological study of 200 workers involved in the production of ethylbenzene, no statistically significant differences in hematological parameters (including red and white blood cell counts, platelet counts) or liver function tests (including bilirubin, LDH and SAP levels) were observed between exposed and non-exposed subjects (Bardodej and Cirek, 1988). Exposure levels were not quantitated, but mean duration of exposure was 12.2 years.

Developmental and Reproductive Toxicity

No data have been located in the scientific literature regarding the developmental and reproductive toxicity of ethylbenzene to humans.

Genetic Toxicity

Ethylbenzene slightly increased the incidence of sister chromatid exchange in human whole blood lymphocyte cultures without metabolic activation (Norppa and Vainio, 1983).

Carcinogenicity

No human data have been located in the scientific literature as supporting evidence for the carcinogenicity of ethylbenzene.

DOSE-RESPONSE ASSESSMENT

Noncarcinogenic Effects

Numerous studies have identified adverse noncarcinogenic effects resulting from exposure to ethylbenzene. However, no suitable data are available from epidemiological studies of human populations or case reports of human exposures for conducting a dose-response analysis. The few case reports which are available, as well as the limited number of chamber studies, are limited by inadequate estimation of exposure levels or by insufficient exposure duration for establishing effects which may result from long-term exposure.

Several studies conducted with experimental animals have established minimum levels of exposure associated with adverse toxicological effects (LOAELs) as well as levels without apparent effect (NOAELs). The only chronic exposure study examining toxicity in experimental animals which included noncarcinogenic endpoints is the NTP (1996) inhalation bioassay. High- and mid-dose mice showed evidence of liver toxicity. The LOAEL established from this study is 250 ppm (164 mg/kg-day; see below) with an NOAEL of 75 ppm (49.3 mg-kg/day). In the analysis of a dose-response for the noncarcinogenic effects of ethylbenzene, the inhalation dose rate was converted to an equivalent dose rate in units of mg/kg-day. For rats, this unit conversion was based on 4.42 mg/m^3 per ppm ethylbenzene (at 20°C), a rat breathing rate of 0.26 m^3/day (adjusted for experimental conditions of six hours/day, five days/week) and a rat body weight of 0.35 kg. A reasonable estimate of the fractional absorption of ethylbenzene from inhalation exposure of 50% was used based upon experimental findings in both animals and humans (see "Metabolism and Excretion" above). For mice, the conversion was based on the same defaults with the exception of a mouse breathing rate of 0.05 m^3/day and a mouse mean body weight of 0.03 kg. Therefore, the inhalation doses of 75, 250 and 750 ppm ethylbenzene were converted to daily dose rates of 22.0, 73.3 and 220 mg/kg-day, respectively, for rats and 49.3, 164 and 493 mg/kg-day for mice.

Only two subchronic studies of the toxicity of ethylbenzene by the oral route are available. One is a six-month study with rabbits administered (presumably orally) a single dose level of 400 mg/kg-day showing hematological effects (Pokkrovskii and Volchkova, 1968). The second (Wolf *et al.*, 1956) provided evidence for liver and kidney effects in rats at doses (administered by intubation) as low as 408 mg/kg-day (the LOAEL), with no effects observed at the next lowest dose of 136 mg/kg-day (the NOAEL).

Subchronic inhalation studies have demonstrated a number of effects for ethylbenzene exposure in experimental animals. Cragg *et al.*(1989) observed adverse effects (organ weight changes) in rats exposed to ethylbenzene levels as low as 382 ppm (LOAEL), with no effects observed at 99 ppm (NOAEL). The NTP (1992) studies showed organ weight changes among rats in four dose groups exposed to 250 ppm ethylbenzene (LOAEL) and higher with no effects observed at 100 ppm (NOAEL). Inhalation studies by Wolf *et al.* (1956) showed organ weight effects among rats exposed to 400 ppm ethylbenzene (LOAEL), the lowest dose tested. Clark (1983) observed no

adverse effects in rats exposed to 100 ppm ethylbenzene for 12 weeks. Metabolic enzyme and mild subcellular changes to the liver were observed in the 16-week study by Elovaara *et al.* (1985) at exposure levels as low as 50 ppm ethylbenzene, however it is not clear that the nature of these changes was adverse.

From animal developmental and reproductive toxicity studies, evidence of maternal toxicity was observed in inhalation exposures at 1,000 ppm ethylbenzene (Andrew *et al.*, 1981) with no adverse effects observed at 100 ppm (NOAEL). In the study by Ungváry and Tátrai (1981), the LOAEL was taken to be 543 ppm ethylbenzene for developmental effects in the offspring of exposed rats, with an NOAEL of 271 ppm. Among mice in the same study low-dose (113 ppm) offspring showed weight retardation (LOAEL).

The NOAEL in mice derived from the chronic inhalation studies (NTP, 1996) was selected as the most sensitive endpoint for noncarcinogenic effects despite some uncertainty regarding the route-to-route conversion. Inhalation studies have provided the most consistent evaluation of the toxicity of ethylbenzene, also evidenced by the fairly consistent dose level (when accounting for the exposure regimen) which is without adverse effect in experimental animals in the subchronic exposure studies. There is also the question as to whether the endpoints observed are route-specific. However, the evidence from both inhalation and oral studies suggests there are common endpoints of toxicity, including liver and kidney toxicity. Furthermore, broad toxicity was observed by both routes. While several subchronic studies provided comparable NOAELs, the NTP (1996) chronic exposure study is the most suitable evaluation of noncarcinogenic endpoints for purposes of developing a PHG for ethylbenzene in drinking water because of the chronic nature of the exposure. The value from this study (and the route-converted dose of 49.3 mg/kg-day) has been selected as the overall NOAEL for adverse noncarcinogenic effects from exposure to ethylbenzene in experimental animals.

Carcinogenic Effects

A dose-response evaluation for the carcinogenic effects of ethylbenzene is not presented because of the preliminary nature of the findings of the NTP (1996) study. However, because of the potential for a carcinogenic effect from ethylbenzene exposure, an additional uncertainty factor (UF) of 10-fold has been included in the calculation of the PHG level (see below).

CALCULATION OF PHG

A public health-protective concentration (C, in mg/L) for ethylbenzene in drinking water can be calculated based on the general equation for noncarcinogenic endpoints:

$$C = \frac{NOAEL \times BW \times RSC}{UF \times L/day} = mg/L$$

where,

NOAEL	=	No-observed-adverse-effect-level (49 mg/kg-day)
BW	=	Adult male body weight (70 kg)
RSC	=	Relative source contribution of 20% (0.2)
UF	=	Uncertainty factor of 1,000 (see text)
L/day	=	Volume of water consumed daily by an adult (2 L/day).

In the case of ethylbenzene, the experimental NOAEL for the principle study (NTP, 1996) was determined to be 49 mg/kg-day. The adult human body weight default is 70 kg for a male. An RSC of 20% was used in the calculation in the absence of more specific information on exposures to other sources of ethylbenzene exposure in addition to drinking water. A cumulative uncertainty factor of 1,000 has been applied which incorporates uncertainty contributions for inter-species extrapolation (10) and potentially sensitive human subpopulations (10), plus an additional factor of 10 for uncertainty from potential severe endpoints (carcinogenicity). U.S. EPA has applied a similar safety factor in establishing a long-term health advisory for drinking water when preliminary evidence has suggested a carcinogenic effect from a chemical (Anonymous, 1988). The adult human water consumption default value is 2 L/day.

Therefore,

$$C = \frac{49 \text{ mg/kg-day} \times 70 \text{ kg} \times 0.2}{1{,}000 \times 2 \text{ L/day}}$$

$$= 0.343 \text{ mg/L} = 0.3 \text{ mg/L (rounded)} = 300 \text{ ppb.}$$

Based on this calculation, OEHHA proposes a PHG of 0.3 mg/L (300 ppb) for ethylbenzene in drinking water.

RISK CHARACTERIZATION

There are a number of areas of uncertainty in regard to the development of the PHG for ethylbenzene in drinking water including route-to-route dose extrapolation (see discussion above), as well as the general toxicological concerns regarding extrapolation to humans of data from experimental animals which are acknowledged in the use of uncertainty factors. In addition, for volatile chemicals such as ethylbenzene exposures through food are unlikely, so the relative source contribution from water could perhaps be set higher than the default value of 0.2. However, net exposures to ethylbenzene in water could also be higher than estimated using the default 2 L/day of water consumption because of inhalation of the solvent vapors during showering and other household activities. The magnitude of these factors has not been estimated for ethylbenzene. It has been assumed that the factors would tend to offset each other (e.g., RSC = 40%, drinking water equivalent = 4 L/day), so the defaults have been retained for this calculation.

Several subpopulations in the general population who may be especially sensitive to the noncarcinogenic effects of ethylbenzene have been identified (HSDB, 1997). They include: individuals whose pulmonary function may be impaired (obstructive airway disease) and individuals with existing skin, liver, kidney, nervous system, blood and hematopoietic, ovulation and or menstrual disorders. The pulmonary and skin disorders are relevant for inhalation and dermal exposures, while the other disorders may be relevant for effects from drinking water exposure. No special sensitivity of infants and children has been noted for ethylbenzene. It is considered that the UF of 10-fold to account for human variability plus another 10-fold for uncertainty about a possible severe endpoint (cancer) should be adequate to protect potentially sensitive subpopulations. No evidence of synergy with other chemicals in the toxicity of ethylbenzene was found in the literature.

OTHER STANDARDS AND REGULATORY LEVELS

U.S. EPA has established a Maximum Contaminant Level Goal (MCLG) and a Maximum Contaminant Level (MCL) of 0.7 mg/L for ethylbenzene which U.S. EPA concluded would protect against the potential health problems identified in its report and is "the lowest level to which water systems can reasonably be required to remove this contaminant should it occur in drinking water" (U.S. EPA, 1991b; U.S. EPA, 1991c). This value was based on histopathological changes observed in a six-month rat study yielding a Drinking Water Equivalent Level (DWEL) of 3.4 mg/L assuming a drinking water contribution of 20%. U.S. EPA stated that the DWEL for ethylbenzene is "a lifetime exposure concentration protective of adverse, non-cancer health effects, that assumes all of the exposure to a contaminant is from a drinking water source." (U.S. EPA, 1996). The availability of new data regarding the chronic toxicity of ethylbenzene (NTP, 1996) since U.S. EPA's evaluation is the source of the departure from this value with OEHHA's proposed PHG. The current California MCL is also 0.7 mg/L (700 ppb).

U.S. EPA also established an ambient water quality criterion of 1.4 mg/L for ethylbenzene ingested through water and contaminated aquatic organisms and an ambient water quality criterion of 3.28 mg/L for ethylbenzene ingested through contaminated aquatic organisms alone (U.S. EPA, 1980).

The Occupational Safety and Health Administration (OSHA) established a workplace exposure standard of 100 ppm ethylbenzene in air for an eight-hour workday. The American Congress of Governmental Industrial Hygienists (ACGIH) has established a threshold limit value of 100 ppm and a short-term exposure limit of 125 ppm in air.

Various states have set guidelines for drinking water concentrations and acceptable ambient air concentrations. These are shown in Tables 4 and 5 (HSDB, 1997; ATSDR, 1990).

Table 4. State Drinking Water Guidelines

State	Drinking Water Guideline
Arizona	680 ppb
California	680 ppb
Illinois	1 ppb
Kansas	680 ppb
Maine	700 ppb
Minnesota	680 ppb
New Mexico	750 ppb
Rhode Island	680 ppb
Vermont	1,400 ppb
Wisconsin	700 ppb

Table 5. State Ambient Air Guidelines

State	Ambient Air Guideline
Connecticut	8,700 $\mu g/m^3$ (8 hours)
Massachusetts	118 $\mu g/m^3$ (24 hours)
Nevada	10,357$\mu g/m^3$ (8 hours)
New York	1,450 $\mu g/m^3$ (1 year)
North Dakota	4,350 $\mu g/m^3$ (8 hours)
South Carolina	4,350 $\mu g/m^3$ (24 hours)
Virginia	7,250 $\mu g/m^3$ (24 hours)

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A PBPK Modeling-Based Approach to Account for Interactions in the Health Risk Assessment of Chemical Mixtures

Sami Haddad, Martin Béliveau, Robert Tardif, and Kannan Krishnan[1]

Groupe de recherche en toxicologie humaine (TOXHUM), Faculté de médecine, Université de Montréal, Case Postale 6128, Succursale centre-ville, Montréal, Québec H3C 3J7, Canada

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The objectives of the present study were: (1) to develop a risk assessment methodology for chemical mixtures that accounts for pharmacokinetic interactions among components, and (2) to apply this methodology to assess the health risk associated with occupational inhalation exposure to airborne mixtures of dichloromethane, benzene, toluene, ethylbenzene, and *m*-xylene. The basis of the proposed risk assessment methodology relates to the characterization of the change in tissue dose metrics (e.g., area under the concentration-time curve for parent chemical in tissues [AUC_{tissue}], maximal concentration of parent chemical or metabolite [C_{max}], quantity metabolized over a period of time) in humans, during mixed exposures using PBPK models. For systemic toxicants, an interaction-based hazard index was calculated using data on tissue dose of mixture constituents. Initially, the $AUC_{target\ tissue}$ (AUC_n) corresponding to guideline values (e.g., threshold limit value [TLV]) of individual chemicals were obtained. Then, the AUC_n for each chemical during mixed exposure was obtained using a mixture PBPK model that accounted for the binary and higher order interactions occurring within the mixture. An interaction-based hazard index was then calculated for each toxic effect by summing the ratio of AUC_n obtained during mixed exposure (predefined mixture) and single exposure (TLV). For the carcinogenic constituents of the mixture, an interaction-based response additivity approach was applied. This method consisted of adding the cancer risk for each constituent, calculated as the product of $q^*_{chosen\ dose}$ and AUC_n. The AUC_n during mixture exposures was obtained using an interaction-based PBPK model. The approaches developed in the present study permit, for the first time, the consideration of the impact of multichemical pharmacokinetic interactions at a quantitative level in mixture risk assessments.

Key Words: mixtures; PBPK modeling; risk assessment; VOCs; pharmacokinetic interactions; hazard index.

Single chemical exposure is an exception rather than the rule in the general and occupational environments. The currently used default mixture risk assessment methodologies do not take into account the consequences of potential interactions occurring between components (U.S. EPA, 1986). The occurrence of pharmacokinetic and pharmacodynamic interactions can result in lower toxicity (antagonism) or greater toxicity (synergism) of mixtures than would be expected based on the knowledge of the potency and dose of the constituents (Calabrese, 1991). Whereas a mechanistic risk assessment framework for single chemical exposure is fairly well developed (Andersen *et al.*, 1987), such a framework for characterizing health risk associated with mixture exposure is still in development.

Recent advances in physiologically based pharmacokinetic (PBPK) modeling have demonstrated the feasibility of predicting the change in tissue dose of the components of complex mixtures, due to multiple pharmacokinetic interactions occurring among the constituents (Haddad *et al.*, 1999a, 2000b; Tardif *et al.*, 1997). In this modeling framework, information on the pharmacokinetic interactions at the binary level alone are sufficient to predict the magnitude of the interactions occurring in mixtures of greater complexity.

The use of such mixture PBPK models, along with the currently used dose addition and response addition approaches should facilitate the consideration of the consequences of pharmacokinetic interactions for a scientifically sound characterization of risk associated with mixture exposures. The objectives of the present study were: (1) to develop a pharmacokinetic interaction-based risk assessment methodology for mixtures containing systemic toxicants and/or carcinogens, and (2) to apply this methodology to assess the health risk associated with occupational inhalation exposure to mixtures of five volatile organic chemicals (VOCs): dichloromethane, benzene, toluene, ethylbenzene, and *m*-xylene.

METHODS

Pharmacokinetic interaction-based risk assessment of mixtures of systemic toxicants. The dose addition or the hazard index (HI) approach is currently used to characterize the risk associated with exposure to noncarcinogenic chemical mixtures (ACGIH, 1999; USEPA, 1986). In this approach, the doses of the mixture components are standardized using health-based values (e.g., acceptable daily intake [ADI], reference dose [RfD], threshold limit values [TLVs]) and are summed as follows:

[1] To whom correspondence should be addressed at Département de santé environnementale et santé au travail, Université de Montréal, 2375 Côte Ste-Catherine Bureau 4105, Montréal, Québec H3T 1A8, Canada. Fax: (514) 343-2200. E-mail: kannan.krishnan@umontreal.ca.

$$HI = \sum_{i=1}^{n} \frac{\text{exposure dose}_i}{\text{health based value}_i} \quad (1)$$

where i refers to individual mixture components and n is the number of components in the mixture (Mumtaz and Hertzberg, 1993; Mumtaz *et al.*, 1993; U.S. EPA, 1986).

This approach has been recommended and applied appropriately for components that induce the same toxic effect by identical mechanism of action. In cases where the mixture components act by different mechanisms or affect different target organs, a separate HI calculation is performed for each end point of concern. This approach lacks 2 important notions that should be considered in mechanistic mixture risk assessment: (1) tissue dosimetry of toxic moiety, and (2) possible pharmacokinetic interactions. The denominator and numerator of Equation 1 can be transformed to reflect tissue dose measures that can in turn be obtained using PBPK models. The resulting equation is similar to that proposed by Haddad *et al.* (1999b) for calculating biological hazard indices for use in biological monitoring of worker exposure to contaminant mixtures at workplaces. Accordingly, the interaction-based HI for systemic toxicant mixtures, based on tissue doses, can be calculated as follows:

$$HI_{\text{(interaction-based)}} = \sum_{i=1}^{n} \frac{TM_i}{TR_i} \quad (2)$$

where TR_i is the tissue dose estimated by PBPK models for human exposure to guideline values of individual mixture constituents, and TM_i refers to the tissue dose of each mixture constituent during human exposure to mixtures as provided by PBPK models. The TM_i can be obtained with mixture PBPK models that account for multiple pharmacokinetic interactions occurring among the mixture constituents (Haddad *et al.*, 1999a; Tardif *et al.*, 1997).

Pharmacokinetic interaction-based risk assessment of mixtures of carcinogens. ACGIH (1999) addresses neither the methodological issues related to the cancer risk assessment of chemical mixtures nor uses of quantitative approaches for the risk assessment of carcinogens. However, the current state of knowledge dictates that the risk assessment of carcinogenic chemical mixtures be conducted per response additivity approach, which involves the summation of excess risk attributed to each carcinogenic mixture constituent (U.S. EPA, 1986):

$$CRM = \sum_{i=1}^{n} (\text{Exposure dose}_i \times q^*_i) \quad (3)$$

where CRM is the carcinogenic risk related to mixture exposure, and q^*_i is the carcinogenic potential of chemical i expressed as risk per unit dose.

Like the dose addition approach, the currently used response addition approach neither considers the information on target tissue dose of mixture constituents nor accounts for potential interactions occurring at the pharmacokinetic level. Andersen *et al.* (1987) developed an approach to incorporate tissue dosimetry into cancer risk assessment of individual chemicals using PBPK modeling. Along those lines, the information on altered tissue dose simulated by mixture PBPK models can be used to account for pharmacokinetic interactions in the calculation of CRM as follows:

$$CRM = \sum_{i=1}^{n} (TM_i \times q^*_{mi}) \quad (4)$$

where q^*_{mi} is the tissue dose-based unit risk for each carcinogen in the mixture.

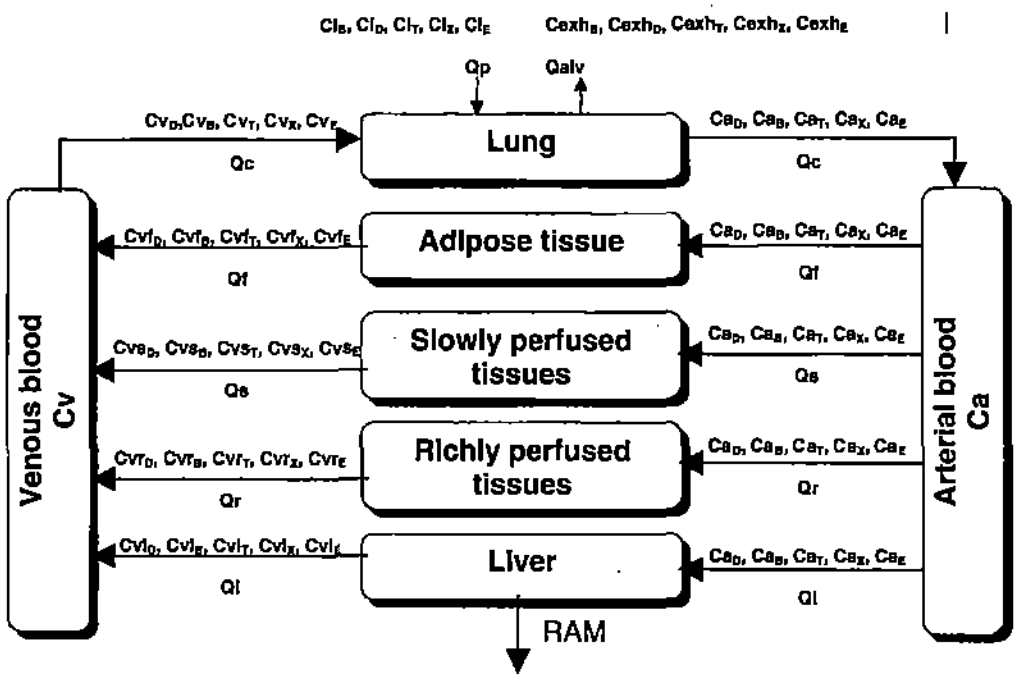
The use of q^*_{mi} in Equation 4 enables us to calculate the CRM from knowledge of the target tissue dose of mixture components (TM_i), which can vary due to pharmacokinetic interactions. PBPK models for individual mixture constituents can be used for estimating q^*_{mi}, whereas the mixture PBPK models are of use in estimating TM_i by accounting for the interactions occurring among mixture constituents.

Estimating target tissue exposure. Equations 2 and 4 represent essentially the proposed manner of conducting interaction-based risk assessment of exposure to chemical mixtures. These 2 equations, corresponding to noncancer and cancer risk assessments, require that the estimate of TM_i be obtained with PBPK models for mixture exposures. The estimation of the target tissue dose during individual and mixed exposures, in fact, is the crucial step of the proposed risk assessment approach. The appropriate tissue dose metric (e.g., area under the concentration-time curve [AUC] for parent chemical or metabolite, maximal concentration [Cmax] of metabolite or parent chemical in tissues, amount metabolized over a period of time, and average concentration of metabolite in target tissue) should be chosen based on the state of knowledge of the mechanism of toxicity of the mixture constituents (e.g., Andersen *et al.*, 1987).

Tissue dose can be estimated from knowledge of external exposure or administered dose, using PBPK models. These models can adequately simulate the uptake, disposition, and tissue dose of chemicals in various conditions (i.e., species, dose, scenario, and exposure route), because they are based on the mechanisms that account for the biology and chemistry of the organism, and the characteristics of the chemical. During mixed exposures, the pharmacokinetics and tissue dose of a chemical may be modified in the presence of other chemicals. When the mechanisms of interactions are known or hypothesized, it is possible, with PBPK models, to predict the altered pharmacokinetics and tissue dose of the components of a chemical mixture. It has been done for several binary mixtures (reviewed in Krishnan and Brodeur, 1994; Simmons, 1995), and recently for more complex mixtures (Haddad *et al.* 1999a, 2000b; Tardif *et al.* 1997). The methodology involves linking binary interactions within a PBPK model framework (Fig. 1) to simulate the kinetics and tissue dose of constituents of mixtures regardless of their complexity (Haddad *et al.*, 2000b; Haddad and Krishnan, 1998).

Interaction-based risk assessment of hypothetical exposure to a chemically defined mixture. The health risk assessment for occupational inhalation exposure to mixtures of dichloromethane (D), benzene (B), toluene (T), ethylbenzene (E), and *m*-xylene (X) was performed by considering the pharmacokinetic interactions among them. An interaction-based mixture PBPK model (Fig. 1) was used to simulate the internal dose of D, B, T, E, and X in workers exposed to these chemicals alone or as a mixture. The structure of the human model used in this study was essentially the same as the rat model developed and validated for this mixture by Haddad *et al.* (2000b). This PBPK model describes the organism as a set of four compartments (liver, richly perfused tissues, slowly perfused tissues, and adipose tissue) interconnected by systemic circulation. The tissue uptake of the mixture components is described as a perfusion-limited process. Metabolism of individual chemicals and metabolic interactions among them are described at the level of liver. The model simulates the kinetics of all mixture components by taking into account the metabolic and physicochemical characteristics, as well as the consequence of interactions among chemicals occurring at various levels. The mixture PBPK model of Haddad *et al.* (2000b) uniquely simulates the kinetics of D, B, T, E, and X on the basis of the mechanisms of binary level interactions and the characterization of the interconnections among them.

The rat model for DBTEX mixture developed and validated by Haddad *et al.* (2000b) was scaled to a human model by changing the rat physiological (tissue blood flow, alveolar ventilation rate, and cardiac output) and physicochemical (partition coefficients) parameters to human values (Tables 1 and 2) (Andersen *et al.*, 1991). The biochemical parameters (i.e., inhibition constants, maximal velocity for metabolism scaled to the body weight$^{2/3}$ and Michaelis affinity constant [K_m]) were kept species-invariant, except for the K_m of D, which was changed to the human value specified by Andersen *et al.* (1991; see Table 2). The D submodel also contained parameters and equations essential for simulating the percent carboxyhemoglobin in blood that resulted from D exposure (Andersen *et al.*, 1991). The consideration of the species-invariant nature of metabolic interaction constants was based on the previous observations of a



$$RAM_D = \frac{Vmax_D \cdot Cvl_D}{Km_D\left(1 + \frac{Cvl_D}{Ki_{DD}} + \frac{Cvl_T}{Ki_{TD}} + \frac{Cvl_X}{Ki_{XD}} + \frac{Cvl_E}{Ki_{ED}}\right) + Cvl_D}$$

$$RAM_B = \frac{Vmax_B \cdot Cvl_B}{Km_B\left(1 + \frac{Cvl_D}{Ki_{DB}} + \frac{Cvl_T}{Ki_{TB}} + \frac{Cvl_B}{Ki_{BB}} + \frac{Cvl_X}{Ki_{XB}}\right) + Cvl_B}$$

$$RAM_T = \frac{Vmax_T \cdot Cvl_T}{Km_T\left(1 + \frac{Cvl_D}{Ki_{DT}} + \frac{Cvl_B}{Ki_{BT}} + \frac{Cvl_T}{Ki_{TT}} + \frac{Cvl_X}{Ki_{XT}}\right) + Cvl_T}$$

$$RAM_E = \frac{Vmax_E \cdot Cvl_E}{Km_E\left(1 + \frac{Cvl_D}{Ki_{DE}} + \frac{Cvl_B}{Ki_{BE}} + \frac{Cvl_T}{Ki_{TE}} + \frac{Cvl_X}{Ki_{XE}}\right) + Cvl_E}$$

$$RAM_X = \frac{Vmax_X \cdot Cvl_X}{Km_X\left(1 + \frac{Cvl_D}{Ki_{DX}} + \frac{Cvl_B}{Ki_{BX}} + \frac{Cvl_T}{Ki_{TX}} + \frac{Cvl_E}{Ki_{EX}}\right) + Cvl_X}$$

FIG. 1. Conceptual representation of a physiologically based pharmacokinetic model for a mixture of VOCs (dichloromethane [D], benzene [B], toluene [T], ethylbenzene [E], and *m*-xylene [X]). Pharmacokinetic interactions between the components of this mixture occur at the level of hepatic metabolism. Ci and Cexh refer to inhaled and exhaled chemical concentrations. Cv and Ca refer to venous and arterial blood concentrations. Cvi and Qi refer to venous blood concentrations leaving tissue compartments and blood flow to tissues (i.e., f: adipose tissue, s: slowly perfused tissues, r: richly perfused tissues, and l: liver). Ki_{ij} is the constant describing competitive inhibition of the metabolism of chemical *i* by chemical *j*. Vmax, K_m, and RAM refer to the maximal velocity of metabolism, Michaëlis affinity constant, and rate of the amount metabolized, respectively.

mixture PBPK modeling study (Tardif *et al.*, 1997) in which the rat-human extrapolation of the occurrence of interactions among T, E, and X was validated with experimental data.

The noncancer risk assessment for the DBTEX mixture was conducted by calculating the hazard index for 2 endpoints (central nervous system [CNS] effects, hypoxia). For this purpose, the AUC of carboxyhemoglobin in blood and the AUC of D, B, T, E, and X in parental form in the richly perfused tissue compartment (i.e., brain) were simulated using the individual chemical and mixture PBPK models. The choice of dose metrics reflects our working hypothesis of the mode of action of these chemicals. The exposure scenarios simulated with the PBPK models corresponded to an 8-h inhalation exposure and a 24-h simulation period. For calculating $HI_{\text{interaction-based}}$, the $AUC_{\text{target tissue}}$ of D, B, T, E, and X were estimated for their exposure guidelines (TLVs) and for various exposure concentration combinations of these chemicals in mixtures. The various combinations represent hypothetical cases of worker exposure and they were chosen to reflect situations where the conventional and interaction-based assessments are likely to yield similar, or very different, results. For performing interaction-based cancer risk assessment for this mixture, change in the risk level due to mixture exposure was estimated by integrating the concentration of GSH conjugate formed from D over 24 h and by calculating the total amount of benzene metabolites in liver during mixture exposures (Andersen *et al.*, 1987; Cox and Ricci, 1992). Since Equation 4 represents a

TABLE 1
Human Physiological Parameters Used in this Study

Parameters	Values
Alveolar ventilation rate (l/h/kg)	18
Cardiac output (l/h/kg)	18
Blood flow rate (fraction of cardiac output)	
Fat	0.05
Slowly perfused tissues	0.25
Richly perfused tissues	0.44
Liver	0.26
Volume (fraction of body weight)*	
Fat	0.19
Slowly perfused tissues	0.62
Richly perfused tissues	0.05
Liver	0.026

Note. Parameters from Tardif *et al.* (1997).
*For carboxyhemoglobin estimation; blood volume was set equal to 6% of body weight (Andersen *et al.*, 1991).

linear model, the carcinogenic risk is essentially proportional to the change in tissue dose metric of B and D during mixed exposures, particularly at low doses. Therefore, the ratios of tissue dose metric during mixed and single exposures to D and B were calculated to indicate the change in risk level during mixed exposures. Calculations of HI and CRM, according to the conventional approach (i.e., without the consideration of the possible occurrence of metabolic interactions) were also performed for comparison purposes.

RESULTS

Systemic Risk Assessment for DBTEX Mixtures

The conventional and interaction-based hazard indices for CNS effects and hypoxia for various DBTEX mixtures are presented in Tables 3 and 4. The conventional HI calculations for CNS effects were done using the exposure concentrations of D, T, E, and X, whereas such calculations for hypoxia were done using the exposure concentrations of D. Examining the data for CNS effect, it can be noticed that at high concentrations the HI values, calculated with the consideration of interactions, are greater than those obtained according to the dose-addition approach that did not account for the occurrence of interactions (Table 3). At lower exposure concentrations of DBTEX in mixtures, the difference between the conventional and interaction-based HI is smaller.

The interaction-based estimate of HI for hypoxia, however, was lower than that calculated without consideration of the occurrence of interactions at high exposure concentrations (Table 4). The presence of competitive inhibitors such as the T, E, B, and X reduces the rate of D metabolism by P450, resulting in a diminution of the formation of carboxyhemoglobin. As seen in Table 4, the greater the relative concentration of the inhibitors, the greater the discrepancy between the conventional and interaction-based HI.

Cancer Risk Assessment for DBTEX Mixtures

According to the methodology used in the present study, the relative change in cancer risk associated with D and B during mixture exposures is a direct consequence of the change in their tissue dose metrics. The change in risk level during mixture exposures compared to single chemical exposures, as calculated using PBPK model-simulated changes in the tissue doses of D and B, is shown in Table 5. In the case of D, the GSH conjugate is the relevant dose surrogate (Andersen *et al.*, 1991). In the presence of competitive inhibitors (i.e., BTEX) of P450 metabolism of D, the flux of D through the GSH conjugation pathway increases, thus contributing to a greater risk level during mixed than during single exposures. For the mixture exposure scenarios considered in the present study, the cancer risk attributed to D could increase by up to a factor of 4 compared to single chemical exposure situations (Table 5). The cancer risk attributed to B exposure, however, would decrease during mixed exposures compared to single chemical exposures, since the rate of formation of oxidative metabolites from B is reduced during concurrent exposure to DTEX (Table 5). The simulation results presented in Table 5 indicate that the relative cancer risk due to B in DBTEX mixtures approaches unity (i.e., close to the absolute risk level associated with a single exposure to B) as the concentration of DTEX in the mixture decreases.

DISCUSSION

The occupational and environmental health risk assessments of chemical mixtures do not account for the quantitative impact of possible interactions among mixture components, which

TABLE 2
Physicochemical and Biochemical Parameters for PBPK Modeling of Dichloromethane (D), Benzene (B), Toluene (T), Ethylbenzene (E), and *m*-Xylene (X)

Parameters	D	B	T	E	X
Blood:air	8.94	7.4	15.6	28.0	26.4
Fat:air	111	406	1021.0	1556.0	1859.0
SPT:air	7.3	15	27.7	26.0	41.9
RPT:air	13.1	11	83.6	60.3	90.9
Liver:air	13.1	11	83.6	83.8	90.9
V_{max} (mg/h/kg)	6.25	2.11	3.44	6.39	6.49
K_m (mg/l)	0.75	0.10	0.13	1.04	0.45
Ki (mg/l)					
αD	—	0.08	0.16	0.11	0.32
αB	0.30	—	0.14	0.26	0.22
αT	0.35	0.22	—	0.17	0.33
αE	0.99	0.63	0.95	—	1.67
αX	0.45	0.23	0.36	0.51	0.35
Kf ($h^{-1} \times kg^{-1}$)	2.0		—	—	—

Note. Parameters from Travis *et al.* (1988), Andersen *et al.* (1991), Tardif *et al.* (1997), and Haddad *et al.* (1999a; 2000b). Kf, first order constant for GSH conjugation; SPT, slowly perfused tissues; RPT, richly perfused tissues; Ki, inhibition constant; α, inhibitor acting on the substrate's metabolism; V_{max}, body surface-normalized maximal velocity of metabolism; K_m, Michaelis-Menten affinity constant.

TABLE 3
Comparison of Interaction-Based and Conventional Hazard Index (HI) for Central Nervous System Effect Calculated for Different Mixtures of Dichloromethane (D), Benzene (B), Toluene (T), Ethylbenzene (E), and *m*-Xylene (X)

Exposure concentration (ppm)					AUC_{BPT}, mg/l × h				HI	
D	B	T	E	X	D	T	E	X	Interaction-based[a]	Conventional[b]
50	0.5	50	100	100	13.9	54.4	64.0	94.4	6.8	4.0
25	0.5	25	50	50	5.99	21.0	24.6	35.2	2.7	2.0
16	0.5	16	33	33	3.36	11.3	14.1	19.5	1.5	1.3
12.5	0.5	12.5	25	25	2.41	8.06	10.1	13.6	1.1	1.0
10	0.5	5	40	20	2.04	3.35	16.5	11.1	0.94	0.90
20	0.5	10	10	10	3.32	5.77	3.79	4.98	0.82	0.80
10	0.5	10	10	10	1.62	5.64	3.71	4.85	0.58	0.60

Note. Exposure scenario: 8 h inhalation per day, simulation period: 24 h. AUC_{BPT}, mg/l × h, area under the parent chemical concentration-time curve (richly perfused tissues).

[a]Calculated as the sum of the ratio of the exposure concentration to the TLVs of D (50 ppm), T (50 ppm), E (100 ppm), and X (100 ppm).

[b]Calculated as the sum of the ratio of the AUC_{BPT} determined during mixture exposure to that associated with single exposure to the TLV of D (7.39 mg/l × h), T (28.6 mg/l × h), E (46.5 mg/l × h), and X (57.3 mg/l × h).

may vary as a function of dose and exposure scenario in animals and humans. Depending on the relative and absolute concentrations of the chemicals present in the mixture, they may result in interactions that cause departure from additivity. Interactions may be pharmacokinetic or pharmacodynamic in nature. The pharmacokinetic interactions result in a change in tissue dose of chemicals during mixture exposures compared to single exposures, and represent the most common type of interaction observed and reported in the literature (reviewed in Krishnan and Brodeur, 1991, 1994). The relative change in tissue dose of chemicals due to pharmacokinetic interactions during mixture exposures depends on the relative concentrations of components and the mechanism(s) of interactions. PBPK models are unique tools that facilitate the consideration of interaction mechanisms at the binary level to simulate the change in tissue dose of chemicals present in complex mixtures. The present study, for the first time, demonstrates the use of PBPK models in quantifying the change in the tissue dose metrics of chemicals during mixture exposures and in improving the mechanistic basis of mixture risk assessment. The application of PBPK models in mixture risk assessment has been demonstrated in this study using DBTEX mixture, for which an interaction-based PBPK model has recently been developed and validated (Haddad *et al.*, 2000b).

According to the proposed approach, it is possible that $HI_{interaction\text{-}based}$ exceeds 1 while the conventional HI value is less than unity, or *vice versa*. The interaction-based HI values developed in the present study are more relevant than the conventional HI because internal concentrations of the toxic entities (and not external exposure concentrations) are used for

TABLE 4
Comparison of Interaction-Based and Conventional Hazard Index (HI) for Hypoxia Calculated for Different Mixtures of Dichloromethane (D), Benzene (B), Toluene (T), Ethylbenzene (E) and *m*-Xylene (X)

Exposure concentration (ppm)						HI	
D	B	T	E	X	$AUC_{COHb,24h}$ (% × h)	Interaction-based[a]	Conventional[b]
50	0.5	50	100	100	8.86	0.29	1.00
25	0.5	25	50	50	7.66	0.25	0.50
16	0.5	16	33	33	6.50	0.21	0.32
12.5	0.5	12.5	25	25	5.80	0.19	0.25
10	0.5	5	40	20	4.26	0.14	0.20
20	0.5	10	10	10	11.1	0.37	0.40
10	0.5	10	10	10	5.69	0.19	0.20

Note. Exposure scenario: 8-h inhalation per day, simulation period: 24 h. Area under the carboxyhemoglobin concentration-time curve associated with D exposure, calculated by subtracting the background $AUC_{COHb,24h}$ value (36.97 % × h).

[a]Calculated as the ratio of $AUC_{COHb,24h}$ associated with exposure to D in mixtures, to that associated with single exposure to TLV of D (30.3 % × h).

[b]Calculated as the ratio of the exposure concentration of D to its TLV (50 ppm).

TABLE 5
Effect of Pharmacokinetic Interactions on the Cancer Risk Level Associated with Dichloromethane (D) and Benzene (B) Present in Mixtures along with Toluene (T), Ethylbenzene (E), and *m*-Xylene (X)

Exposure concentration (ppm)					$A_{GSH(D+B)}$ (mg/l × h)[a]		A_{met} (mg)[b]		Change in cancer risk[c]	
D	B	T	E	X	Mixture	D single	Mixture	B single	D	B
50	0.5	50	100	100	110	26.2	1.19	3.14	4.20	0.38
25	0.5	25	50	50	42.9	10.9	1.93	3.14	3.94	0.61
16	0.5	16	33	33	21.4	6.53	2.39	3.14	3.28	0.76
12.5	0.5	12.5	25	25	13.9	4.98	2.61	3.14	2.79	0.83
10	0.5	5	40	20	12.6	3.92	2.57	3.14	3.21	0.82
20	0.5	10	10	10	15.2	8.39	2.87	3.14	1.81	0.91
10	0.5	10	10	10	7.06	3.92	2.93	3.14	1.80	0.93

Note. Exposure scenario: 8-h inhalation per day, simulation period: 24 h.
[a]Integrated amount of D conjugated with GSH per tissue volume over 24 h.
[b]Amount metabolized during 24 h.
[c]Calculated as the ratio of the tissue dose metric for mixed exposure to that for single exposures.

the calculation. The computed $HI_{interaction\text{-}based}$ will not always be different from the conventional HI because its magnitude depends on the relative concentrations of all mixture constituents and the quantitative nature of the interaction mechanisms as included in the PBPK models. When both the $HI_{interaction\text{-}based}$ and conventional HI values exceed 1, the interpretation should be limited to a qualitative indication of health risk being associated with exposure to the given chemical mixture. The difference in numerical values obtained, once they are above 1, should not be interpreted in quantitative risk terms. This is consistent with the current practice of risk assessment for systemic toxicants, either present individually or as mixtures.

The interaction-based PBPK model facilitates the prediction of the change in tissue dose of the toxic moiety of chemicals during mixture exposures to assess the cancer risk for chemical mixtures. In this approach, the potency of the mixture constituents does not change between single and mixture exposures, but it is the tissue dose that changes according to the interaction mechanism and the exposure concentration of interacting chemicals. The proposed approach then improves upon the currently used response-addition methodology by facilitating the incorporation of data on the tissue dose of chemicals in mixtures (instead of their external concentration), and by accounting for the extent of their modulation due to interactions during mixed exposures. During coexposures to chemicals that interact at the metabolic level, the tissue dose and associated cancer risk of mixture constituents may either be decreased or increased (compared to single exposures) as exemplified in this study. The magnitude and direction of the change in tissue dose during mixed exposures depend on the mechanism of pharmacokinetic interactions (e.g., metabolic inhibition or enzymatic induction) and the identity of the putative toxic moiety (e.g., parent chemical, metabolite).

The present study applied the validated rodent PBPK model to characterize the cancer and noncancer risk associated with occupational exposure to the DBTEX mixture of varying compositions by accounting for the change in tissue dose due to metabolic interactions. The simulated changes in tissue dose and risk levels for occupational mixture exposures do not necessarily reflect those that are expected in environmental exposure situations. While comparing the occupational and environmental exposure to mixtures, the interaction mechanisms are likely to remain the same in both situations, whereas the concentrations of the inhibitors differ markedly. With decreasing blood concentrations of the inhibitors, their effect on the metabolism of other mixture components becomes smaller and smaller. Using the mixture PBPK model developed in the current study, a threshold of interactions in multichemical mixtures can be established following the simulation of the exposure level impact on the magnitude of interactions. Such studies should facilitate a better understanding of the relative importance and relevance of specific interactions and interaction mechanisms in occupational and environmental exposure situations.

Even though the mixture model used in the present study accounted for the occurrence of metabolic inhibition as the interaction mechanism (Haddad *et al.* 2000b), induction of metabolism may occur during repeated exposure scenarios, complicating the PBPK model calculation of the magnitude of net change in tissue dose during mixed exposures. However, experimental studies have shown the absence of induction effects on D, B, T, E, and X during repeated exposures (Haddad *et al.*, 2000a). Therefore, the assessment presented in this paper, based on the consideration of the inhibition mechanism, is likely to describe adequately the pharmacokinetic interactions occurring in the DBTEX mixture and ensuing changes in tissue dose of the mixture constituents. The possible impact of pharmacodynamic interactions on the mixture risk was not evaluated in the present study, but it can be performed

if quantitative mechanistic data on binary level interactions are available/generated. Overall, the modeling and risk assessment frameworks outlined in this study should be amenable to the use of data on other mechanisms of interactions, toxic endpoints, and dose-response relationships, if intended and if the required data are available.

An advantage of the PBPK model-based risk assessment methodology developed in this study is that the combinations of exposure concentrations of individual chemicals that will not deviate significantly from the conventional HI (i.e., < 1) or the CRM (i.e., $< 1 \times 10^{-6}$) can be determined by iterative simulation. The proposed approach should then be useful from health protection and prevention perspectives, particularly where there is a possibility of pharmacokinetic interactions among chemicals present as mixtures in the occupational environment.

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Catechol and Hydroquinone Have Different Redox Properties Responsible for Their Differential DNA-damaging Ability

Kazutaka Hirakawa,[†] Shinji Oikawa,[‡] Yusuke Hiraku,[‡] Iwao Hirosawa,[§] and Shosuke Kawanishi*,[‡]

Radioisotope Center, and Department of Hygiene, Mie University School of Medicine, Edobashi 2-174, Tsu, Mie 514-8507, Japan, and Department of Hygiene, Akita University School of Medicine, Akita 010-8543, Japan

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We examined the redox properties of the "carcinogenic" catechol and the "noncarcinogenic" hydroquinone in relation to different DNA damaging activities and carcinogenicity using ^{32}P-labeled DNA fragments obtained from the human genes. In the presence of endogenous NADH and Cu^{2+}, catechol induces stronger DNA damage than hydroquinone, although the magnitudes of their DNA damaging activities were reversed in the absence of NADH. In both cases, DNA damage resulted from base modification at guanine and thymine residues in addition to strand breakage induced by Cu^{+} and H_2O_2, generated during the oxidation of catechol and hydroquinone into 1,2-benzoquinone and 1,4-benzoquinone, respectively. EPR and ^{1}H NMR studies indicated that 1,2-benzoquinone is converted directly into catechol through a nonenzymatic two-electron reduction by NADH whereas 1,4-benzoquinone is reduced into hydroquinone through a semiquinone radical intermediate through two cycles of one-electron reduction. The reduction of 1,2-benzoquinone by NADH proceeds more rapidly than that of 1,4-benzoquinone. This study demonstrates that the rapid 1,2-benzoquinone two-electron reduction accelerates the redox reaction turnover between catechol and 1,2-benzoquinone, resulting in the enhancement of DNA damage. These results suggest that the differences in NADH-mediated redox properties of catechol and hydroquinone contribute to their different carcinogenicities.

Introduction

Quinones shuffle electrons enzymatically (*1*−*6*) or nonenzymatically (*1*−*3*, *7*−*11*) among their reduced forms, oxidized forms, and/or their semiquinone radicals to construct redox cycles. The toxicity of quinones results from the formation of reactive oxygen species, including superoxide ($O_2^{\bullet-}$),[1] hydrogen peroxide (H_2O_2), and ultimately the hydroxyl radical ($OH^{\bullet}$) (*1*−*3*), during these redox processes. Reactive oxygen species, implicated in the pathogenesis of cancer, are produced from hydroquinone (1,4-BQH_2, 1,4-benzenediol) (*8*, *9*) and its derivatives, dichlorohydroquinone (*10*), tetrachlorohydroquinone (*11*), and phenylhydroquinone (*12*), during autoxidation into the corresponding benzoquinones (1,4-BQ and its derivatives) to induce oxidative DNA damage. These experiments suggest that the carcinogenicity of quinones arises from the redox reactions mediated by metal ion and NADH.

Catechol (1,2-BQH_2; 1,2-benzenediol), a reduced form of 1,2-benzoquinone (1,2-BQ), occurs in foods and cigarette smoke (*13*). With the discovery that 1,2-BQH_2 is carcinogenic in rodents (*14*−*21*), the IARC (International Agency for Research on Cancer) has evaluated that 1,2-BQH_2 is possibly carcinogenic to humans (*13*). The carcinogenicity of 1,2-BQH_2 is thought to result from DNA damage induced by reactive oxygen species (*22*). The isomer 1,4-BQH_2, however, has not been evaluated as a carcinogen, even though 1,4-BQH_2 is known to induce DNA damage (*8*, *9*) and mutations (*23*). The difference between the carcinogenic potentials of 1,2-BQH_2 and 1,4-BQH_2 may be determined by the redox properties of 1,2-BQH_2/1,2-BQ and 1,4-BQH_2/1,4-BQ in conjunction with endogenous NADH and metal ions.

In this paper, we investigate the mechanism and site specificity of DNA damage induced by 1,2-BQH_2, 1,4-BQH_2, 1,2-BQ, and 1,4-BQ using ^{32}P-labeled DNA fragments obtained from human genes. We examined redox properties of these isomers by EPR, ^{1}H NMR, and measurement of UV−vis. These experiments revealed different redox properties responsible for distinct DNA damaging activities.

Experimental Procedures

Materials. The restriction enzymes (*Ava*I and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. [γ-^{32}P]ATP (222 TBq/mmol) was obtained from DuPont New England Nuclear. Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemical Co. (Kumamoto, Japan). Superoxide dismutase (SOD, 3000 units/mg from bovine erythro-

* To whom correspondence should be addressed. E-mail: kawanisi@doc.medic.mie-u.ac.jp. Phone: +81-59-231-5011. Fax: +81-59-231-5011.

† Radioisotope Center, Mie University School of Medicine.

‡ Department of Hygiene, Mie University School of Medicine.

§ Department of Hygiene, Akita University School of Medicine.

[1] Abbreviations: $O_2^{\bullet-}$, superoxide anion radical; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, free hydroxyl radical; 1,4-BQH_2, hydroquinone; 1,4-BQ, 1,4-benzoquinone; 1,2-BQH_2, catechol; 1,2-BQ, 1,2-benzoquinone; IARC, International Agency for Research on Cancer; UV−vis, UV−visible absorption spectrum; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase; TMS, tetramethylsilane.

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cytes) and catalase (45 000 units/mg from bovine liver) were obtained from Sigma Chemical Co. 1,2-BQH_2, 1,4-BQH_2, and 1,4-BQ were procured from Wako Pure Chemical Ind. Copper chloride ($CuCl_2 \cdot 2H_2O$) and NADH were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). 1,2-BQ was synthesized from 1,2-BQH_2 through oxidation by ceric sulfate (WAKO Pure Chemical Ind.) and then confirmed by ^1H NMR and UV–Vis absorption spectrum as previously described (*24*, *25*). The ^1H NMR ($CDCl_3$, tetramethylsilane (TMS)) spectrum gave δ 6.42 (2H, J_{H-H} = 12 Hz, J_{H-H} = 4.2 Hz, J_{H-H} = 1.5 Hz, 3,6-H), 7.05 (2H, J_{H-H} = 12 Hz, J_{H-H} = 4.2 Hz, J_{H-H} = 1.5 Hz, 4,5-H). UV–vis absorption at λ_{max} = 379 nm was used to determine the concentration of 1,2-BQ according to its molar absorption coefficient (ϵ = 1700 M^{-1} cm^{-1} at λ_{max} in chloroform) (*26*).

Detection of DNA Damage Using ^{32}P-Labeled DNA Fragments. DNA fragments were prepared from the pbcNI plasmid, which contains a 6.6-kb *Bam*HI chromosomal DNA segment with the human c-Ha-*ras*-1 protooncogene (*27*). Singly labeled 341-bp (*Xba*I 1906–*Ava*I* 2246), 261-bp (*Ava*I* 1645–*Xba*I 1905), and 337-bp fragments (*Pst*I 2345–*Ava*I* 2681) were obtained previously described (*27*, *28*). The asterisk indicates ^{32}P-labeling. Nucleotide numbering begins at the *Bam*HI site (*29*).

The standard reaction mixture (1.5-mL Eppendorf) contained the ^{32}P-labeled DNA fragments, quinones, sonicated calf thymus DNA (10 μM/base), and $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA in a microtube. After a 60 min incubation at 37 °C, the DNA fragments were heated for 20 min at 90 °C in 1 M piperidine where indicated, then treated as previously described (*28*).

Preferred cleavage sites were determined by a direct comparison of the chemical reaction products of the Maxam–Gilbert procedure (*30*) using a DNA sequencing system (LKB 2010 Macrophor) with the positions of the oligonucleotides utilized. A laser densitometer (LKB 2222 UltroScan XL) measured the relative quantities of the relative amounts of oligonucleotides from the treated DNA fragments.

Spectroscopic Measurements. ^1H NMR spectra were performed on a JNM-A 500 (500 MHz) FT-NMR spectrometer (JEOL) in chloroform-*d* (Aldrich). The chemical shifts of ^1H were measured in δ (ppm) units relative to a TMS internal standard. Absorption spectra were obtained on a Shimadzu UV-2500PC spectrophotometer. EPR spectra using a JES-FE-3XG spectrometer (JEOL) with 100 kHz field modulation detect the free radicals derived from 1,2-BQH_2, 1,4-BQH_2, and their oxidized products. The spectra were recorded utilizing a microwave power of 4 mW and a modulation amplitude of 10 G.

Measurement of $O_2^{\bullet-}$ Generation. The quantity of $O_2^{\bullet-}$ generated by the reactions of 1,2-BQH_2 and 1,4-BQH_2 with Cu^{2+} was determined by cytochrome *c* reduction. The reaction mixture, containing 50 μM ferricytochrome *c*, 20 μM 1,2-BQH_2 or 1,4-BQH_2, 20 μM Cu^{2+}, and 5 μM DTPA in 1.2 mL of 10 mM sodium phosphate buffer (pH 7.8) with and without SOD (150 units/mL), was incubated at 37 °C. We recorded the absorption at 550 nm (ϵ = 21 100 M^{-1} cm^{-1}) at 2 min intervals for 10 min using a UV–Vis absorption spectrophotometer; the quantity of reduced cytochrome *c* was then calculated to determine total generation of $O_2^{\bullet-}$.

Results

Damage to ^{32}P-Labeled DNA Fragments. Autoradiography of DNA cleavage induced by 1,2-BQH_2 and 1,4-BQH_2 in the presence of Cu^{2+} ion demonstrates that the DNA damage induced by 1,4-BQH_2 was greater than that induced by 1,2-BQH_2 (Figure 1). DNA damage was enhanced by the addition of NADH. When NADH was added, however, the DNA damage induced by 1,2-BQH_2 was much stronger than that induced by 1,4-BQH_2. The treatment of damaged DNA with piperidine significantly enhanced DNA cleavage, suggesting that the DNA damage resulted from base modification in conjugation with strand breakage. In the absence of NADH, neither 1,2-BQ and 1,4-BQ could induce DNA damage (data not shown); upon addition of NADH, both 1,2-BQ and 1,4-BQ could induce Cu^{2+}-mediated DNA damage. The extent of DNA damage induced by 1,2-BQ exceeded that induced by 1,4-BQ (Figure 2, panels A and B).

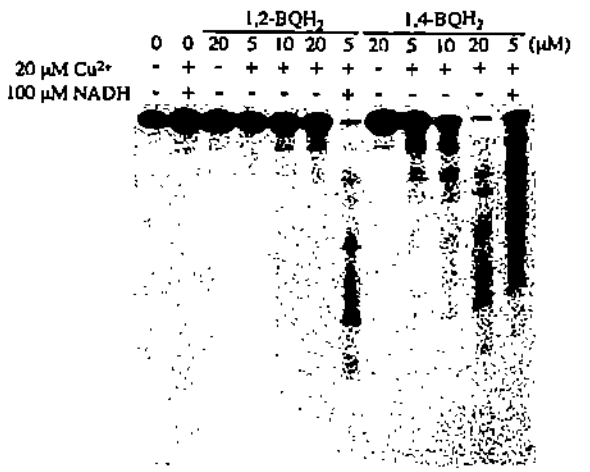

Figure 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with 1,2-BQH_2 and 1,4-BQH_2. Reaction mixtures contained the ^{32}P-5′-end-labeled 341-bp DNA fragment, 10 μM/base sonicated calf thymus DNA, and 20 μM $CuCl_2$ in 200 μL of 10 mM phosphate buffer (pH 7.8) and 5 μM DTPA with or without 100 μM NADH. Mixtures were incubated for 60 min at 37 °C. The DNA fragments were then treated with 1 M piperidine for 20 min at 90 °C and electrophoresed on an 8% polyacrylamide/8 M urea gel.

Site Specificity of DNA Damage. The DNA cleavage patterns induced by these quinones were determined by both the Maxam–Gilbert procedure (*30*) and scanning autoradiography utilizing a laser densitometer. Similar DNA cleavage patterns were observed with 1,2-BQH_2, 1,4-BQH_2, 1,2-BQ plus NADH, and 1,4-BQ plus NADH, suggesting that DNA damage is induced in a similar manner by all the quinones. DNA cleavage was frequently observed at guanine and thymine residues within the DNA fragments obtained from the human c-Ha-*ras*-1 protooncogene (data not shown).

Reactive Species Causing DNA Damage. To investigate the identity of the reactive species involved in DNA damage, we evaluated the ability of scavengers of reactive oxygen species and metal chelators to inhibit DNA damage induced by these quinones (Figure 3). The DNA damage induced by 1,2-BQH_2 and 1,4-BQH_2 was inhibited by catalase and bathocuproine, a specific chelator of Cu^+. Neither $OH^\bullet$ scavengers, such as ethanol, mannitol, sodium formate, and DMSO, nor SOD could inhibit this DNA damage, suggesting the induction of DNA damage mediated cooperatively by H_2O_2 and Cu^+.

Generation of the Reactive Species for DNA Damage. Cu^+ is generated by the reduction of Cu^{2+} by 1,2-BQH_2.[2] Cu^+ ion produces $O_2^{\bullet-}$ through its reaction with oxygen; this $O_2^{\bullet-}$ is easily dismutated into H_2O_2. We measured $O_2^{\bullet-}$ generation using a cytochrome *c* reduction read-out (Figure 4). Cytochrome *c* reduction, mediated by 1,2-BQH_2 and 1,4-BQH_2 in the presence of Cu^{2+}, decreased after treatment with SOD due to reduction of available $O_2^{\bullet-}$ because of its dismutation into H_2O_2. We estimated the amount of $O_2^{\bullet-}$ generation from the difference of cytochrome *c* reduction with and without SOD.

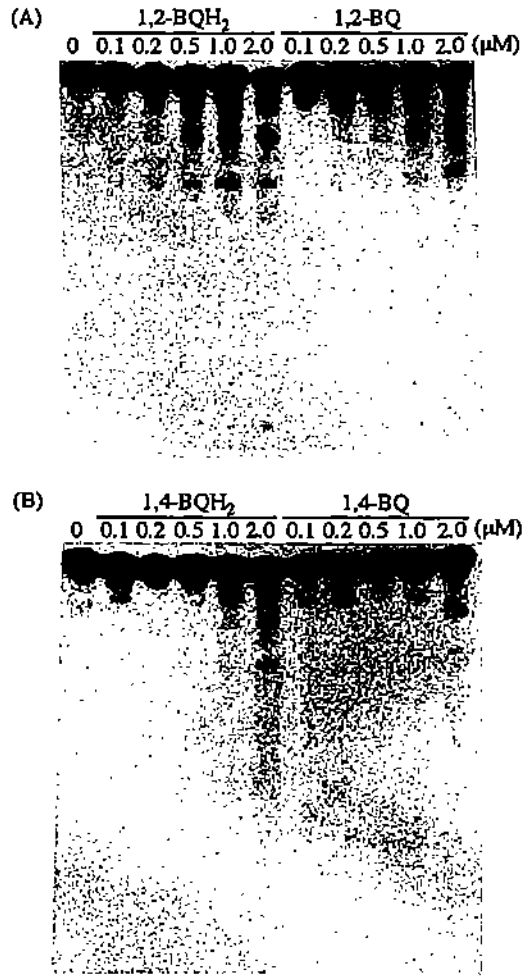

Figure 2. Autoradiogram of ^{32}P-labeled DNA fragments incubated with quinones. The reaction mixture contained the ^{32}P-5′-end-labeled 261-bp DNA fragment, 10 μM/base sonicated calf thymus DNA, the indicated concentration of quinones [(A) 1,2-BQH_2 and 1,2-BQ. (B) 1,4-BQH_2 and 1,4-BQ], 100 μM NADH, and 20 μM $CuCl_2$ in 200 μL of 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA. The mixtures were incubated for 60 min at 37 °C. The DNA fragments were then treated with 1 M piperidine for 20 min at 90 °C and electrophoresed on an 8% polyacrylamide/8 M urea gel.

The initial generation of $O_2^{\bullet-}$ by 1,4-BQH_2 proceeded faster than the reaction mediated by 1,2-BQH_2, indicating 1,4-BQH_2 is oxidized by Cu^{2+} at a faster rate than 1,2-BQH_2.

Semiquinone Radical Formation from 1,2-BQH_2, 1,4-BQH_2, 1,2-BQ, and 1,4-BQ. We performed EPR measurements to investigate the redox process of these quinones. As the semiquinone radical of 1,2-BQH_2 is difficult to detect, we attempted to detect the resulting complex with Mg^{2+} (*32*). The EPR signal was measured in the oxidation of 1,2-BQH_2 by Cu^{2+} in the presence of

[2] The generation of Cu^+ ions was confirmed by the formation of complexes with bathocuproine using absorption spectra. The absorption spectra of the Cu^+-bathocuproine complex has a characteristic absorption peak at 480 nm (*31*), which appeared after the addition of either 1,2-BQH_2 or 1,4-BQH_2 into a solution of Cu^{2+} ions and bathocuproine. These results indicate that Cu^{2+} is reduced into Cu^+ by 1,2-BQH_2 and 1,4-BQH_2. The complex absorbance did not increase with the addition of 1,2-BQH_2 or 1,4-BQH_2 amounts greater than two times the amount of Cu^{2+} present, indicating that one 1,2-BQH_2 and 1,4-BQH_2 molecule reduces two Cu^{2+} ions into Cu^+ in the process of oxidation into BQs.

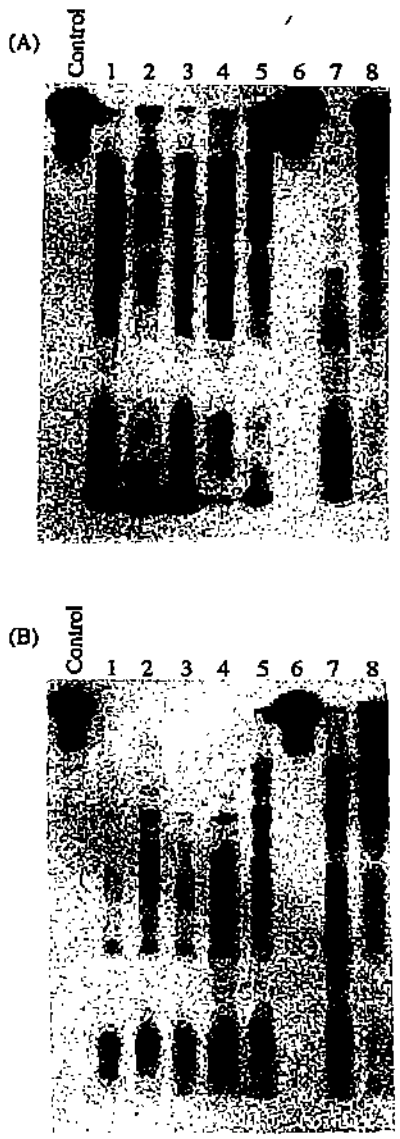

Figure 3. Effects of scavengers on DNA damage induced by quinones and NADH in the presence of Cu^{2+}. Reactions contained the ^{32}P-5′-end labeled 261-bp fragment, 10 μM/base of calf thymus DNA, 5 μM 1,2-BQH_2 (A) or 20 μM 1,4-BQH_2 (B), 20 μM $CuCl_2$, 100 μM NADH, and scavenger in 200 μL of 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA. Mixtures were incubated for 50 min at 37 °C. The DNA fragments were then treated with 1 M piperidine for 20 min at 90 °C and electrophoresed on an 8% polyacrylamide/8 M urea gel. Scavenger or bathocuproine was added as follows: lane 1, no scavenger; lane 2, 0.8 M ethanol; lane 3, 0.2 M mannitol; lane 4, 0.2 M sodium formate; lane 5, 0.8 M DMSO; lane 6, 50 units of catalase; lane 7, 50 units of SOD; lane 8, 50 μM bathocuproine. Control did not contain quinone, NADH, and $CuCl_2$.

Mg^{2+} and bathocuproine, added to remove Cu^+ from the chelate complex of Cu^+ and 1,2-BQH_2. The EPR signal observed following 1,2-BQH_2 oxidation by Cu^{2+} was recognized as a 1,2-semiquinone radical (*32*) (Figure 5), confirming the generation of semiquinone radical through the Cu^{2+}-mediated oxidation of 1,2-BQH_2. The EPR signal of a 1,4-semiquinone radical (*9*) was also observed in the oxidation of 1,4-BQH_2 by Cu^{2+}. The EPR signals of semiquinone radicals were immediately quenched by the addition of NADH, suggesting their reduction into 1,2-BQH_2 and 1,4-BQH_2. In the EPR signals obtained fol-

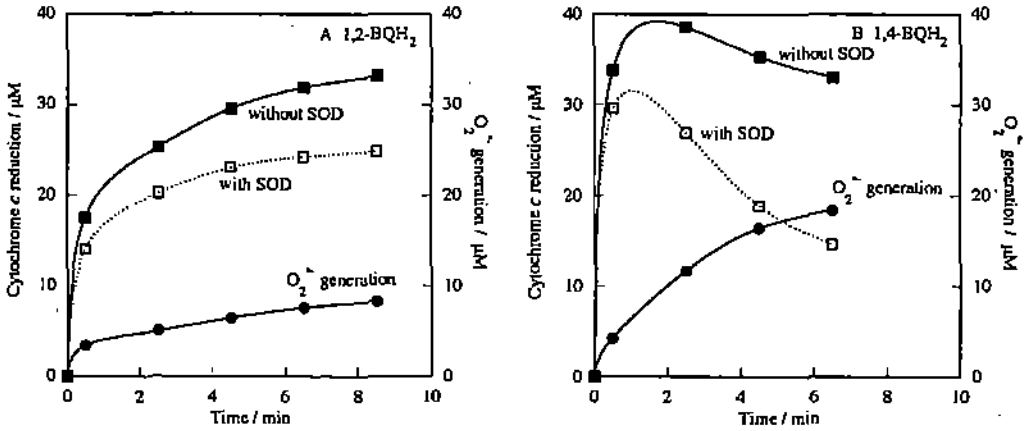

Figure 4. Time course of cytochrome *c* reduction by 1,2-BQH_2 and 1,4-BQH_2 plus 20 μM Cu^{2+}. Reactions were performed with 20 μM 1,2-BQH_2 (A) or 1,4-BQH_2 (B), plus 20 μM Cu^{2+} and 50 μM cytochrome *c* with or without 150 units/mL SOD in 1.2 mL of 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA. The amount of $O_2^{\cdot-}$ generation (●) was estimated by subtracting the amount of reduced cytochrome *c* with SOD (□) from that without SOD (■).

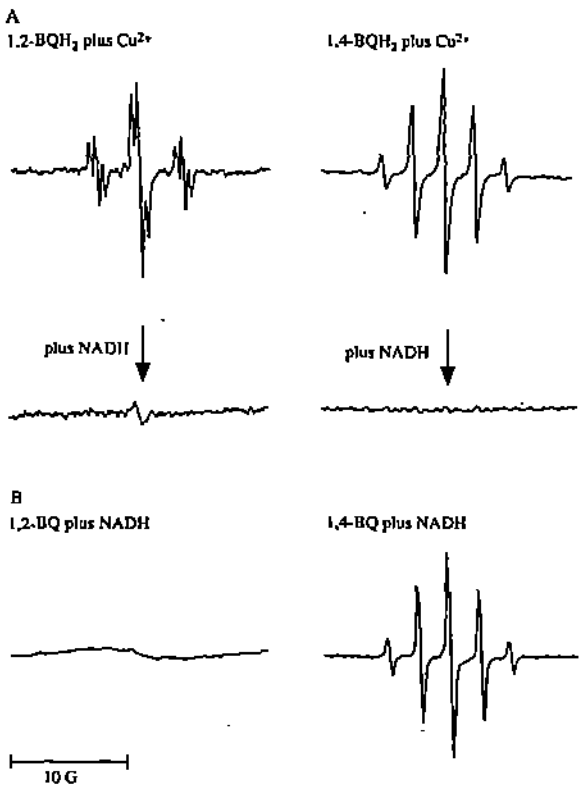

Figure 5. EPR spectra of semiquinone radicals. (A) 1,2-Semiquinone radical ($a_{H3,6}$ = 0.51 G and $a_{H4,5}$ = 3.29 G) was generated by the oxidation of 10 mM 1,2-BQH_2 by 2.5 mM $CuCl_2$ in the presence of 100 mM $MgCl_2$ and 5 mM bathocuproine; a 1,4-semiquinone radical (a_H = 2.3 G) was generated by the oxidation of 10 mM 1,4-BQH_2 by 2.5 mM $CuCl_2$. These agents were incubated for 1 min at 37 °C in 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA. These EPR signals were quenched by the addition of 10 mM NADH. (B) EPR signals of 10 mM 1,2-BQ (plus 100 mM Mg^{2+}) and 10 mM 1,4-BQ following treatment with 10 mM NADH. These agents in 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA were incubated for 1 min at 37 °C.

lowing the reduction of 1,2-BQ and 1,4-BQ by NADH, no signal was observed when 1,2-BQ was treated with NADH (Figure 5B). The EPR signal characteristic of the semiquinone radical was clearly observed following the reduction of 1,4-BQ by NADH, suggesting a one-electron reduction of 1,4-BQ by NADH.

^{1}H NMR Studies of Redox Reactions in 1,2-BQH_2/1,2-BQ and 1,4-BQH_2/1,4-BQ. The oxidized products of 1,2-BQH_2 and 1,4-BQH_2 by Cu^{2+} were extracted in chloroform-*d* to allow measurement of ^{1}H NMR spectra (Figure 6, panels A and B). When 1,2-BQH_2 and 1,4-BQH_2 were treated with Cu^{2+}, the spectra assigned to 1,2-BQ and 1,4-BQ were observed, respectively. 1,2-BQ was reduced to 1,2-BQH_2 by equimolar quantities of NADH within 10 s (Figure 6C), suggesting a two-electron reduction of a 1,2-BQ molecule by a single NADH molecule. This result is consistent with lack of an EPR signal following the reduction of 1,2-BQ by NADH. In the reduction of 1,4-BQ into 1,4-BQH_2 (Figure 6D), the ^{1}H NMR spectra indicated that approximately one-half of the 1,4-BQ was reduced into 1,4-BQH_2 in the presence of an equimolar quantity of NADH. These results indicate that one 1,4-BQ molecule is reduced into 1,4-BQH_2 through two one-electron reduction reactions mediated by two NADH molecules.

NADH Consumption in the Redox Reaction. To investigate the kinetics of these quinone redox reactions, we measured the consumption rate of NADH through a time course examining the absorption spectral changes in air-saturated conditions. In the presence of Cu^{2+}, NADH was consumed by the autoxidation of 1,2-BQH_2 in a time-dependent manner (Figure 7 A). NADH consumption by 1,2-BQH_2 was significantly faster than that of 1,4-BQH_2 (Figure 7B). 1,2-BQH_2 and 1,4-BQH_2 demonstrated similar time courses to 1,2-BQ and 1,4-BQ, respectively (Figure 7B). Although the kinetics of the redox cycle is complex, NADH consumption indicates the turnover frequency of the redox cycle and the rate of generation of reactive species. The number of cycle turnovers, estimated from the NADH consumption in 60 min in the presence of a set amount of quinones, are summarized in Table 1 along with the relative extent of DNA damage.

Discussion

In this study, we examined the ability of 1,2-BQH_2 and 1,4-BQH_2 to cause DNA damage using an in vitro system of DNA fragments obtained from human genes. In the

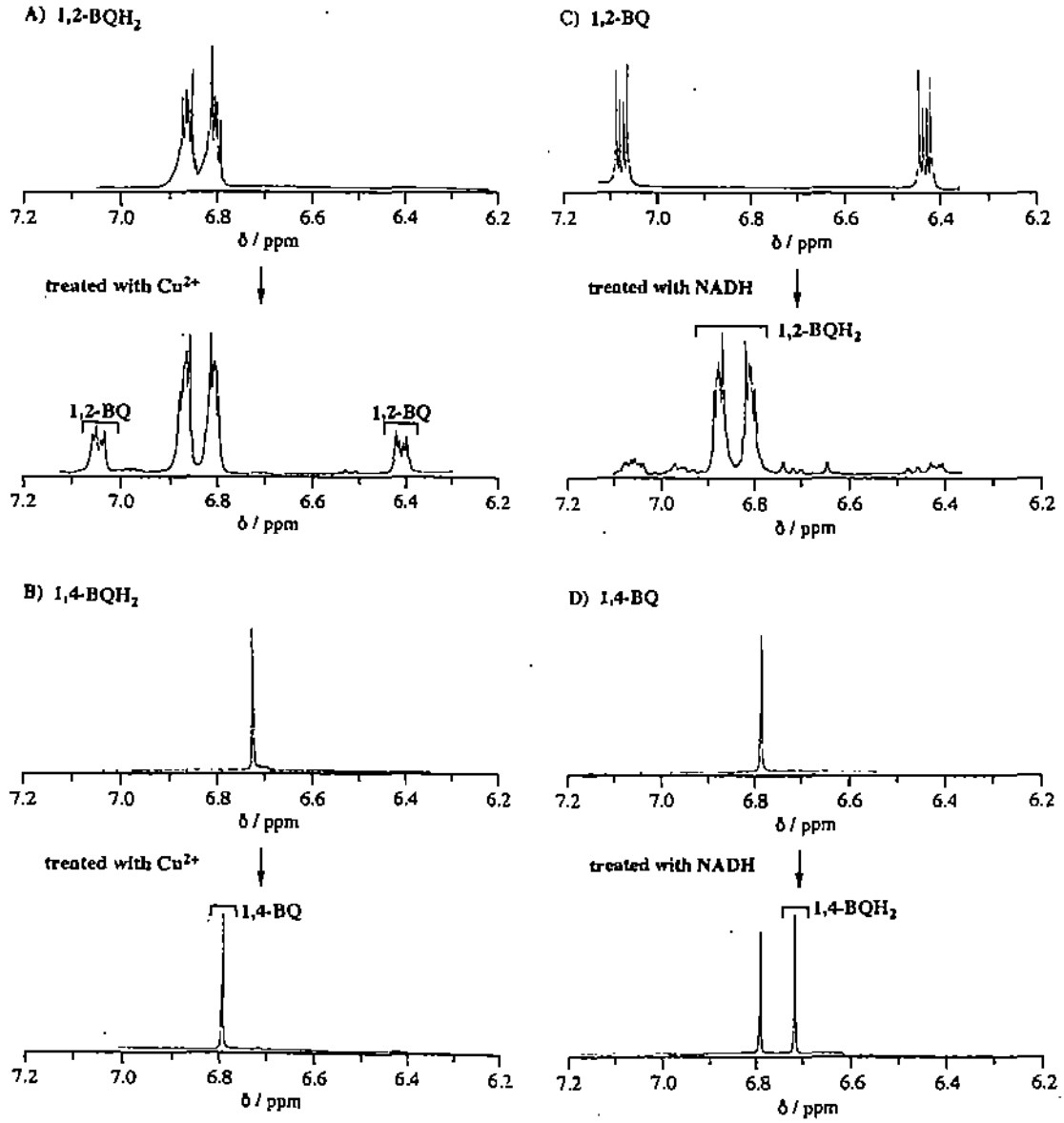

Figure 6. ^{1}H NMR spectra of 1,2-BQH_2 and 1,4-BQH_2 treated with Cu^{2+}, and 1,2-BQ and 1,4-BQ treated with NADH. An aqueous solution (0.75 mL) containing 27 mM 1,2-BQH_2 (A) or 1,4-BQH_2 (B), 27 mM $CuCl_2$, and 27 mM bathocuproine was shaken vigorously for 10 s; the organic compounds were extracted in chloroform-*d* to measure the spectrum. The chloroform (0.75 mL) of 74 mM 1,2-BQ (C) or 1,4-BQ (B) and aqueous (0.75 mL) solution of 74 mM NADH were mixed vigorously for 10 s; the chloroform phase was measured.

presence of NADH, the "carcinogenic" 1,2-BQH_2 induced stronger Cu^{2+}-mediated DNA damage than the related "noncarcinogenic" 1,4-BQH_2. In the absence of NADH, the potency of their DNA damaging activities was reversed. This result suggests that the reduction rate by NADH of the oxidized products of 1,2-BQH_2 and 1,4-BQH_2 determines their abilities to cause DNA damage, leading to carcinogenesis. This idea is supported by the observation that NADH consumption in the presence of 1,2-BQH_2/1,2-BQ was faster than that in the 1,4-BQH_2/1,4-BQ system. ^{1}H NMR and EPR studies revealed that the redox cycle of 1,2-BQH_2/1,2-BQ system involves one 1,2-BQ molecule converted into 1,2-BQH_2 through a two-electron reduction by one NADH molecule; one 1,4-BQ molecule is converted into a semiquinone radical through a one-electron reduction mediated by a single NADH molecule. These results suggest the structure of 1,2-BQ may facilitate the two-electron reduction by NADH better than 1,4-BQ. The two-electron reduction of BQ into a diol by NADH requires a greater free-energy change ($-\Delta G$) than the one-electron reduction creating a semiquinone radical. The faster reduction rate of 1,2-BQ by NADH than 1,4-BQ may be attributed to the ease of the interaction between 1,2-BQ and NADH and the differences in $-\Delta G$. Therefore, the reduction of 1,2-BQ accelerates the total turnover frequency of the redox cycle in 1,2-BQH_2/1,2-BQ greater than 1,4-BQH_2/1,4-BQ.

The DNA damage induced by 1,2-BQH_2 and 1,4-BQH_2 in the presence of Cu^{2+} and NADH results from base modification at guanine and thymine residues in conjunction with strand breakage. This result suggests the involvement of reactive species other than $OH^{\bullet}$; $OH^{\bullet}$ causes DNA damage without site specificity (*33*, *34*). In addition, $OH^{\bullet}$ scavengers demonstrated no inhibitory

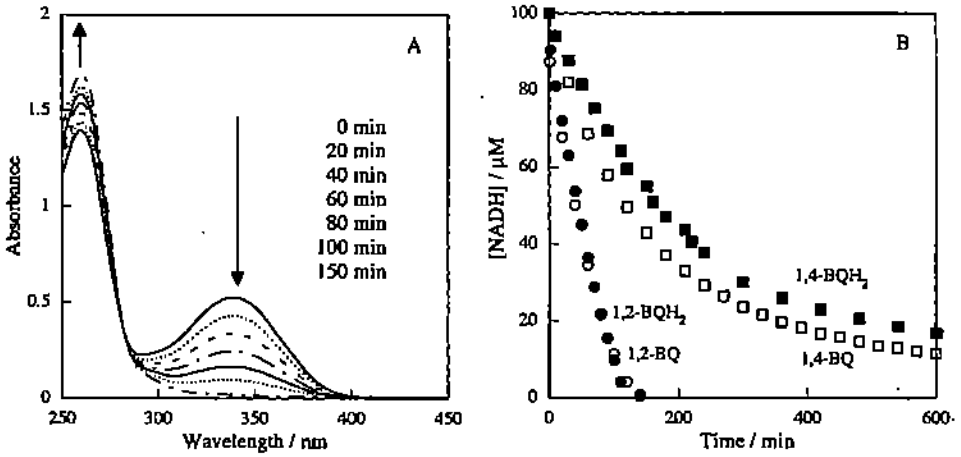

Figure 7. NADH consumption by quinones. The reaction mixture, containing 20 μM quinone, 20 μM $CuCl_2$, and NADH (initial concentration: 100 μM) in 10 mM phosphate buffer (pH 7.8) with 5 μM DTPA solution were incubated at 37 °C. (A) The absorption spectral change in the presence of 1,2-BQH_2 and $CuCl_2$. (B) The time course of NADH consumption in the presence of 1,2-BQH_2 (●), 1,2-BQ (○), 1,4-BQH_2 (■), and 1,4-BQ (□).

Table 1. Relative Extent of DNA Damage and NADH Consumption in the Redox Cycle[a]

	DNA damage	TON_{60}	half-life (min)
1,2-BQH_2	+		
1,2-BQ plus NADH	++++	3.3	40
1,4-BQH_2	++		
1,4-BQ plus NADH	+++	1.6	120

[a] The number of "+": relative extent of DNA damage estimated on the basis of results in Figures 1 and 2. TON_{60}: turnover number of NADH consumption in 60 min. half-life: half-life of NADH in the redox cycles.

effect on Cu^{2+}-mediated DNA damage by 1,2-BQH_2 and 1,4-BQH_2 in the presence of NADH. The inhibitory effects of bathocuproine and catalase on DNA damage indicate Cu^+ and H_2O_2 aid the production of the reactive species responsible for causing DNA damage (*34*–*36*).

The mechanisms of DNA damage induced by 1,2-BQH_2 and 1,4-BQH_2, summarized in Figure 8, propose that 1,2-BQH_2 and 1,4-BQH_2 are oxidized into the corresponding semiquinone radicals by Cu^{2+}, which is subsequently reduced into Cu^+. Cu^+ then reduces oxygen into $O_2^{\cdot-}$, which is easily converted into H_2O_2. Semiquinone radicals are oxidized into 1,2-BQ or 1,4-BQ, producing more Cu^+, $O_2^{\cdot-}$, and H_2O_2 in the process. Cu^+ and H_2O_2 induce oxidative DNA damage through the formation of a copper-hydroperoxo complex (Cu(I)OOH).[3] NADH non-enzymatically reduces 1,2-BQ directly into 1,2-BQH_2 through a two-electron reduction; 1,4-BQ is reduced to 1,4-BQH_2 through a semiquinone radical intermediate. Thus, the NADH-dependent redox cycle may continuously generate reactive oxygen species, resulting in the enhancement of oxidative DNA damage. NADH, a reductant existing at high concentrations (100–200 μM) in certain tissues (*37*), could facilitate the NADH-mediated DNA damage observed in this study under physiological conditions.

[3] The copper-hydroperoxo complex [Cu(I)OOH] induces DNA damage by the generation of $OH^{\cdot}$ through a Cu^+-induced, Fenton-like reaction (*34*–*36*). Copper ions have a high affinity for DNA, promoting the formation of DNA–Cu(I)OOH complexes. As this complex can generate $OH^{\cdot}$ in the vicinity of DNA, $OH^{\cdot}$ scavengers cannot inhibit DNA damage.

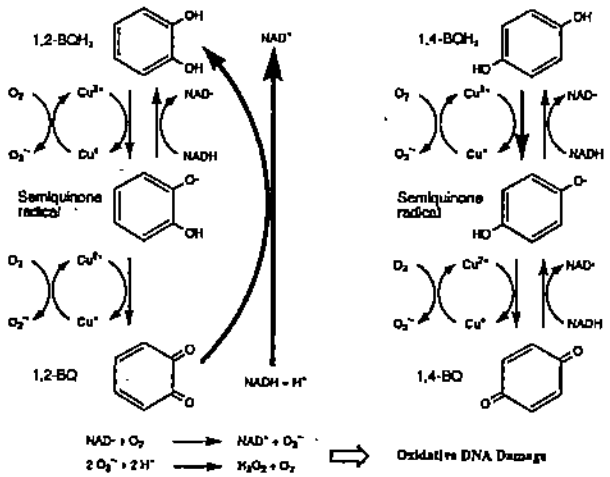

Figure 8. Proposed mechanisms of DNA damage induced by 1,2-BQH_2/1,2-BQ (left) and 1,4-BQH_2/1,4-BQ (right) in the presence of Cu^{2+} and NADH.

In summary, 1,2-BQH_2-induced DNA damage is dramatically enhanced by NADH, exceeding that induced by 1,4-BQH_2. The turnover frequency of 1,2-BQH_2/1,2-BQ redox cycle is significantly greater than that of 1,4-BQH_2/1,4-BQ, possibly resulting from the different NADH-mediated redox properties of 1,2-BQH_2 and 1,4-BQH_2. 1,2-BQH_2 is possibly carcinogenic to human (*13*), whereas 1,4-BQH_2 is not. The difference of NADH-mediated DNA damage induced by 1,2-BQH_2 and 1,4-BQH_2 is noteworthy in relation to their carcinogenic potentials. The carcinogenicity of 1,2-BQH_2 may be associated with the rapid two-electron reduction of 1,2-BQ by endogenous NADH.

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Metabolic activation of carcinogenic ethylbenzene leads to oxidative DNA damage

Kaoru Midorikawa[a], Takafumi Uchida[b], Yoshinori Okamoto[b], Chitose Toda[b], Yoshie Sakai[b], Koji Ueda[b], Yusuke Hiraku[a], Mariko Murata[a], Shosuke Kawanishi[a,*], Nakao Kojima[b,*]

[a] *Department of Environmental and Molecular Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan*
[b] *Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Nagoya 468-8503, Japan*

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Abstract

Ethylbenzene is carcinogenic to rats and mice, while it has no mutagenic activity. We have investigated whether ethylbenzene undergoes metabolic activation, leading to DNA damage. Ethylbenzene was metabolized to 1-phenylethanol, acetophenone, 2-ethylphenol and 4-ethylphenol by rat liver microsomes. Furthermore, 2-ethylphenol and 4-ethylphenol were metabolically transformed to ring-dihydroxylated metabolites such as ethylhydroquinone and 4-ethylcatechol, respectively. Experiment with ^{32}P-labeled DNA fragment revealed that both ethylhydroquinone and 4-ethylcatechol caused DNA damage in the presence of Cu(II). These dihydroxylated compounds also induced the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in calf thymus DNA in the presence of Cu(II). Catalase, methional and Cu(I)-specific chelator, bathocuproine, significantly ($P < 0.05$) inhibited oxidative DNA damage, whereas free hydroxyl radical scavenger and superoxide dismutase did not. These results suggest that Cu(I) and H_2O_2 produced via oxidation of ethylhydroquinone and 4-ethylcatechol are involved in oxidative DNA damage. Addition of an endogenous reductant NADH dramatically enhanced 4-ethylcatechol-induced oxidative DNA damage, whereas ethylhydroquinone-induced DNA damage was slightly enhanced. Enhancing effect of NADH on oxidative DNA damage by 4-ethylcatechol may be explained by assuming that reactive species are generated from the redox cycle. In conclusion, these active dihydroxylated metabolites would be involved in the mechanism of carcinogenesis by ethylbenzene.

Keywords: Ethylbenzene; Metabolic activation; Oxidative DNA damage; Carcinogenesis; Hydrogen peroxide; Copper

1. Introduction

Ethylbenzene is contained in crude petroleum, mobile fuel, paint solvent and cigarette smoke. Because of

* Corresponding authors. Tel.: +81 59 231 5011 (S. Kawanishi)/+81 52 832 1781 (N. Kojima); fax: +81 59 231 5011 (S. Kawanishi)/+81 52 834 8090 (N. Kojima).
E-mail addresses: kawanisi@doc.medic.mie-u.ac.jp (S. Kawanishi), kojiman@ccmfs.meijo-u.ac.jp (N. Kojima).

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its high volatility, ethylbenzene is widely distributed in the environment. Ethylbenzene has been issued as an air pollutant, especially an indoor pollutant. Ethylbenzene is readily absorbed via inhalation [1–3] as well as oral administration [4]. Ethylbenzene causes carcinoma in the kidney and testis of rats, and in the lung and liver of mice by inhalation [5]. Thus, ethylbenzene has been categorized as a group 2B carcinogen (possibly carcinogenic to humans) by the International Agency of Research on Cancer [6]. However, the mechanism of carcinogenesis by ethylbenzene remains to be clarified. Ethylbenzene itself has no mutagenic activity [6]. Most of the Ames test-negative chemicals exert their carcinogenicity via oxidative DNA damage [7–9]. In the present study, we examined whether the metabolites of ethylbenzene are capable of causing DNA damage through generation of reactive oxygen species. A main metabolite of ethylbenzene, 1-phenylethanol, induced renal tubular adenomas at high dose in male rats [10]. In addition, alternative pathways including ring-hydroxylation are also possible [11]. The resulting metabolites might be responsible for the ethylbenzene-mediated carcinogenesis.

To confirm this hypothesis, we analyzed ethylbenzene metabolites formed by rat liver microsomes and their ability to cause oxidative DNA damage. The metabolites were identified by high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). We investigated the ability of ethylbenzene metabolites to induce DNA damage using ^{32}P-labeled DNA fragments obtained from the human *p53* tumor suppressor gene. This gene is known to be the most important target for chemical carcinogens [12]. Moreover, mutations in the *p53* gene have been frequently found in cancer patients [13]. Effect of these metabolites on the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a characteristic oxidative product of DNA, was analyzed using an HPLC equipped with an electrochemical detector (ECD).

2. Materials and methods

2.1. Chemicals

Ethylbenzene, acetophenone, 2-ethylphenol and 4-ethylphenol were purchased from Aldrich Chemical (Milwaukee, WI). 1-Phenylethanol was from Fluka Chemie GmbH (Buchs, Switzerland). 4-Ethylcatechol (EC) was from Tokyo Kasei Kogyo (Tokyo, Japan). Ethylhydroquinone (EHQ), bis(trimethylsilyl)trifluoroacetamide (BSTFA) and calf thymus DNA were from Sigma Chemical (St. Louis, MO). Glucose 6-phosphate dehydrogenase, β-nicotinamide-adenine dinucleotide phosphate ($NADP^+$), D-glucose 6-phosphate, dimethyl sulfoxide (DMSO) and superoxide dismutase (SOD) were from Wako Pure Chemical (Osaka, Japan). Nuclease P_1 was from Yamasa Shoyu (Choshi, Chiba, Japan). Calf intestine alkaline phosphatase (CIP) was purchased from Roche Diagnostics (Mannheim, Germany). Phenobarbital was from Hoei (Osaka, Japan). Ethylbenzene, 2-ethylphenol, 4-ethylphenol, EHQ and EC were of the highest purity available (>95%). Other chemicals used were of the highest quality commercially available.

2.2. Preparation of rat liver microsomes

We prepared microsomes from the liver of male Sprague–Dawley rats (5 weeks of age, Clea Japan, Tokyo, Japan) as described previously [14]. These rats were given oral administration of phenobarbital (60 mg/kg body weight) daily for 3 days before use. The livers of rats were excised from exsanguinated rats and immediately perfused with 1.15% KCl. The livers were homogenized in four volumes of the KCl solution using a homogenizer. The microsomal fraction was obtained from the homogenate by successive centrifugation at 9000 × *g* for 20 min and 105,000 × *g* for 60 min. The fraction was washed by resuspension in the same solution and recentrifugation. The pellets of microsomes were resuspended in 1 ml of the solution for 1 g of liver. Protein amount was quantified by the Bradford method using Biorad protein assay dye reagent (Biorad, Hercules, CA). Quantity of cytochrome P450 (0.68 ± 0.04 nmol/mg protein) was determined by the method of Omura and Sato [15].

2.3. Microsomal reaction

Microsomal reaction mixture contains 2 mg/ml of microsomal protein, 1 mM $NADP^+$, 10 mM glucose 6-phosphate, 1 unit/ml of glucose 6-phosphate dehydrogenase and 10 mM $MgCl_2$ in 1 ml of 100 mM

phosphate buffer (pH 7.4). Substrate (ethylbenzene, 2-ethylphenol or 4-ethylphenol) dissolved in DMSO was added into the mixture (5 mM, final concentration of substrate) and incubated for 30 min at 37 °C. After incubation, 200 mM HCl was added and the products were extracted with diethyl ether three times. The pooled diethyl ether extract was dehydrated with sodium sulfate anhydride and evaporated.

2.4. HPLC analysis

The dried extract of microsomal reaction mixture was dissolved in 40% methanol–water containing 0.1% trifluoroacetic acid (TFA) and applied to an HPLC system (LC-VP, Shimadzu, Kyoto, Japan) equipped with a diode array detector and Develosil packed column (4.6 mm i.d. × 250 mm, Nomura Chemical, Aichi, Japan) and eluted with 40% methanol–water containing 0.1% TFA at a flow rate of 1 ml/min at 40 °C.

2.5. GC/MS analysis

For GC/MS analysis, the extract was dissolved in 100 μl of methanol. Some compounds, such as EHQ and EC, were purified by HPLC, evaporated and incubated for 30 min at 60 °C in BSTFA for a derivatization before the analysis. The samples were injected into a GC (HP 6890 GC System Plus, Agilent Technologies, Palo Alto, CA) equipped with a MS (JMS-700 MStation, JEOL, Tokyo, Japan), using electron impact ionization at 70 eV. Helium was used as carrier gas at a flow rate of 0.5 ml/min. Temperature of injector, interface and ion source was 200 °C. The temperature program for an HP Ultra 2 column (0.2 mm i.d. × 25 m × 0.33 μm film thickness, Agilent Technologies) was as follows: 40 °C (2 min isothermal), 40–120 °C (2 °C/min), and 120 °C (5 min isothermal). For derivatized compounds, temperature of injector, interface and ion source was 250 °C, and the temperature program was as follows: 70 °C (2 min isothermal), 70–280 °C (10 °C/min), and 280 °C (5 min isothermal).

2.6. Detection of damage to ^{32}P-5′-end labeled DNA

DNA fragments obtained from the human *p53* tumor suppressor gene [16] containing exons were prepared, as described previously [17]. The 5′-end labeled 650 bp fragment (*Hin*dIII* 13972–*Eco*RI* 14621) was obtained by dephosphorylation with CIP and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The asterisk (*) indicates ^{32}P-labeling. The 650 bp fragment was further digested with *Apa*I to obtain a singly labeled 443 bp fragment (*Apa*I 14179–*Eco*RI* 14621). The standard reaction mixtures (1.5 ml in an Eppendorf microtube) containing ethylbenzene metabolites, ^{32}P-5′-end labeled DNA fragments, calf thymus DNA (50 μM/base), 100 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated at 37 °C for 1 h. Then, the DNA fragments were treated in 10% (v/v) piperidine at 90 °C for 20 min. The treated DNA was electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel.

2.7. Analysis of 8-oxodG formation in calf thymus DNA

Calf thymus DNA (50 μM/base) was incubated with ethylbenzene metabolites, and 20 μM $CuCl_2$ in 4 mM sodium phosphate buffer (pH 7.8) for 1 h at 37 °C. In a certain experiment, 100 μM NADH was added. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and CIP, and then 8-oxodG content was measured with an HPLC-ECD as described previously [18]. To examine the reactive species involved in 8-oxodG formation, scavengers (ethanol, methional, SOD and catalase) and a metal chelator (bathocuproine) were added before addition of ethylbenzene metabolites to reaction mixtures. The reaction mixtures were incubated and 8-oxodG content was measured as described above.

2.8. Detection of O_2^- derived from ethylbenzene metabolites

The amount of O_2^- generated by the reaction of EHQ or EC with Cu(II) was determined by the measurement of cytochrome *c* reduction. The reaction mixture containing 40 μM ferricytochrome *c*, 100 μM EHQ or EC, 2.5 μM DTPA in 1 ml of

10 mM sodium phosphate buffer (pH 7.8) with or without SOD (100 units) was incubated at 37 °C. Maximum absorption of reduced cytochrome *c* at 550 nm ($\varepsilon_{550} = 21{,}100\ M^{-1}\ cm^{-1}$) was recorded at 1 min intervals for 6 min, using a UV–visible absorption spectrophotometer (Hitachi 228A, Tokyo, Japan). The content of $O_2^{\cdot-}$ was calculated by subtracting absorbance with SOD from that without SOD.

2.9. Measurement of Cu(I)–bathocuproine complex in the reaction of ethylbenzene metabolites and Cu(II)

Cu(I) was quantified by measuring characteristic absorption of Cu(I)–bathocuproine complex at 480 nm [19]. The reaction mixture containing 200 μM bathocuproine, 50 μM $CuCl_2$ and a test compound (EHQ or EC, 0–40 μM) dissolved in DMSO was analyzed by a spectrophotometer (Hitachi) immediately after addition of these reagents. The amount of Cu(I) was calculated using molar absorbance coefficient of the complex ($\varepsilon_{480} = 13{,}900\ M^{-1}\ cm^{-1}$).

3. Results

3.1. Metabolism of ethylbenzene by rat liver microsomes

Ethylbenzene was treated with rat liver microsomes and the metabolites were analyzed by HPLC and GC/MS. As side chain-oxidized metabolites, 1-phenylethanol and acetophenone were detected (Fig. 1). 2-Ethylphenol and 4-ethylphenol were detected as benzene ring-hydroxylated metabolites (Fig. 1). Approximately 3% of ethylbenzene was converted to 1-phenylethanol, and smaller amounts of 2-ethylphenol (0.0048%) and 4-ethylphenol (0.014%) were generated under the conditions used. The major peaks, which appeared before the retention time of 5 min, are attributed to microsomal components rather than ethylbenzene metabolites (Fig. 1). HPLC retention times and UV spectra of four metabolites were consistent with those of each authentic compound. Moreover, structures of these metabolites were confirmed by GC/MS analysis as estimated in the HPLC analysis (data not shown).

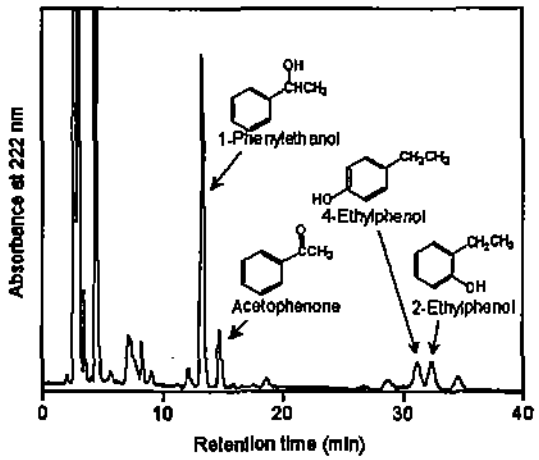

Fig. 1. HPLC profile of ethylbenzene metabolites formed by phenobarbital-treated rat liver microsomes. The conditions for the microsomal reaction and HPLC are as described in Section 2. Peaks without arrows are observed even in the absence of ethylbenzene.

3.2. 2-Ethylphenol and 4-ethylphenol-derived ethylbenzene metabolites

When 2-ethylphenol and 4-ethylphenol were treated with microsomes, ethylhydroquinone and 4-ethylcatechol were detected, respectively. Identification of these metabolites was estimated by the identical HPLC retention time and UV spectrum to that of each authentic standard (Fig. 2A and B) and confirmed by GC/MS analysis (data not shown). The major peaks, which appeared before 5 min in Fig. 2A, are attributed to microsomal components rather than ethylbenzene metabolites. The peak near 10 min in Fig. 2B is attributed to an unknown metabolite yet to be identified.

3.3. Damage to ^{32}P-labeled DNA fragments by ethylbenzene metabolites in the presence of NADH and Cu(II)

As shown in Fig. 3, both EHQ and EC induced DNA damage in the presence of Cu(II). The intensity of DNA damage increased with increasing concentrations of the metabolites (Fig. 3). Addition of an endogenous reductant NADH enhanced DNA damage by EHQ slightly. On the other hand, EC-induced DNA damage was dramatically enhanced by the addition of NADH (Fig. 3).

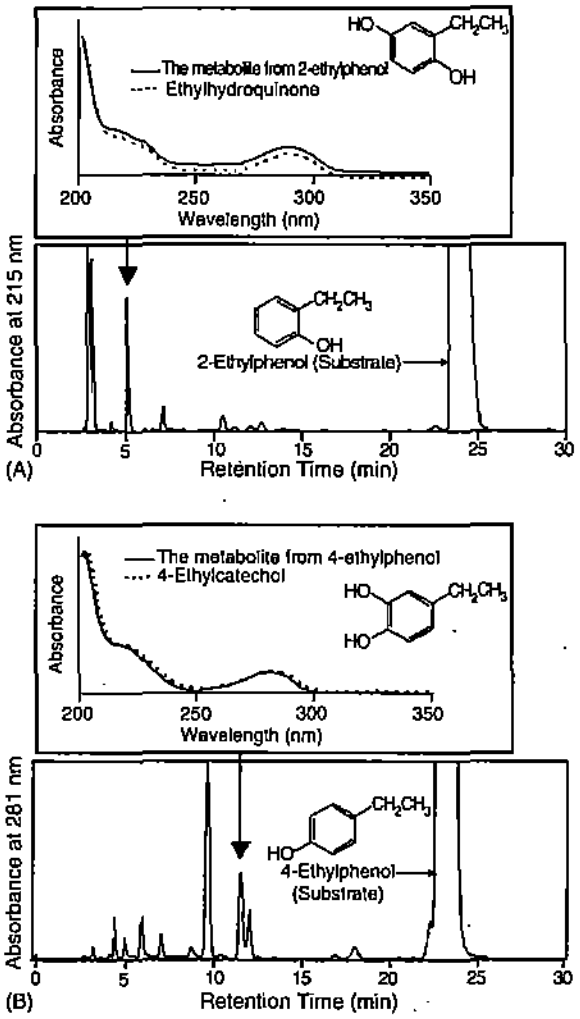

Fig. 2. HPLC profiles of 2-ethylphenol, 4-ethylphenol and their metabolites and UV spectra of the metabolites. The conditions for the microsomal reaction and HPLC are as described in Section 2. (Bottom) HPLC profiles of 2-ethylphenol (A) and 4-ethylphenol metabolites (B); (top) UV spectra of EHQ (A) and EC (B).

3.4. Formation of 8-oxodG in calf thymus DNA by ethylbenzene metabolites

Ethylbenzene metabolites, EHQ and EC, induced 8-oxodG formation in calf thymus DNA in the presence of Cu(II) in a dose-dependent manner (Fig. 4A and B). EHQ generated approximately two-fold larger amount of 8-oxodG compared with EC. Furthermore, EC-induced 8-oxodG formation increased three- to four-fold by the addition of NADH (Fig. 4B). In the case of EHQ, a slight increase in 8-oxodG formation was observed by the addition of NADH (Fig. 4A). When Cu(II) was omitted, the amounts of 8-oxodG induced by EHQ and EC were similar to the negative control (data not shown). Other ethylbenzene metabolites, 1-phenylethanol, acetophenone, 2-ethylphenol and 4-ethylphenol, did not exert 8-oxodG formation activity, under the conditions used (data not shown).

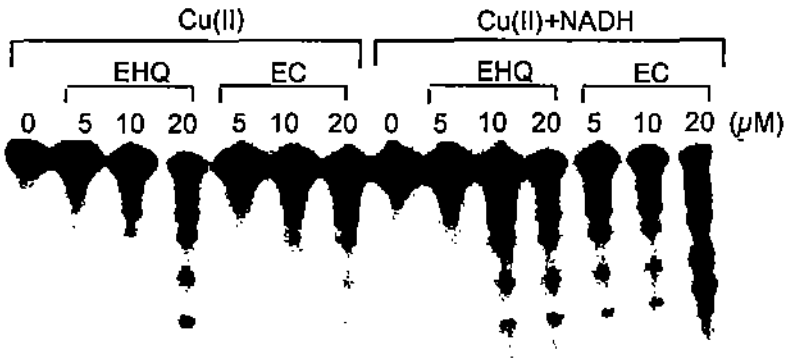

Fig. 3. Autoradiogram of ^{32}P-labeled DNA fragments incubated with ethylbenzene metabolites in the presence of NADH and Cu(II). The reaction mixtures containing each ethylbenzene metabolite (concentration as indicated), ^{32}P-5′-end labeled 443 bp DNA fragments, calf thymus DNA (50 μM/base), 100 μM NADH and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated at 37 °C for 1 h. After the incubation, the DNA fragments were treated with hot piperidine, and electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel.

3.5. Effects of radical scavengers on the formation of 8-oxodG induced by ethylbenzene metabolites

To identify the reactive species responsible for DNA damage, we investigated the inhibitory effect of reactive oxygen species scavengers and Cu(I)-specific chelator bathocuproine on the 8-oxodG formation induced by EHQ and EC (Fig. 5). Typical hydroxyl radical scavenger ethanol did not show an inhibitory effect on 8-oxodG formation. Catalase significantly inhibited 8-oxodG formation in both cases. Relatively strong inhibition was observed with methional, a wide range scavenger, which reacts with various less reactive species other than hydroxyl radical. In addition, Cu(I)-specific chelator bathocuproine completely suppressed the 8-oxodG for-

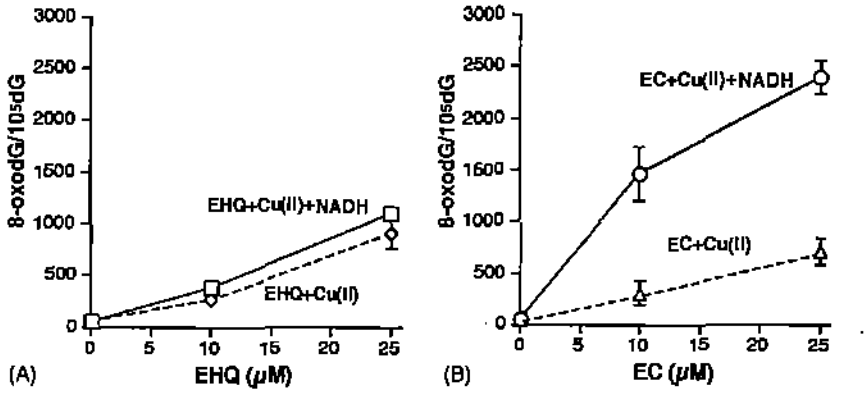

Fig. 4. Formation of 8-oxodG induced by EHQ or EC in the presence of Cu(II). Reaction mixture contained calf thymus DNA (50 μM/base), 20 μM $CuCl_2$, 100 μM NADH and EHQ (A) or EC (B) in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The reaction and measurement were performed as described in Section 2. Results were obtained from two independent experiments. Values are expressed as means ± S.D.

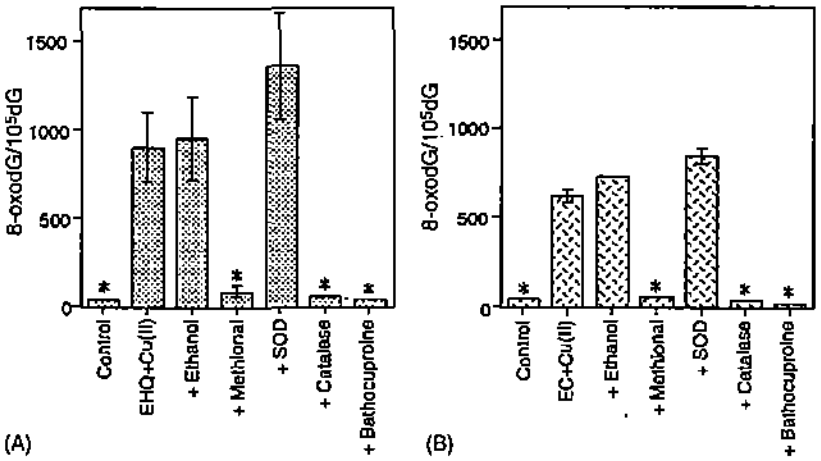

Fig. 5. Effects of reactive oxygen species scavengers and bathocuproine on 8-oxodG formation induced by EHQ or EC. Reaction mixture contained calf thymus DNA (50 μM/base), 20 μM $CuCl_2$, 25 μM EHQ (A) or EC (B) and scavenger in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. Scavenger was added to the positive control (EHQ + Cu(II) (A) and EC + Cu(II) (B)) where indicated. The concentration of scavengers and metal chelator was as follows: 5% (v/v) ethanol; 0.1 M methional; 50 units of SOD; 50 units of catalase; 50 μM bathocuproine. Control does not contain EHQ or EC. Results were obtained from three independent experiments. $^*P < 0.05$, significant decrease compared with the positive control, evaluated by *t*-test or Welch test. Values are expressed as means ± S.D.

mation. However, SOD slightly facilitated 8-oxodG formation.

3.6. Involvement of O_2^- in 8-oxodG formation

Generation of O_2^- in the 8-oxodG formation was investigated by measuring the extent of cytochrome *c* reduction. As shown in Fig. 6, O_2^- was generated in the reaction system containing EHQ or EC under the aerobic condition, suggesting the involvement of O_2^- in the mechanism of oxidative DNA damage. EHQ induced about two-fold larger generation of O_2^- compared with EC.

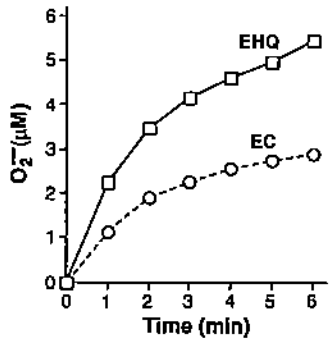

Fig. 6. O_2^--generation from EHQ or EC. The reaction mixture containing 40 μM ferricytochrome *c*, 100 μM EHQ or EC, 2.5 μM DTPA in 1 ml of 10 mM sodium phosphate buffer (pH 7.8) with or without SOD (100 units) was incubated at 37 °C. The amount of O_2^- was determined by the measurement of cytochrome *c* reduction as described in Section 2.

3.7. Stoichiometry of the reaction between ethylbenzene metabolites and Cu(II)

We investigated molar ratio of these ring-dihydroxylated metabolites (EHQ or EC) and Cu(II) in the redox reaction by measuring the absorbance of Cu(I)-bathocuproine complex (Fig. 7). Twenty-five micromolars of EHQ or EC were necessary for the complete reduction of 50 μM of Cu(II). This result implies a 2:1 stoichiometry for the reduction of Cu(II) by ethylbenzene metabolites.

4. Discussion

The present study has firstly demonstrated that EHQ and EC are formed by microsomes from rat liver as novel metabolites of ethylbenzene via generation of 2-ethylphenol and 4-ethylphenol, respectively. EC has been detected as a metabolite of ethylbenzene in bacteria [20]. Recent studies have suggested that CYP2E1 is

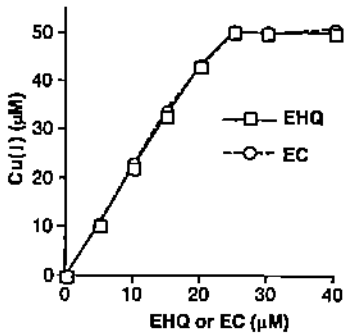

Fig. 7. Stoichiometry of the reaction between ethylbenzene metabolites and Cu(II). Calculated amounts of reduced Cu against EHQ and EC are shown based on the absorbance of the Cu(I)–bathocuproine complex as described in Section 2. The reaction mixture contains 50 μM $CuCl_2$.

the major enzyme to metabolize ethylbenzene [21,22]. Sams et al. have demonstrated that CYP2E1 catalyzes the side-chain hydroxylation of ethylbenzene to produce 1-phenylethanol [21]. 2-Ethylphenol and 4-ethylphenol are minor metabolites of ethylbenzene formed through ring hydroxylation [6,11,23]. Previous studies have demonstrated that benzene undergoes CYP2E1-catalyzed ring hydroxylation to generate toxic metabolites, such as hydroquinone, catechol and benzoquinone [24,25]. Therefore, the generation of two ring-dihydroxylated metabolites, EHQ and EC, may be primarily catalyzed by CYP2E1. This idea is supported by a recent study showing that CYP2E1 is involved in ethylbenzene metabolism to form the compounds capable of generating reactive oxygen species [22]. In this study, EHQ and EC caused oxidative DNA damage including 8-oxodG in the presence of Cu(II). EHQ formed approximately two-fold larger amount of 8-oxodG compared with EC. This result can be explained by the observation that EHQ generated about two-fold larger amount of O_2^- compared with EC. Furthermore, NADH enhanced Cu(II)-mediated DNA damage and the 8-oxodG formation induced by EC. 8-OxodG is not only as a significant biomarker for oxidative DNA damage, but also as an inducer for another intramolecular base damage in the DNA strand under oxidative stress [26]. Numerous studies have indicated that the formation of 8-oxodG causes misreplication of DNA, leading to mutation and cancer [27,28]. The kidney and testis are target organs for carcinoma induction by ethylbenzene. This can be explained by assuming that ethylbenzene metabolites produced in the liver are transported to target organs. CYPs are also expressed in the kidney [29], where toxic metabolites can be produced. The testis is highly susceptible to oxidative damage, since this organ has low activity of catalase [30]. Thus, it would be possible that the oxidative DNA damage mediated by CYP-catalyzed metabolites of ethylbenzene is involved in carcinogenesis in these organs.

Copper is present in nucleus and closely associated with chromosomes and bases [31,32]. Although mammals have evolved means of minimizing levels of free copper ions and most copper ions bind to protein carriers and transporters [33], free copper ions may participate in ROS generation under certain conditions. The level (20 μM) of free copper ions used in this study may be higher than the physiological concentrations. In our experimental conditions, DNA was treated with ethylbenzene metabolites and Cu(II) for a short time. The conditions would be relevant to the lifetime exposure of human to low level of free copper ions. NADH concentration in tissues was estimated to be as high as that applied in our in vitro system [34]. Therefore, Cu(II) and NADH may play significant roles in the mechanism of ethylbenzene metabolites-mediated DNA damage in vivo. Complete inhibition of 8-oxodG formation by Cu(I)-specific chelator suggests Cu(II) reduction coupled to the autoxidation of EHQ and EC. The significant inhibition by catalase indicates the participation of H_2O_2 in DNA damage. Generation of O_2^- in the reaction system containing the ring-dihydroxylated metabolites is consistent with the result that SOD enhances 8-oxodG formation, assuming that DNA damage is caused by H_2O_2 derived from O_2^- generated concomitantly in the reaction of these metabolites and Cu(II). Inhibitory effect of methional supports the formation of a reactive species other than hydroxyl radical, such as copper–hydroperoxo complex (Cu(I)OOH). Based on these results, we propose a possible mechanism of the Cu(II)-mediated DNA damage by ethylbenzene metabolites as shown in Fig. 8. EHQ undergoes Cu(II)-mediated autoxidation to the corresponding semiquinone radical and subsequently to ethylbenzoquinone. Similarly, EC undergoes Cu(II)-mediated autoxidation to semiquinone radical and then 4-ethyl-1,2-benzoquinone. The hypothesis that these ethylbenzene metabolites undergo two steps of Cu(II)-mediated autoxidation is confirmed by a 2:1 stoichiometry for

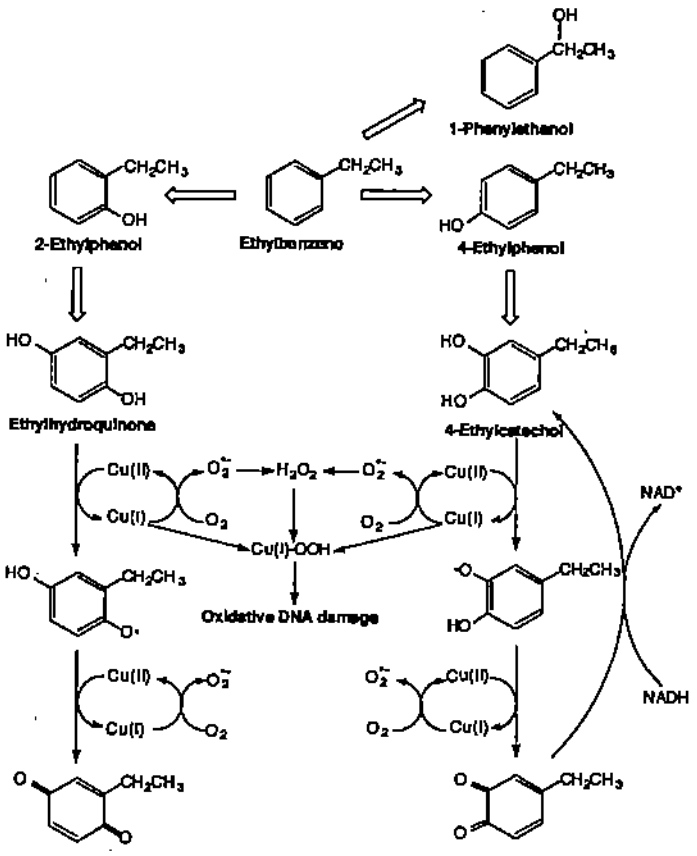

Fig. 8. Possible mechanism of oxidative DNA damage induced by EHQ and EC.

the reduction of Cu(II) by ethylbenzene metabolites. This result simply reflects the mechanism by which the maximum number of electrons are transferred to Cu(II) during autoxidation of EHQ and EC. On the other hand, abilities to cause DNA damage may depend on redox potentials of EHQ and EC. During the autoxidation, Cu(II) is reduced to Cu(I) and O_2^- is generated from O_2 by the reaction with the Cu(I). O_2^- is dismutated into H_2O_2 and interact with Cu(I) to form DNA–Cu(I)OOH complex [35]. There remains a possibility that DNA damage is induced by hydroxyl radical generated in very proximity to the DNA, i.e. in the DNA–Cu(I)OOH complex, before being scavenged [36].

We showed that EC-induced Cu(II)-mediated DNA damage more efficiently than EHQ in the presence of NADH. This result suggests that 4-ethyl-1,2-benzoquinone is again reduced to EC by NADH, and forms a redox cycle in which large amount of O_2^- is produced. Similar effects of NADH were observed with benzene metabolites, catechol. Hirakawa et al. [37] indicated that 1,2-benzoquinone was converted directly into catechol through a nonenzymatic two-electron reduction by NADH. The reduction of 1,2-benzoquinone by NADH proceeds more rapidly than that of 1,4-benzoquinone. Similarly, 4-ethyl-1,2-benzoquinone appears to be converted to 1,2-benzoquinone through NADH-mediated two-electron reduction. This reduction of 4-ethyl-1,2-benzoquinone accelerates the redox reaction, resulting in the enhancement of DNA damage. We previously demonstrated that methylcatechols, toluene metabolites, caused Cu(II)-mediated DNA damage, which was more efficiently enhanced by NADH compared with methylhydroquinone, an another toluene metabolite [38,39]. The generation of reactive oxygen

species from redox reaction contributes to carcinogenesis caused by a variety of air pollutants, such as ethylbenzene, benzene and toluene.

This is the first report that DNA damaging active compounds, EHQ and EC were detected in ethylbenzene metabolism. These metabolites have not been noticed as the causes of ethylbenzene-mediated carcinogenesis probably because of their limited production. Nevertheless, oxidative stress provided in the redox cycle containing these metabolites would be a key in the carcinogenesis mechanism of ethylbenzene.

Acknowledgement

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Metabolism of ethylbenzene by human liver microsomes and recombinant human cytochrome P450s (CYP)

Craig Sams[a,*], George D. Loizou[a], John Cocker[a], Martin S. Lennard[b]

[a] Health and Safety Laboratory, Broad Lane, Sheffield S3 7HQ, UK
[b] Academic Unit of Molecular Pharmacology and Pharmacogenetics, University of Sheffield, Sheffield S10 2JF, UK

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Abstract

The enzyme kinetics of the initial hydroxylation of ethylbenzene to form 1-phenylethanol were determined in human liver microsomes. The individual cytochrome P450 (CYP) forms catalysing this reaction were identified using selective inhibitors and recombinant preparations of hepatic CYPs.

Production of 1-phenylethanol in hepatic microsomes exhibited biphasic kinetics with a high affinity, low K_m, component (mean $K_m = 8\,\mu M$; $V_{max} = 689$ pmol/min/mg protein; $n = 6$ livers) and a low affinity, high K_m, component ($K_m = 391\,\mu M$; $V_{max} = 3039$ pmol/min/mg protein; $n = 6$). The high-affinity component was inhibited 79%–95% (mean 86%) by diethyldithiocarbamate, and recombinant CYP2E1 was shown to metabolise ethylbenzene with low K_m (35 μM), but also low V_{max} (7 pmol/min/pmol P450), indicating that this isoform catalysed the high-affinity component. Recombinant CYP1A2 and CYP2B6 exhibited high V_{max} (88 and 71 pmol/min/pmol P450, respectively) and high K_m (502 and 219 μM, respectively), suggesting their involvement in catalysing the low-affinity component.

This study has demonstrated that CYP2E1 is the major enzyme responsible for high-affinity side chain hydroxylation of ethylbenzene in human liver microsomes. Activity of this enzyme in the population is highly variable due to induction or inhibition by physiological factors, chemicals in the diet or some pharmaceuticals. This variability can be incorporated into the risk assessment process to improve the setting of occupational exposure limits and guidance values for biological monitoring.

Keywords: Biological monitoring; Cytochrome P450; Ethylbenzene

1. Introduction

Ethylbenzene is a commonly used chemical with several industrial applications. It is a solvent (often mixed with other aromatic solvents such as xylene and toluene), an intermediate in the synthesis of styrene, a raw material for the production of rubber and plastics, and an additive to some fuels (Cavender, 1994; WHO, 1996). The absorption of ethylbenzene in exposed workers arises mainly via inhalation, and to a much lesser extent through the dermal route (Gromiec and Piotrowski, 1984; Fishbein, 1985). Ethylbenzene has low acute and chronic toxicity, but it acts as a central nervous system depressant at high doses and

* Corresponding author. Tel.: +44-114-289-2725; fax: +44-114-289-2768.
E-mail address: craig.sams@hsl.gov.uk (C. Sams).

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can cause mild irritation of the mucous membranes and eyes (WHO, 1996). Therefore, it is important to control and minimise worker exposure to ethylbenzene. Its metabolites can be detected in urine, and the major product, mandelic acid, is recommended for biological monitoring of ethylbenzene (ACGIH, 2000).

Human in vivo studies have shown that mandelic acid and phenylglyoxylic acid are the major urinary metabolites following inhalation exposure to ethylbenzene (Bardodej and Bardodejova, 1970; Engstrom et al., 1984). These metabolites result from initial hydroxylation of the side chain of ethylbenzene, followed by further oxidation. Some minor ring oxidation metabolites have also been detected in urine, but these account for less than 5% of total metabolites (Engstrom et al., 1984).

Knowledge of the toxicokinetics of a chemical and the individual enzymes involved in its metabolism can improve interpretation of biological monitoring results and risk assessment by predicting the range of biotransformation rates that might be expected in the general population. In vitro metabolic systems, such as liver microsomes, have been extensively employed to study rates of biotransformation and data can be scaled to predict in vivo clearances (Houston, 1994; MacGregor et al., 2001). As part of ongoing studies of industrial solvents, the In vitro metabolism of ethylbenzene has been investigated in human liver microsomes obtained from individual donors. The aims of this study were to determine the kinetic parameters for the initial oxidation of ethylbenzene to 1-phenylethanol and to characterise the forms of cytochrome P450 (CYP) involved in this reaction.

2. Methods

2.1. Chemicals

Ethylbenzene and its metabolite, 1-phenylethanol, were obtained from Sigma–Aldrich (Gillingham, UK). All other chemicals used were of analytical grade or better.

2.2. Human liver microsomes and recombinant human cytochrome P450 isoforms

Human liver microsomes were obtained from TCS Cellworks (Botolph Claydon, UK; distributors for BioPredic, Rennes, France). Collection and processing of human tissue was conducted in compliance with all current regulatory and ethical requirements. Microsomes were characterised for activity towards a range of model CYP substrates. Donor demographics are presented in Table 1. Microsomes prepared from Baculovirus-infected insect cells expressing recombinant human CYP1A2, 2A6, 2B6, 2C9*1 (Arg_{144}), 2C19, 2D6*1, 2E1 and 3A4 co-expressed with cytochrome P450 reductase/cytochrome b_5 (Supersomes^{TM}) were obtained from Cambridge Bioscience (Cambridge, UK; distributors for Gentest Corp., Woburn, USA).

2.3. In vitro metabolism of ethylbenzene

Kinetic experiments were performed by incubation of ethylbenzene (final concentration range 10–5000 μM) with human liver microsomes. Incubation mixtures contained 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 5 mM magnesium chloride,

Table 1
Human liver microsomes: donor demographics

Patient ID	Age	Sex	Diagnosis	Total CYP (pmol/mg protein)	Rate of chlorzoxazone hydroxylation (nmol/min/mg)
MIC259015	62	F	Liver metastasis	659	2.1
MIC259018	–	F	–	434	2.1
MIC259021	45	F	Angioma	571	1.6
MIC259006	53	M	Myocardial infarction	434	1.4
MIC259007	52	M	Angioma	539	1.4
MIC259009	74	M	Liver metastasis	629	1.6
MIC259002	37	M	Steatosis	663	7.1

0.4 U/ml glucose-6-phosphate dehydrogenase, 50 mM phosphate buffer (pH 7.4) and 0.25 mg microsomal protein in a total volume of 0.5 ml. Incubations were performed in duplicate at each concentration. Incubation mixtures were pre-incubated at 37 °C for 3 min and reactions were started by addition of ethylbenzene dissolved in acetonitrile. The final acetonitrile content of the incubation mixtures was 1%, which has been previously shown to have no (Chauret et al., 1998) or minimal (Busby et al., 1999) effect on the activity of a range of CYP isoforms, including CYP2E1. Reactions were performed at 37 °C for 20 min and stopped by addition of 50 μl 3 M H_2SO_4.

For chemical inhibition studies, selective inhibitors of specific CYP isoforms were used at concentrations to obtain maximum inhibition of the CYP activity of interest with minimal effect on other CYP activities (Newton et al., 1995; Eagling et al., 1998; Sai et al., 2000). Inhibitors and their concentrations were furafylline (CYP1A2) (5 μM), sulfaphenazole (CYP2C9) (20 μM), quinidine (CYP2D6) (5 μM), diethyldithiocarbamate (DDC) (CYP2E1) (50 μM) and ketoconazole (CYP3A4) (1 μM). Furafylline and DDC were pre-incubated with microsomes for 15 min before addition of substrate. All inhibitors were added dissolved in water so that the organic solvent concentration of the incubation mixture was not altered.

Incubations of ethylbenzene with Supersomes™ expressing individual CYP isoforms were conducted using the same incubation conditions described above at a concentration of 50 pmol CYP/ml, which has previously been shown to be within the linear range with respect to rate of metabolism for model substrates. In initial experiments metabolite formation at 500 μM ethylbenzene was monitored to identify the CYP isoforms that contribute to ethylbenzene metabolism. In subsequent experiments the kinetics of 1-phenylethanol production by selected CYP isoforms over a range of ethylbenzene concentrations (10–500 μM) were determined.

2.4. Metabolite analysis

After addition of 3 M H_2SO_4, incubations were centrifuged to pellet the microsomal protein, and the supernatant was transferred to clean vials. Standards of 1-phenylethanol in 50 mM phosphate buffer (pH 7.4) were prepared in the same volume as the samples, over the concentration range 0–50 μM, and 50 μl 3 M H_2SO_4 was added. A linear relationship between metabolite concentration and chromatographic peak height was achieved over this range (data not shown). Samples were analysed by HPLC using a Hewlett-Packard model 1050 fitted with a diode array detector. A Sphereclone ODS(2) 5 μm column (250 mm × 4.6 mm) (Phenomenex, Macclesfield, UK) was used in conjunction with a Bondapak C18 guard column. The mobile phase consisted of 20% acetonitrile in water containing 0.25% (v/v) orthophosphoric acid, at a flow rate of 1 ml/min. The injection volume was 100 μl and the metabolite was detected at 200 nm.

2.5. Data analysis

Analysis of the kinetics of metabolite formation was performed by inspection of Eadie–Hofstee plots and by iterative non-linear least squares regression analysis using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA, USA). One- and two-site models incorporating the Michaelis–Menten equation were fitted to the data and the goodness of fit of each model evaluated.

3. Results

3.1. Analytical and experimental precision

The limit of detection for 1-phenylethanol was 50 pmol (0.5 μM). At 500 pmol (5 μM), the coefficient of variation of the assay was 4.5% within batch ($n = 6$) and 14% between batches ($n = 14$). Experimental precision for duplicate microsomal incubations from separate experiments analysed independently was 7% at 1 mM ($n = 3$) and 20% at 50 μM ethylbenzene ($n = 5$).

3.2. Ethylbenzene metabolism by human liver microsomes

The microsomal protein concentration and incubation time used in this study were both within linear ranges determined in preliminary experiments. No metabolite was detected when incubations were carried out in the absence of an NADPH-generating system.

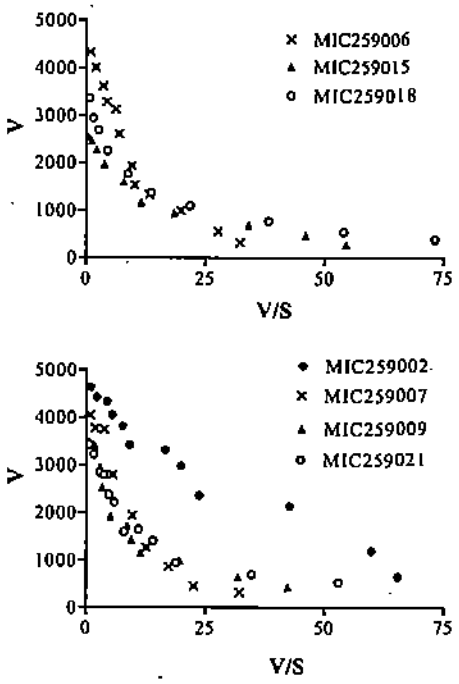

Fig. 1. Eadie–Hofstee plots for the human liver microsomal metabolism of ethylbenzene to 1-phenylethanol.

The kinetics of ethylbenzene metabolism to 1-phenylethanol were investigated in human liver microsomes from seven donors. Eadie–Hofstee plots indicated that the reaction exhibited biphasic kinetics, with a high and low-affinity component (Fig. 1). Fits of the two-site Michaelis–Menten equation to the data are shown in Fig. 2 and kinetic constants obtained by regression analysis are presented in Table 2. For the high-affinity component, the individual kinetic constants K_m and V_{max} had coefficients of variation (CV) = 36% and 40%, respectively. However variation in intrinsic clearance (V_{max}/K_m) for this component was considerably lower (CV = 18%).

One microsomal preparation (MIC259002) was found to give a noticeably less curved Eadie–Hofstee plot and also metabolised ethylbenzene at a considerably higher rate than the other preparations (high-affinity V_{max} = 2922 pmol/min/mg). It also showed an increased K_m for the high-affinity component (33 μM). However, this liver displayed steatosis, which can progress to cirrhosis, and is often a result of alcoholism, obesity or diabetes mellitus. Because of these anomalies, data obtained using this preparation were omitted from the descriptive statistics.

3.3. Correlation and inhibitor studies

Kinetic constant V_{max} for high-affinity ethylbenzene metabolism was found to correlate significantly with chlorzoxazone hydroxylase activity (Spearman r_s = 0.83, $P < 0.05$, $n = 7$).

Addition of DDC (50 μM) to microsomal incubations inhibited 1-phenylethanol production by 79%–95% at an ethylbenzene concentration of 20 μM (mean inhibition = 86%; $n = 4$). The IC_{50} for DDC inhibition of 1-phenylethanol production was determined to be 6.2 μM in a single microsome preparation (MIC259018). However, at an ethylbenzene concentration of 1 mM, DDC inhibition of 1-phenylethanol production decreased to between 57% and 82% (mean = 68%, $n = 4$).

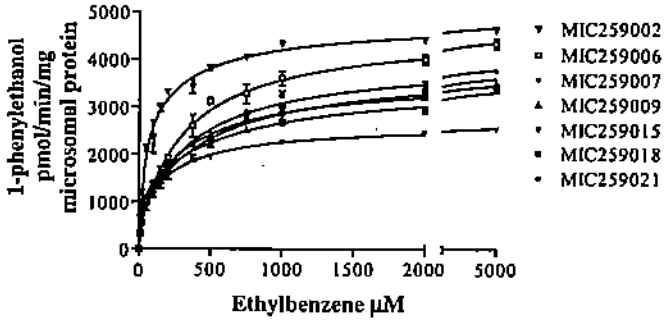

Fig. 2. Michaelis–Menten plots for the human liver microsomal metabolism of ethylbenzene to 1-phenylethanol (mean ± S.E.M., $n = 2$): r^2 of the lines was ≥0.987.

Table 2
Kinetic data for metabolism of ethylbenzene to 1-phenylethanol in human liver microsomes

	High affinity			Low affinity		
	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m[a]	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m[a]
MIC259015	8.2	707	86.2	251	1941	7.7
MIC259018	13.4	1167	87.1	582	2394	4.1
MIC259021	6.4	717	112.0	355	2923	8.2
MIC259006	5.4	359	66.5	327	4259	13.0
MIC259007	6.1	473	77.5	364	3554	9.8
MIC259009	8.5	708	83.3	467	3160	6.8
MIC259002	[33]	[2922]	[88.5]	[391]	[1901]	[4.9]
Mean	8.0	689	85.4	391	3039	8.3
S.D.	2.9	278	15.1	117	825	3.0
n	6	6	6	6	6	6

Figures in brackets were omitted from the descriptive statistics.
[a] In μl/min/mg microsomal protein.

Even in the presence of DDC the microsomal metabolism of ethylbenzene gave non-linear Eadie–Hofstee plots. Further investigation using pooled human liver microsomes (from 22 individual donors) and a range of selective CYP inhibitors showed that at low ethylbenzene concentrations (20 μM) only DDC significantly inhibited production of 1-phenylethanol. However, at high ethylbenzene concentrations (1 mM), furafylline and sulfaphenazole, as well as DDC, inhibited 1-phenylethanol production (Table 3).

3.4. Ethylbenzene metabolism by recombinant human CYPs

Preliminary experiments using 500 μM ethylbenzene showed that CYP1A2 and 2B6 exhibited the highest rate of metabolism (43.5 and 48 pmol/min/pmol P450, respectively). Less activity was detected with the other CYP isoforms investigated (CYP2A6 = 9.5 pmol/min/pmol P450, CYP2C9 = 3.0, CYP2C19 = 19.5, CYP2D6 = 10.5, CYP2E1 = 7.0, CYP3A4 = 0.5) while control Supersomes™ (containing no recombinant CYP) did not produce any 1-phenylethanol. The kinetics of 1-phenylethanol production were subsequently determined for CYP1A2, 2A6, 2B6, 2C19 and 2E1. Eadie–Hofstee plots for individual recombinant CYPs are presented in Fig. 3. Kinetic constants derived by fitting the Michaelis–Menten equation to the experimental data are shown in Table 4. The rate of chorzoxazone oxidation by the recombinant CYP2E1 preparation was determined to be 9.0 pmol/min/pmol P450.

Table 3
Inhibition of 1-phenylethanol production by selective chemical inhibitors in pooled human liver microsomes (mean of duplicate data) at two concentrations of ethylbenzene, corresponding to the high and low-affinity components of metabolism

Inhibitor	Concentration (μM)	CYP isoform	Inhibition (%)	
			20 μM ethylbenzene	1000 μM ethylbenzene
Furafylline	5	1A2	7	28
Sulfaphenazole	20	2C9	0	14
Quinidine	5	2D6	0	4
DDC	50	2E1	84	70
Ketoconazole	1	3A4	0	7

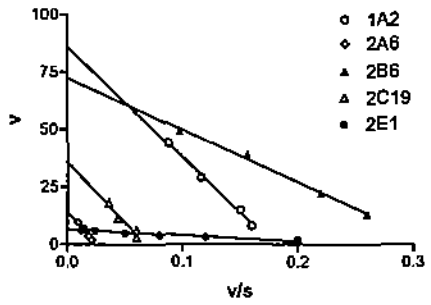

Fig. 3. Eadie–Hofstee plots of ethylbenzene metabolism to 1-phenylethanol catalysed by individually expressed recombinant CYP1A2, 2A6, 2B6, 2C19 and 2E1 (mean of duplicate data points).

Table 4
Enzyme kinetic constants for metabolism of ethylbenzene to 1-phenylethanol by individually expressed human CYP1A2, 2A6, 2B6, 2C19 and 2E1

CYP	K_m (μM)	V_{max} (pmol/min/pmol P450)	V_{max}/K_m
1A2	502	88	0.18
2A6	622	15	0.02
2B6	219	71	0.32
2C19	650	41	0.06
2E1	35	7	0.20

4. Discussion

We present human liver microsomal data for the initial step of ethylbenzene metabolism, namely side chain oxidation to form 1-phenylethanol. Our findings suggest strongly that, like many other low molecular weight chemicals, ethylbenzene is predominantly metabolised by CYP2E1 (Guengerich et al., 1991). However, in common with other structurally related compounds including xylene and toluene, ethylbenzene metabolism exhibited biphasic kinetics characterised by a high- and a low-affinity component (Tassaneeyakul et al., 1996). The high-affinity component of ethylbenzene metabolism was almost completely inhibited by the CYP2E1 inhibitor DDC. The concentration of DDC used in this study (50 μM) has been shown previously to inhibit CYP2E1 activity in human liver microsomes by about 80% while causing less than 20% inhibition of other CYPs including CYP1A2 and 2C9 (Eagling et al., 1998). This concentration of DDC was also found to cause 50% inhibition of CYP3A4 (Eagling et al., 1998). However, the lack of inhibition by ketoconazole found in the present study indicates that CYP3A4 is not active in ethylbenzene metabolism. A study using recombinant CYP isoforms indicated that DDC was not a selective inhibitor of CYP2E1 (Sai et al., 2000); since CYP2A6 and CYP2C19 were also significantly inhibited at the concentration of DDC. Thus, the panel of inhibitors used in the present study cannot eliminate the possible contribution of these two CYP isoforms to high-affinity ethylbenzene metabolism. Inhibitor studies indicated that CYP1A2, and to a lesser degree CYP2C9, may be responsible for catalysing the low affinity, high K_m, component of ethylbenzene metabolism.

Subsequent investigations using microsomes prepared from insect cells expressing recombinant human CYP isoforms showed that CYP1A2, 2B6, 2C19, and to a much lesser degree other CYPs, were able to metabolise ethylbenzene to 1-phenylethanol, in addition to CYP2E1. Kinetic constants derived from incubations using recombinant CYPs showed that CYP2E1 had between 6- and 19-fold lower K_m for ethylbenzene than the other CYPs investigated but V_{max} was also approximately 10-fold lower than that for CYP1A2 and 2B6 and six-fold lower than that for CYP2C19. This finding is consistent with CYP2E1 possessing high affinity but low capacity to metabolise ethylbenzene to 1-phenylethanol.

The rate of ethylbenzene metabolism by recombinant CYP2E1 appears to be low in comparison to some of the other CYP isoforms investigated. However, similar activity was found for the model CYP2E1 substrate chlorzoxazone. We determined the rate of chlorzoxazone hydroxylation to be 1.3-fold higher than that of ethylbenzene hydroxylation in microsomes containing recombinant CYP2E1. This difference is similar to human liver microsome preparations, where the mean rate of chlorzoxazone hydroxylation was 2.5-fold greater than the high-affinity V_{max} for ethylbenzene metabolism.

Data obtained using recombinant CYP1A2, together with inhibition by furafylline of 1-phenylethanol production at high ethylbenzene concentrations, indicated a role for CYP1A2 as a low affinity, high capacity enzyme. CYP2B6 and to a lesser degree, CYP2C19 also appeared to contribute to the low-affinity component of ethylbenzene metabolism. The different K_m values observed for these three CYPs explain the

non-linear Eadie–Hofstee plots obtained for ethylbenzene metabolism under conditions of CYP2E1 inhibition by DDC. Data obtained using recombinant CYP2A6 indicate that this isoform has low activity and low affinity for ethylbenzene, thus it makes a negligible contribution to overall metabolism. This finding has significance for the interpretation of chemical inhibition by DDC. Potential non-specific inhibition of CYP2A6 by DDC (Sai et al., 2000) can be discounted in this study, as ethylbenzene is not a good substrate for the enzyme. These data, together with a significant correlation between the high-affinity component of ethylbenzene metabolism and chlorzoxazone hydroxylation, support the conclusion that CYP2E1 is the major isoform catalysing the high-affinity component of ethylbenzene metabolism.

Scaling data from recombinant CYP isoforms to intact liver microsomes can be problematic, due to differences in the degree of expression of individual CYPs and in turnover numbers between the two systems (Venkatakrishnan et al., 2000). However, involvement of CYP2B6 and 1A2 in the low-affinity component of metabolism of structurally related chemicals to ethylbenzene has been previously demonstrated. Thus, CYP1A2 was shown to catalyse the side chain oxidation of toluene to benzylalcohol at high concentrations (Kim et al., 1997) and the side chain oxidation of styrene to styrene glycol at high concentrations has been shown to be catalysed by CYP2B6 (Nakajima et al., 1994; Kim et al., 1997).

Despite the finding that CYP1A2 and 2B6 can contribute to ethylbenzene metabolism, in practice only CYP2E1 metabolism is likely to be physiologically relevant because of the higher affinity of this isoform and the improbability of saturation occurring during occupational or environmental exposure. Exposure to ethylbenzene vapour at 100 ppm for 8 h gave rise to a concentration of 1.5 mg/l (14 μM) in blood (Lauwerys and Hoet, 2001). The involvement of CYP2E1 in the initial step of ethylbenzene metabolism has significance for the interpretation of biological monitoring data. CYP2E1 is known to have wide variation within human populations, primarily due to enzyme induction in response to fasting, diabetes or alcohol consumption (Kadlubar and Guengerich, 1992). CYP2E1 activity can also be inhibited in vivo either by dietary intake of alcohol and chemicals such as diallyl sulphate from garlic (Loizou and Cocker, 2001), or by pharmaceuticals such as chlormethiazole (Gebhardt et al., 1997) and disulfiram (Kharasch et al., 1993). In the present work, six of the microsomal preparations appear to be from normal individuals constitutively expressing CYP2E1, whereas one preparation (MIC259002) was obtained from a donor showing early symptoms of liver cirrhosis, a disease commonly resulting from alcoholism, obesity or diabetes mellitus where induction of CYP2E1 may be expected (Farrell, 1999; de la Maza et al., 2000). The V_{max} value for the CYP2E1-catalysed high-affinity component was greater than four-fold in this microsomal preparation compared to the other preparations. More kinetic data using microsomes prepared from steatotic livers or from donors with diabetes mellitus or alcoholics would help to assess further the effect of CYP2E1 induction on ethylbenzene metabolism.

The kinetic data presented in this paper are suitable for incorporation into mathematical models, such as PBPK models, to describe the fate of ethylbenzene in vivo. These models allow incorporation of biokinetic data, such as enzyme kinetic constants, with physiological parameters, such as organ size and perfusion rate (Hissink et al., 2002; Lipscomb and Kedderis, 2002; Blaauboer, 2003). Thus, the variability in the rate of CYP2E1-catalysed high-affinity metabolism of ethylbenzene to 1-phenylethanol observed in this study can be used to improve the setting of occupational exposure limits and guidance values for biological monitoring. In addition, these data contribute to the more general understanding of population variability in enzyme kinetics for low molecular weight solvents.

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Metabolism of ethylbenzene by human liver microsomes and recombinant human cytochrome P450s (CYP)

Craig Sams[a,*], George D. Loizou[a], John Cocker[a], Martin S. Lennard[b]

[a] *Health and Safety Laboratory, Broad Lane, Sheffield S3 7HQ, UK*
[b] *Academic Unit of Molecular Pharmacology and Pharmacogenetics, University of Sheffield, Sheffield S10 2JF, UK*

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Abstract

The enzyme kinetics of the initial hydroxylation of ethylbenzene to form 1-phenylethanol were determined in human liver microsomes. The individual cytochrome P450 (CYP) forms catalysing this reaction were identified using selective inhibitors and recombinant preparations of hepatic CYPs.

Production of 1-phenylethanol in hepatic microsomes exhibited biphasic kinetics with a high affinity, low K_m, component (mean K_m = 8 μM; V_{max} = 689 pmol/min/mg protein; n = 6 livers) and a low affinity, high K_m, component (K_m = 391 μM; V_{max} = 3039 pmol/min/mg protein; n = 6). The high-affinity component was inhibited 79%–95% (mean 86%) by diethyldithiocarbamate, and recombinant CYP2E1 was shown to metabolise ethylbenzene with low K_m (35 μM), but also low V_{max} (7 pmol/min/pmol P450), indicating that this isoform catalysed the high-affinity component. Recombinant CYP1A2 and CYP2B6 exhibited high V_{max} (88 and 71 pmol/min/pmol P450, respectively) and high K_m (502 and 219 μM, respectively), suggesting their involvement in catalysing the low-affinity component.

This study has demonstrated that CYP2E1 is the major enzyme responsible for high-affinity side chain hydroxylation of ethylbenzene in human liver microsomes. Activity of this enzyme in the population is highly variable due to induction or inhibition by physiological factors, chemicals in the diet or some pharmaceuticals. This variability can be incorporated into the risk assessment process to improve the setting of occupational exposure limits and guidance values for biological monitoring.

Keywords: Biological monitoring; Cytochrome P450; Ethylbenzene

1. Introduction

Ethylbenzene is a commonly used chemical with several industrial applications. It is a solvent (often mixed with other aromatic solvents such as xylene and toluene), an intermediate in the synthesis of styrene, a raw material for the production of rubber and plastics, and an additive to some fuels (Cavender, 1994; WHO, 1996). The absorption of ethylbenzene in exposed workers arises mainly via inhalation, and to a much lesser extent through the dermal route (Gromiec and Piotrowski, 1984; Fishbein, 1985). Ethylbenzene has low acute and chronic toxicity, but it acts as a central nervous system depressant at high doses and

* Corresponding author. Tel.: +44-114-289-2725; fax: +44-114-289-2768.
E-mail address: craig.sams@hsl.gov.uk (C. Sams).

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can cause mild irritation of the mucous membranes and eyes (WHO, 1996). Therefore, it is important to control and minimise worker exposure to ethylbenzene. Its metabolites can be detected in urine, and the major product, mandelic acid, is recommended for biological monitoring of ethylbenzene (ACGIH, 2000).

Human in vivo studies have shown that mandelic acid and phenylglyoxylic acid are the major urinary metabolites following inhalation exposure to ethylbenzene (Bardodej and Bardodejova, 1970; Engstrom et al., 1984). These metabolites result from initial hydroxylation of the side chain of ethylbenzene, followed by further oxidation. Some minor ring oxidation metabolites have also been detected in urine, but these account for less than 5% of total metabolites (Engstrom et al., 1984).

Knowledge of the toxicokinetics of a chemical and the individual enzymes involved in its metabolism can improve interpretation of biological monitoring results and risk assessment by predicting the range of biotransformation rates that might be expected in the general population. In vitro metabolic systems, such as liver microsomes, have been extensively employed to study rates of biotransformation and data can be scaled to predict in vivo clearances (Houston, 1994; MacGregor et al., 2001). As part of ongoing studies of industrial solvents, the In vitro metabolism of ethylbenzene has been investigated in human liver microsomes obtained from individual donors. The aims of this study were to determine the kinetic parameters for the initial oxidation of ethylbenzene to 1-phenylethanol and to characterise the forms of cytochrome P450 (CYP) involved in this reaction.

2. Methods

2.1. Chemicals

Ethylbenzene and its metabolite, 1-phenylethanol, were obtained from Sigma–Aldrich (Gillingham, UK). All other chemicals used were of analytical grade or better.

2.2. Human liver microsomes and recombinant human cytochrome P450 isoforms

Human liver microsomes were obtained from TCS Cellworks (Botolph Claydon, UK; distributors for BioPredic, Rennes, France). Collection and processing of human tissue was conducted in compliance with all current regulatory and ethical requirements. Microsomes were characterised for activity towards a range of model CYP substrates. Donor demographics are presented in Table 1. Microsomes prepared from Baculovirus-infected insect cells expressing recombinant human CYP1A2, 2A6, 2B6, 2C9*1 (Arg_{144}), 2C19, 2D6*1, 2E1 and 3A4 co-expressed with cytochrome P450 reductase/cytochrome b_5 (Supersomes™) were obtained from Cambridge Bioscience (Cambridge, UK; distributors for Gentest Corp., Woburn, USA).

2.3. In vitro metabolism of ethylbenzene

Kinetic experiments were performed by incubation of ethylbenzene (final concentration range 10–5000 μM) with human liver microsomes. Incubation mixtures contained 1.3 mM NADP, 3.3 mM glucose-6-phosphate, 5 mM magnesium chloride,

Table 1
Human liver microsomes: donor demographics

Patient ID	Age	Sex	Diagnosis	Total CYP (pmol/mg protein)	Rate of chlorzoxazone hydroxylation (nmol/min/mg)
MIC259015	62	F	Liver metastasis	659	2.1
MIC259018	–	F	–	434	2.1
MIC259021	45	F	Angioma	571	1.6
MIC259006	53	M	Myocardial infarction	434	1.4
MIC259007	52	M	Angioma	539	1.4
MIC259009	74	M	Liver metastasis	629	1.6
MIC259002	37	M	Steatosis	663	7.1

0.4 U/ml glucose-6-phosphate dehydrogenase, 50 mM phosphate buffer (pH 7.4) and 0.25 mg microsomal protein in a total volume of 0.5 ml. Incubations were performed in duplicate at each concentration. Incubation mixtures were pre-incubated at 37 °C for 3 min and reactions were started by addition of ethylbenzene dissolved in acetonitrile. The final acetonitrile content of the incubation mixtures was 1%, which has been previously shown to have no (Chauret et al., 1998) or minimal (Busby et al., 1999) effect on the activity of a range of CYP isoforms, including CYP2E1. Reactions were performed at 37 °C for 20 min and stopped by addition of 50 μl 3 M H_2SO_4.

For chemical inhibition studies, selective inhibitors of specific CYP isoforms were used at concentrations to obtain maximum inhibition of the CYP activity of interest with minimal effect on other CYP activities (Newton et al., 1995; Eagling et al., 1998; Sai et al., 2000). Inhibitors and their concentrations were furafylline (CYP1A2) (5 μM), sulfaphenazole (CYP2C9) (20 μM), quinidine (CYP2D6) (5 μM), diethyldithiocarbamate (DDC) (CYP2E1) (50 μM) and ketoconazole (CYP3A4) (1 μM). Furafylline and DDC were pre-incubated with microsomes for 15 min before addition of substrate. All inhibitors were added dissolved in water so that the organic solvent concentration of the incubation mixture was not altered.

Incubations of ethylbenzene with Supersomes™ expressing individual CYP isoforms were conducted using the same incubation conditions described above at a concentration of 50 pmol CYP/ml, which has previously been shown to be within the linear range with respect to rate of metabolism for model substrates. In initial experiments metabolite formation at 500 μM ethylbenzene was monitored to identify the CYP isoforms that contribute to ethylbenzene metabolism. In subsequent experiments the kinetics of 1-phenylethanol production by selected CYP isoforms over a range of ethylbenzene concentrations (10–500 μM) were determined.

2.4. Metabolite analysis

After addition of 3 M H_2SO_4, incubations were centrifuged to pellet the microsomal protein, and the supernatant was transferred to clean vials. Standards of 1-phenylethanol in 50 mM phosphate buffer (pH 7.4) were prepared in the same volume as the samples, over the concentration range 0–50 μM, and 50 μl 3 M H_2SO_4 was added. A linear relationship between metabolite concentration and chromatographic peak height was achieved over this range (data not shown). Samples were analysed by HPLC using a Hewlett-Packard model 1050 fitted with a diode array detector. A Spherecione ODS(2) 5 μm column (250 mm × 4.6 mm) (Phenomenex, Macclesfield, UK) was used in conjunction with a Bondapak C18 guard column. The mobile phase consisted of 20% acetonitrile in water containing 0.25% (v/v) orthophosphoric acid, at a flow rate of 1 ml/min. The injection volume was 100 μl and the metabolite was detected at 200 nm.

2.5. Data analysis

Analysis of the kinetics of metabolite formation was performed by inspection of Eadie–Hofstee plots and by iterative non-linear least squares regression analysis using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego CA, USA). One- and two-site models incorporating the Michaelis–Menten equation were fitted to the data and the goodness of fit of each model evaluated.

3. Results

3.1. Analytical and experimental precision

The limit of detection for 1-phenylethanol was 50 pmol (0.5 μM). At 500 pmol (5 μM), the coefficient of variation of the assay was 4.5% within batch ($n = 6$) and 14% between batches ($n = 14$). Experimental precision for duplicate microsomal incubations from separate experiments analysed independently was 7% at 1 mM ($n = 3$) and 20% at 50 μM ethylbenzene ($n = 5$).

3.2. Ethylbenzene metabolism by human liver microsomes

The microsomal protein concentration and incubation time used in this study were both within linear ranges determined in preliminary experiments. No metabolite was detected when incubations were carried out in the absence of an NADPH-generating system.

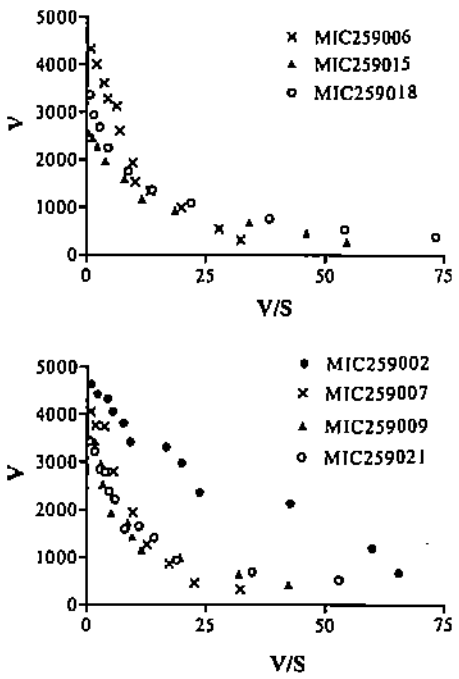

Fig. 1. Eadie–Hofstee plots for the human liver microsomal metabolism of ethylbenzene to 1-phenylethanol.

The kinetics of ethylbenzene metabolism to 1-phenylethanol were investigated in human liver microsomes from seven donors. Eadie–Hofstee plots indicated that the reaction exhibited biphasic kinetics, with a high and low-affinity component (Fig. 1). Fits of the two-site Michaelis–Menten equation to the data are shown in Fig. 2 and kinetic constants obtained by regression analysis are presented in Table 2. For the high-affinity component, the individual kinetic constants K_m and V_{max} had coefficients of variation (CV) = 36% and 40%, respectively. However variation in intrinsic clearance (V_{max}/K_m) for this component was considerably lower (CV = 18%).

One microsomal preparation (MIC259002) was found to give a noticeably less curved Eadie–Hofstee plot and also metabolised ethylbenzene at a considerably higher rate than the other preparations (high-affinity V_{max} = 2922 pmol/min/mg). It also showed an increased K_m for the high-affinity component (33 μM). However, this liver displayed steatosis, which can progress to cirrhosis, and is often a result of alcoholism, obesity or diabetes mellitus. Because of these anomalies, data obtained using this preparation were omitted from the descriptive statistics.

3.3. Correlation and inhibitor studies

Kinetic constant V_{max} for high-affinity ethylbenzene metabolism was found to correlate significantly with chlorzoxazone hydroxylase activity (Spearman r_s = 0.83, $P < 0.05$, $n = 7$).

Addition of DDC (50 μM) to microsomal incubations inhibited 1-phenylethanol production by 79%–95% at an ethylbenzene concentration of 20 μM (mean inhibition = 86%; $n = 4$). The IC_{50} for DDC inhibition of 1-phenylethanol production was determined to be 6.2 μM in a single microsome preparation (MIC259018). However, at an ethylbenzene concentration of 1 mM, DDC inhibition of 1-phenylethanol production decreased to between 57% and 82% (mean = 68%, $n = 4$).

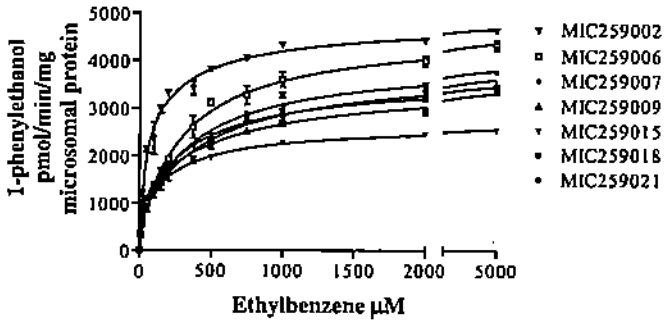

Fig. 2. Michaelis–Menten plots for the human liver microsomal metabolism of ethylbenzene to 1-phenylethanol (mean ± S.E.M., $n = 2$); r^2 of the lines was ≥0.987.

Table 2
Kinetic data for metabolism of ethylbenzene to 1-phenylethanol in human liver microsomes

	High affinity			Low affinity		
	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m[a]	K_m (μM)	V_{max} (pmol/min/mg)	V_{max}/K_m[a]
MIC259015	8.2	707	86.2	251	1941	7.7
MIC259018	13.4	1167	87.1	582	2394	4.1
MIC259021	6.4	717	112.0	355	2923	8.2
MIC259006	5.4	359	66.5	327	4259	13.0
MIC259007	6.1	473	77.5	364	3554	9.8
MIC259009	8.5	708	83.3	467	3160	6.8
MIC259002	[33]	[2922]	[88.5]	[391]	[1901]	[4.9]
Mean	8.0	689	85.4	391	3039	8.3
S.D.	2.9	278	15.1	117	825	3.0
n	6	6	6	6	6	6

Figures in brackets were omitted from the descriptive statistics.
[a] In μl/min/mg microsomal protein.

Even in the presence of DDC the microsomal metabolism of ethylbenzene gave non-linear Eadie–Hofstee plots. Further investigation using pooled human liver microsomes (from 22 individual donors) and a range of selective CYP inhibitors showed that at low ethylbenzene concentrations (20 μM) only DDC significantly inhibited production of 1-phenylethanol. However, at high ethylbenzene concentrations (1 mM), furafylline and sulfaphenazole, as well as DDC, inhibited 1-phenylethanol production (Table 3).

3.4. Ethylbenzene metabolism by recombinant human CYPs

Preliminary experiments using 500 μM ethylbenzene showed that CYP1A2 and 2B6 exhibited the highest rate of metabolism (43.5 and 48 pmol/min/pmol P450, respectively). Less activity was detected with the other CYP isoforms investigated (CYP2A6 = 9.5 pmol/min/pmol P450, CYP2C9 = 3.0, CYP2C19 = 19.5, CYP2D6 = 10.5, CYP2E1 = 7.0, CYP3A4 = 0.5) while control Supersomes™ (containing no recombinant CYP) did not produce any 1-phenylethanol. The kinetics of 1-phenylethanol production were subsequently determined for CYP1A2, 2A6, 2B6, 2C19 and 2E1. Eadie–Hofstee plots for individual recombinant CYPs are presented in Fig. 3. Kinetic constants derived by fitting the Michaelis–Menten equation to the experimental data are shown in Table 4. The rate of chorzoxazone oxidation by the recombinant CYP2E1 preparation was determined to be 9.0 pmol/min/pmol P450.

Table 3
Inhibition of 1-phenylethanol production by selective chemical inhibitors in pooled human liver microsomes (mean of duplicate data) at two concentrations of ethylbenzene, corresponding to the high and low-affinity components of metabolism

Inhibitor	Concentration (μM)	CYP isoform	Inhibition (%)	
			20 μM ethylbenzene	1000 μM ethylbenzene
Furafylline	5	1A2	7	28
Sulfaphenazole	20	2C9	0	14
Quinidine	5	2D6	0	4
DDC	50	2E1	84	70
Ketoconazole	1	3A4	0	7

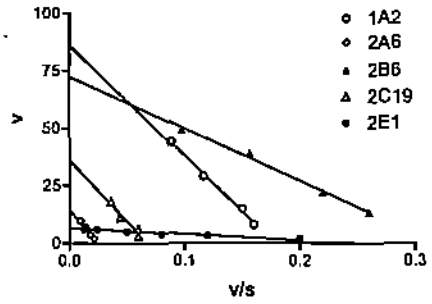

Fig. 3. Eadie–Hofstee plots of ethylbenzene metabolism to 1-phenylethanol catalysed by individually expressed recombinant CYP1A2, 2A6, 2B6, 2C19 and 2E1 (mean of duplicate data points).

Table 4
Enzyme kinetic constants for metabolism of ethylbenzene to 1-phenylethanol by individually expressed human CYP1A2, 2A6, 2B6, 2C19 and 2E1

CYP	K_m (μM)	V_{max} (pmol/min/pmol P450)	V_{max}/K_m
1A2	502	88	0.18
2A6	622	15	0.02
2B6	219	71	0.32
2C19	650	41	0.06
2E1	35	7	0.20

4. Discussion

We present human liver microsomal data for the initial step of ethylbenzene metabolism, namely side chain oxidation to form 1-phenylethanol. Our findings suggest strongly that, like many other low molecular weight chemicals, ethylbenzene is predominantly metabolised by CYP2E1 (Guengerich et al., 1991). However, in common with other structurally related compounds including xylene and toluene, ethylbenzene metabolism exhibited biphasic kinetics characterised by a high- and a low-affinity component (Tassaneeyakul et al., 1996). The high-affinity component of ethylbenzene metabolism was almost completely inhibited by the CYP2E1 inhibitor DDC. The concentration of DDC used in this study (50 μM) has been shown previously to inhibit CYP2E1 activity in human liver microsomes by about 80% while causing less than 20% inhibition of other CYPs including CYP1A2 and 2C9 (Eagling et al., 1998). This concentration of DDC was also found to cause 50% inhibition of CYP3A4 (Eagling et al., 1998). However, the lack of inhibition by ketoconazole found in the present study indicates that CYP3A4 is not active in ethylbenzene metabolism. A study using recombinant CYP isoforms indicated that DDC was not a selective inhibitor of CYP2E1 (Sai et al., 2000); since CYP2A6 and CYP2C19 were also significantly inhibited at the concentration of DDC. Thus, the panel of inhibitors used in the present study cannot eliminate the possible contribution of these two CYP isoforms to high-affinity ethylbenzene metabolism. Inhibitor studies indicated that CYP1A2, and to a lesser degree CYP2C9, may be responsible for catalysing the low affinity, high K_m, component of ethylbenzene metabolism.

Subsequent investigations using microsomes prepared from insect cells expressing recombinant human CYP isoforms showed that CYP1A2, 2B6, 2C19, and to a much lesser degree other CYPs, were able to metabolise ethylbenzene to 1-phenylethanol, in addition to CYP2E1. Kinetic constants derived from incubations using recombinant CYPs showed that CYP2E1 had between 6- and 19-fold lower K_m for ethylbenzene than the other CYPs investigated but V_{max} was also approximately 10-fold lower than that for CYP1A2 and 2B6 and six-fold lower than that for CYP2C19. This finding is consistent with CYP2E1 possessing high affinity but low capacity to metabolise ethylbenzene to 1-phenylethanol.

The rate of ethylbenzene metabolism by recombinant CYP2E1 appears to be low in comparison to some of the other CYP isoforms investigated. However, similar activity was found for the model CYP2E1 substrate chlorzoxazone. We determined the rate of chlorzoxazone hydroxylation to be 1.3-fold higher than that of ethylbenzene hydroxylation in microsomes containing recombinant CYP2E1. This difference is similar to human liver microsome preparations, where the mean rate of chlorzoxazone hydroxylation was 2.5-fold greater than the high-affinity V_{max} for ethylbenzene metabolism.

Data obtained using recombinant CYP1A2, together with inhibition by furafylline of 1-phenylethanol production at high ethylbenzene concentrations, indicated a role for CYP1A2 as a low affinity, high capacity enzyme. CYP2B6 and to a lesser degree, CYP2C19 also appeared to contribute to the low-affinity component of ethylbenzene metabolism. The different K_m values observed for these three CYPs explain the

non-linear Eadie–Hofstee plots obtained for ethylbenzene metabolism under conditions of CYP2E1 inhibition by DDC. Data obtained using recombinant CYP2A6 indicate that this isoform has low activity and low affinity for ethylbenzene, thus it makes a negligible contribution to overall metabolism. This finding has significance for the interpretation of chemical inhibition by DDC. Potential non-specific inhibition of CYP2A6 by DDC (Sai et al., 2000) can be discounted in this study, as ethylbenzene is not a good substrate for the enzyme. These data, together with a significant correlation between the high-affinity component of ethylbenzene metabolism and chlorzoxazone hydroxylation, support the conclusion that CYP2E1 is the major isoform catalysing the high-affinity component of ethylbenzene metabolism.

Scaling data from recombinant CYP isoforms to intact liver microsomes can be problematic, due to differences in the degree of expression of individual CYPs and in turnover numbers between the two systems (Venkatakrishnan et al., 2000). However, involvement of CYP2B6 and 1A2 in the low-affinity component of metabolism of structurally related chemicals to ethylbenzene has been previously demonstrated. Thus, CYP1A2 was shown to catalyse the side chain oxidation of toluene to benzylalcohol at high concentrations (Kim et al., 1997) and the side chain oxidation of styrene to styrene glycol at high concentrations has been shown to be catalysed by CYP2B6 (Nakajima et al., 1994; Kim et al., 1997).

Despite the finding that CYP1A2 and 2B6 can contribute to ethylbenzene metabolism, in practice only CYP2E1 metabolism is likely to be physiologically relevant because of the higher affinity of this isoform and the improbability of saturation occurring during occupational or environmental exposure. Exposure to ethylbenzene vapour at 100 ppm for 8 h gave rise to a concentration of 1.5 mg/l (14 μM) in blood (Lauwerys and Hoet, 2001). The involvement of CYP2E1 in the initial step of ethylbenzene metabolism has significance for the interpretation of biological monitoring data. CYP2E1 is known to have wide variation within human populations, primarily due to enzyme induction in response to fasting, diabetes or alcohol consumption (Kadlubar and Guengerich, 1992). CYP2E1 activity can also be inhibited in vivo either by dietary intake of alcohol and chemicals such as diallyl sulphate from garlic (Loizou and Cocker, 2001), or by pharmaceuticals such as chlormethiazole (Gebhardt et al., 1997) and disulfiram (Kharasch et al., 1993). In the present work, six of the microsomal preparations appear to be from normal individuals constitutively expressing CYP2E1, whereas one preparation (MIC259002) was obtained from a donor showing early symptoms of liver cirrhosis, a disease commonly resulting from alcoholism, obesity or diabetes mellitus where induction of CYP2E1 may be expected (Farrell, 1999; de la Maza et al., 2000). The V_{max} value for the CYP2E1-catalysed high-affinity component was greater than four-fold in this microsomal preparation compared to the other preparations. More kinetic data using microsomes prepared from steatotic livers or from donors with diabetes mellitus or alcoholics would help to assess further the effect of CYP2E1 induction on ethylbenzene metabolism.

The kinetic data presented in this paper are suitable for incorporation into mathematical models, such as PBPK models, to describe the fate of ethylbenzene in vivo. These models allow incorporation of biokinetic data, such as enzyme kinetic constants, with physiological parameters, such as organ size and perfusion rate (Hissink et al., 2002; Lipscomb and Kedderis, 2002; Blaauboer, 2003). Thus, the variability in the rate of CYP2E1-catalysed high-affinity metabolism of ethylbenzene to 1-phenylethanol observed in this study can be used to improve the setting of occupational exposure limits and guidance values for biological monitoring. In addition, these data contribute to the more general understanding of population variability in enzyme kinetics for low molecular weight solvents.

Acknowledgements

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Cytogenetic analysis using fluorescence in situ hybridization (FISH) to evaluate occupational exposure to carcinogens

Radim J. Sram[a,*], Olena Beskid[a], Blanka Binkova[a], Pavel Rossner[b], Zdenek Smerhovsky[a]

[a] *Laboratory of Genetic Ecotoxicology, Institute of Experimental Medicine AS CR and Health Institute of Central Bohemia, 142 20 Prague 4, Czech Republic*
[b] *Laboratory of Genetic Toxicology, National Institute of Public Health, 100 42 Prague 10, Czech Republic*

Abstract

Chromosomal aberrations determined by conventional method or fluorescence in situ hybridization (FISH) technique with whole chromosome painting are used as biomarkers of effect. Groups occupationally exposed to 1,3-butadiene (BD), acrylonitrile, ethyl benzene and benzene in petrochemical industry, and carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) from ambient air were followed by conventional method and FISH painting for chromosomes # 1 and # 4, in total 383 subjects, including controls. No effect was observed by either method with exposure to 1,3-butadiene < 1 mg/m^3 and acrylonitrile < 0.3 mg/m^3. Ethyl benzene and benzene exposure significantly increased chromosomal aberrations by both methods, which decreased after the implementation of preventive measures. The genomic frequency of translocations by FISH calculated as F_G/100 was significantly increased in city policemen versus control group exposed to c-PAHs from ambient air (1.72 ± 1.57 versus 1.25 ± 1.11, $P < 0.05$). The method of FISH with whole chromosome painting seems to be more sensitive to detect chromosomal injury by occupational exposure to carcinogens than conventional method.

Keywords: Chromosomal aberrations; Fluorescence in situ hybridization; 1,3-Butadiene; Acrylonitrile; Ethyl benzene; Carcinogenic polycyclic aromatic hydrocarbons

1. Introduction

The conventional cytogenetic analysis of peripheral blood lymphocytes (PBL) has been accepted as a technique suitable for the biological monitoring of genetic damage in somatic cells since the early 1970s. So far it has remained the only assay broadly accepted for biological monitoring of the genetic damage induced in somatic cells by excessive exposures to physical and chemical clastogenic agents in the workplaces (Carrano and Natarajan, 1988; Albertini et al., 2000). With a pivotal idea to prevent genetic injury in workers and its expected consequences, such as an increase in cancer rates or in the rates of malformations in workers' progeny, the cytogenetic analysis has been implemented as a component of preventive medical check-ups since 1976/1977. The conventional cytogenetic analysis proved its worth for the public health.

* Corresponding author. Tel.: +420-241-062-596; fax: +420-241-062-785.
E-mail address: sram@biomed.cas.cz (R.J. Sram).

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Soon it became a particularly useful tool to test the safety of industrial hygienic standards. In many instances, cytogenetic analysis provided the chief argument used to enforce improved working conditions (Sram and Kulcshov, 1980; Sram, 1981). In the middle of 1980s, the Czech Hygiene Service cytogenetically assayed up to 3000 workers yearly, and there are extensive experiences with this assay in the Czech Republic.

The exceptionally broad use of cytogenetic analysis in the Czech Republic was facilitated by the fact that the Hygiene Service accepted the theoretical assumption that the frequency of aberrant lymphocytes is a surrogate indicator for the genetic damage caused by clastogens in target tissues. Today, chromosomal aberrations in human peripheral lymphocytes are recognized as a valuable biomarker of effect, probably the only one which was internationally standardized and validated (Carrano and Natarajan, 1988; Albertini et al., 2000). Now is generally accepted that a high frequency of chromosomal aberrations in peripheral lymphocytes is predictive of an increased risk of cancer (Hagmar et al., 1998; Bonassi et al., 2000; Smerhovsky et al., 2001).

Cytogenetic analysis of chromosomal aberrations has been suggested to be a useful tool for checking whether a safe maximum allowable concentration (MAC) has really been established (Sram and Kuleshov, 1980; Sram, 1981). The MAC of a chemical is defined as the maximum concentration of a gas, vapor or aerosol in the working atmosphere that, according to contemporary scientific knowledge, does not adversely affect the health of exposed humans.

The curve in Fig. 1 represents a simplified model situation in which the exposure (concentration of the chemical in the working atmosphere integrated over time) is related to a measurable effect such as the frequency of aberrant cells (AB.C.). It is assumed that a clastogenic exposure that does not increase the frequency of adverse effects over the level found in a suitable control (unexposed) population (*B*) is expected to be safe. According to this reasoning, the MAC should be set below the threshold level (*T*) for clastogenic action (Sram, 1981).

The fluorescence in situ hybridization (FISH) technique became available for public health purposes substantially later, in the middle of 1990s. In comparison with conventional cytogenetic analyses, which detects

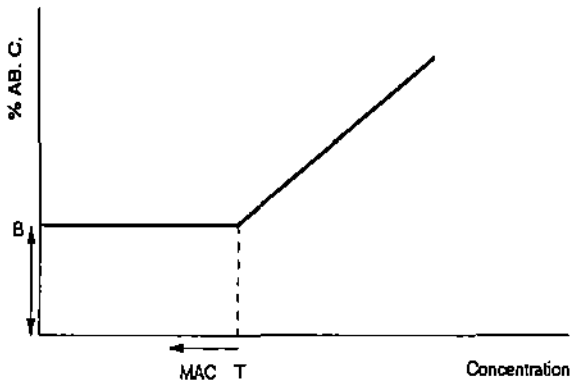

Fig. 1. Principle of establishing the MAC. (%) AB.C.: percentage of aberrant cells; *B*: background frequency of aberrant cells determined in suitable controls; *T*: threshold concentration of pollutants in working atmosphere; MAC: maximum allowable concentration is set below the threshold value.

particularly unstable types of aberrations, the FISH using whole chromosome painting was developed as a rapid and sensitive method of detecting structural rearrangements, especially reciprocal translocations (Swiger and Tucker, 1996; Pressl and Stephan, 1998). Furthermore, this method is substantially more sensitive, reliable and definitely less laborious.

The FISH painting methods have been often applied in the field of ionizing radiation research. Several reports are available on the effects of ionizing radiation on the frequency of chromosomal aberrations determined by FISH (Natarajan et al., 1996; Lindholm et al., 1998; Matsumoto et al., 1998; Hoffmann et al., 1999). As far as the exposures to chemical clastogens are a matter of interest, the available data is still scant. Only few reports were published: Rubeš et al. (1998) investigated association between exposure to cytostatic drugs and the frequency of chromosomal aberrations determined by FISH, Verdorfer et al. (2001) focused on the effect of military waste disposal, and Tucker et al. (2003) studied effects of an exposure to pesticide phosphine. Besides, the aneusomy or translocations of chromosomes expected to be related to leukemogenesis in benzene exposed workers were investigated (e.g. chromosomes # 8 and 21 or # 7 and 8; Smith et al., 1998; Zhang et al., 1999).

Therefore, we decided to carry out a study, the major aim of which was to evaluate advantages and disadvantages of the conventional cytogenetic analysis and

hybridization in situ fluorescence (FISH) technique with whole chromosome painting for chromosomes 1 and 4 from the point of view of a detection of chromosomal damage in occupationally exposed human populations. Both methods were used to analyze the level of genetic damage in subjects exposed to 1,3-butadiene (BD), acrylonitrile, ethyl benzene, and carcinogenic polycyclic aromatic hydrocarbons. Including controls, we analyzed data on 383 subjects.

2. Materials and methods

2.1. Study subjects

The clastogenic effects of 1,3-butadiene were studied in 82 workers recruited in a chemical plant; 23 workers were involved in a monomer production, 34 workers were involved in polymer production and 25 matched control subjects were selected in the plant administration. The exposure to BD was measured by personal monitoring for 8 h work shift using diffuse solid sorbent tubes. Each subject's exposure was measured on ten separate occasions over a 60-day period (Albertini et al., 2003).

The effects of acrylonitrile (ACN) were studied in the 45 males involved in the polymerization of India rubber and 23 matched controls living in the same region (controls I) and additional 33 unexposed controls from Prague (controls II). Workers were exposed in the last 3 months before blood sampling to 0.05–0.3 mg ACN/m^3 according to stationary monitoring (Sram et al., 2001).

The group of 39 male workers exposed to ethylene benzene and benzene was recruited in a production line. The concentrations of ethyl benzene at workplaces ranged from 0.2 to 13.1 mg/m^3 and concentrations of benzene ranged from 0.4 to 15.1 mg/m^3 according to stationary monitoring. The control group consisted of 55 matched control subjects.

The cytogenetic endpoints were studied in 50 male police officers spending > 8 h outdoor and 48 matched controls spending > 90% of daily time indoor. The exposure to air born dust and c-PAHs, respectively, were measured by personal monitors for the collection of PM 2.5. The participants carried the monitors for 8 h working shift. The samples of blood and urine were taken at the end of the shift. The personal monitoring was supplemented with data from stationary air pollution monitors located in Prague. The following levels of air pollution were recorded during the study period: PM10 62.6 μg/m^3, c-PAHs 24.7 ng/m^3, B[a]P 3.50 ng/m^3. Personal monitoring results for exposed population at the days of sampling were c-PAHs 12.04 ± 11.10 ng/m^3, B[a]P 1.79 ± 1.67 ng/m^3, for controls c-PAHs 6.17 ± 3.48, B[a]P 0.84 ± 0.60 ng/m^3 (Binkova et al., 2002).

Peripheral blood samples were taken from healthy subjects, all were males and all were volunteers. The frequency of chromosomal aberrations in an exposed group was compared with a suitably matched control group. All individuals were interviewed and completed questionnaire on demographic data, occupational and environmental exposures, smoking habits, medication, X-ray examinations, viral infections, and alcohol consumption within 3 months preceding blood sampling. The research conformed to the Helsinki Principles.

2.2. Culture conditions

Whole venous blood cultures were established within 24 h after blood collection in the sodium-heparinised tubes, according to the method described by Sorsa et al. (1994). Lymphocyte cultures were set up in the tissue culture flasks, each culture containing 0.6 ml of whole blood and 7.5 ml of medium (cultivation medium for one culture was composed from RPMI 1640 Sevac 1.06 ml, calf serum Opavac 1.80 ml, distilled water 4.24 ml, glutamine 0.10 ml, $NaHCO_3$ (7.5%) 0.16 ml, PHA HA-15 Murex, UK, 0.10 ml). The cultures for chromosomal aberrations were cultivated at 37 °C for 48 h. Two hours before the end of cultivation Colchicine (Fluka) was added in a final concentration of 0.5 μg Colchicine/ml culture. The cells were then treated with a hypotonic solution of KCl (0.075 M) for 10 min and fixed. After processing of the cultures and preparation of slides, slides were dried and stained after 24 h with 5% (pH 6.8) solution of Giemsa-Romanowski, randomly numbered and scored "blind" in numerical order.

The cultures for the FISH were harvested after 72 h of incubation to obtain a sufficient number of mitoses. Colchicine (Fluka) was added to a final concentration of 0.5 μg/ml 2 h before the end of the incubation. The cell suspensions were stored at −20 °C. Slides were

prepared using the air-dry method always day before painting by FISH.

2.3. Conventional chromosomal analysis

Classification of chromosomal aberrations suggested by Carrano and Natarajan (1988) was used. One hundred well spread metaphases with 46 ± 1 centromeres per subject were analyzed in 1000 times magnification. Chromatid plus chromosome breaks and chromatid plus chromosome exchanges were scored as chromosomal aberrations. Aberrant cells were determined as cells bearing breaks and exchanges. Gaps were registered but were not included in the score of aberrant cells. As another measure of aberration frequency the quantity of breaks per cell (B/C) was calculated.

2.4. Fluorescence in situ hybridization (FISH)

FISH analysis using commercial WCP probes differing in colors (Cambio, UK) for chromosomes 1 and 4 was carried out according to the manufacturer's chromosome painting protocol. Unpainted chromosomes were counterstained with DAPI. The slides were mounted in antifade solution and stored at 4° C in the dark. 1000 metaphases were examined for each subject. Zeiss Axioskop equipped with CCD camera and triple-band pass filter for DAPI and fluorochrome tracers (FITC and Cy3) was used for visualization (Rubeš et al., 1998). ISIS software (MetaSystem, Germany) was used for digitalization, detailed scanning and storing of each aberrant metaphase.

All aberrant cells were classified according to the Protocol for Aberration Identification and Nomenclature (PAINT) (Tucker et al., 1995). The Protocol defines translocations as follows: translocation is a rearranged chromosome with a single centromere and is to be counted as an aberration; the translocated chromosome must exhibit at least two colors.

Genomic frequencies (F_G) of stable chromosomal exchanges were calculated according to Lucas and Sachs (1993) using the equation: $F_G = F_{rg}/2.05\,[f_r(1-f_r)+f_g(1-f_g)-f_r f_g$. F_{rg} is the translocation frequency measured by FISH after two-color painting, f_r and f_g are the fractions of the genome painted red and green, respectively.

3. Results

3.1. Occupational exposure to 1,3-butadiene

In case of 1,3-butadiene exposed group neither conventional cytogenetic analysis nor FISH with whole chromosome painting detected statistically significant differences in the frequency of chromosomal aberrations. The results are presented in Table 1. Because the $F_G/100$ of controls (2.67±2.13) was unexpectedly high and significantly different from other control subjects examined in the Czech Republic, we attempt to reanalyze the data after excluding all subjects with suspected clonality. The results of reanalysis are shown in Fig. 2. We found the $F_G/100$ of 1.72 ± 1.14 in the monomer production workers, 2.73 ± 1.51 in workers involved in polymerization, and 2.06 ± 1.31 in controls. The differences between the $F_G/100$ found in the polymerization unit and both remaining groups reached the 0.05 level of statistical significance.

Also chromosomal aberrations determined by conventional method were elevated over historical controls (Rössner, 2000). These controls were obtained from several sources in the Czech Republic and may not be strictly comparable to occupationally exposed subjects. The analysis of questionnaire responses did

Table 1
Occupational exposure to 1,3-butadiene

Group	N	Exposure (mg/m³)	Conventional analysis		N	FISH		t
			AB.C. (%)	B/C		$F_G/100$	AB.C. (%)	
Monomer	23 (0.051–3.515)	0.642	1.52 ± 1.20	0.018 ± 0.015	23	2.35 ± 2.36	0.33 ± 0.24	5.09 ± 4.52
Polymer	34 (0.142–9.245)	1.794	1.54 ± 1.05	0.018 ± 0.014	31	3.10 ± 1.92	0.46 ± 0.33	7.06 ± 5.28
Controls	25 (0–0.084)	0.023	1.56 ± 1.23	0.018 ± 0.015	24	2.67 ± 2.13	0.37 ± 0.27	5.83 ± 4.90

AB.C. (%): aberrant cells carrying chromosomal aberrations, B/C: breaks per cell, $F_G/100$: genomic frequencies of stable chromosomal exchanges, t: translocations/1000 cells.

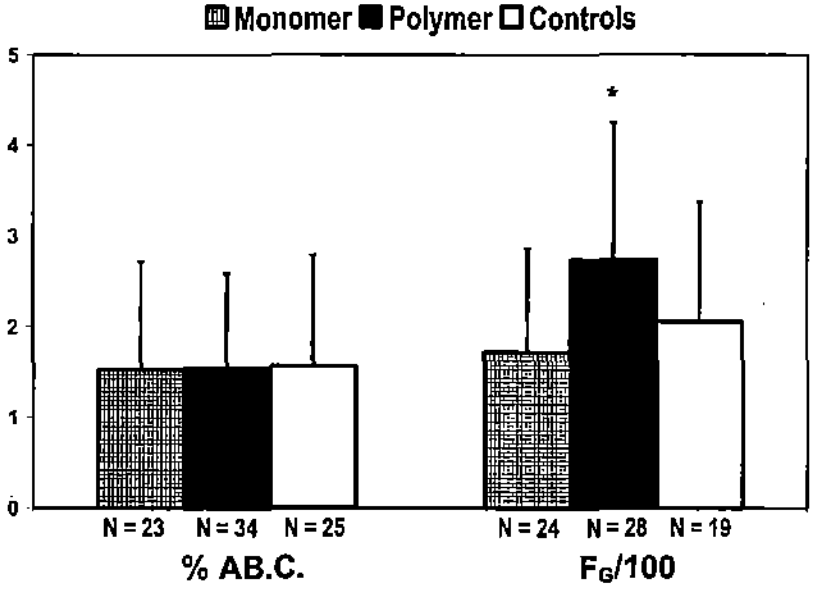

Fig. 2. Effect of 1,3-butadiene exposure. Conventional vs. FISH method. * $P < 0.05$ (comparing polymerization unit vs. monomer production unit and controls). (%) AB.C.: percentage of aberrant cells by conventional method; $F_G/100$: genomic frequency of translocations by FISH. (Exposure to 1,3-butadiene: monomer production unit 0.642 mg/m^3, polymerization unit 1.794 mg/m^3, controls 0.023 mg/m^3.)

not reveal a reason for the elevation of aberrations in control subjects, but we could exclude BD exposure as the cause. So the only effect was seen using FISH analysis comparing polymerization workers and controls as well as monomer unit workers, after the exclusion of subjects with a possible clonality. When this approach would be acceptable, it would indicate a higher sensitivity of FISH to BD exposure compared to conventional method, as well as the conclusion that BD exposure higher than 1 mg/m^3, which is the basic difference between polymerization unit exposure and monomer production unit exposure, could induce stable translocations.

3.2. Occupational exposure to acrylonitrile

The conventional cytogenetic analysis did not reveal any differences in the frequency of chromosomal aberrations determined in the acrylonitrile-exposed group, control group I and control group II, respectively. The FISH technique, however, detected significant differences ($P < 0.05$) between the frequencies of chromosomal aberrations in exposed and control group I when compared with the control group II. The results of this comparison are shown in Table 2. It is also worth to mention that smoking did not influenced the frequencies of translocations in any assayed groups. On the other hand, the frequency of stable aberrations was positively correlated with age ($R = 0.375$, $P < 0.001$), and negatively with the plasma level of Vitamin C ($R = -0.213$, $P < 0.05$) (Sram et al., 2001).

The obtained results indicate that occupational exposure to concentrations of 0.05 – 0.3 mg ACN/m^3 does not represent a significant genotoxic risk. Difference between ACN and control I groups versus controls II by FISH implies a possible effect of other factors in the region of petrochemical industry, which may increase the level of stable translocations.

3.3. Occupational exposure to ethyl benzene

Ethyl benzene is produced by benzene alkylation by ethylene. During the production process, workers are exposed to a mixture of several chemicals, especially ethyl benzene and benzene.

In the ethyl benzene-exposed groups, both compared methods indicated a significant increase in the frequency of the aberrations. The results are presented in Table 3. Unusually high levels of chromosomal damage initiated an implementation of several

Table 2
Occupational exposure to acrylonitrile

Group	N	Conventional analysis		FISH		t
		AB.C. (%)	B/C	F_G/100	AB.C. (%)	
Exposed	45	2.04 ± 1.31	0.022 ± 0.015	2.03 ± 1.18*	0.36 ± 0.21	5.42 ± 3.17*
Smokers	28	2.14 ± 1.30	0.024 ± 0.015	2.22 ± 1.38*	0.38 ± 0.24	5.93 ± 3.71*
Nonsmokers	17	1.88 ± 1.36	0.020 ± 0.015	1.71 ± 0.66	0.31 ± 0.15	4.59 ± 1.77
Controls I	23	2.65 ± 2.29*	0.028 ± 0.025	2.01 ± 1.23	0.35 ± 0.21	5.39 ± 3.30
Smokers	11	2.45 ± 2.81	0.028 ± 0.032	2.00 ± 1.43	0.34 ± 0.23	5.36 ± 3.83
Nonsmokers	12	2.83 ± 1.80*	0.028 ± 0.018	2.02 ± 1.09	0.36 ± 0.19	5.42 ± 2.91
Controls II	33	1.73 ± 1.35	0.019 ± 0.017	1.47 ± 1.50	0.28 ± 0.22	3.94 ± 4.02
Smokers	8	1.88 ± 1.13	0.020 ± 0.013	1.22 ± 0.74	0.23 ± 0.15	3.25 ± 1.98
Nonsmokers	25	1.68 ± 1.44	0.019 ± 0.018	1.55 ± 1.68	0.30 ± 0.24	4.16 ± 4.49

AB.C. (%): aberrant cells carrying chromosomal aberrations, B/C: breaks per cell, F_G/100: genomic frequencies of stable chromosomal exchanges, *t*: translocations/1000 cells.
* $P < 0.05$ (comparing exposed group and controls I vs. controls II).

industrial hygienic measures in the production line, which was followed by a significant decrease in the workers' exposures. A sample of 12 workers, who had originally more than 5% of AB.C., was cytogenetically tested after the 10 months period, and the results confirmed improved working conditions. The frequency of aberrant cells determined by the conventional cytogenetic analysis decreased in these subjects from 4.17 ± 2.08 to 2.75 ± 1.14% AB.C. ($P < 0.05$). The percentage of aberrant cells determined by FISH decreased from 0.79 ± 0.33 to 0.37 ± 0.20 ($P < 0.001$), F_G/100 decreased from 4.57 ± 2.15 to 2.18 ± 1.25 ($P < 0.001$) and the frequency of translocation per 1000 cells from 12.25 ± 5.75 to 5.83 ± 3.35 ($P < 0.001$) (Fig. 3).

3.4. Occupational exposure to carcinogenic PAHs

PAHs are widespread environmental pollutants that are derived mainly from incomplete combustion. The major sources are motor vehicles, residential heating, power plants, etc. PAHs comprise a portion of organic matter adsorbed onto respirable particles (<2.5 μm). As carcinogenic PAHs (c-PAHs) are understood benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene (B[a]P), benzo[g,h,i]perylene, chrysene, dibenzo[a,h]anthracene and indeno[1,2,3-cd]pyrene. Policemen represent a model group, which is highly exposed to ambient air pollution as they spend the most of their working hours outdoors. The use of different biomarkers of exposure,

Table 3
Occupational exposure to ethylbenzene

Group	N	Conventional analysis		FISH		t
		AB.C. (%)	B/C	F_G/100	AB.C. (%)	
Exposed	39	3.28 ± 1.70***	0.036 ± 0.019***	3.06 ± 2.13***	0.56 ± 0.31***	8.21 ± 5.63***
Smokers	25	3.44 ± 1.61*	0.038 ± 0.020**	2.95 ± 2.05*	0.57 ± 0.33**	8.00 ± 5.42*
Nonsmokers	14	3.00 ± 1.88*	0.031 ± 0.019*	3.25 ± 2.33***	0.54 ± 0.30**	8.57 ± 6.20**
Controls	55	2.05 ± 1.53	0.021 ± 0.016	1.63 ± 1.30	0.29 ± 0.21	4.35 ± 3.50
Smokers	13	2.08 ± 1.80	0.021 ± 0.018	1.81 ± 1.07	0.31 ± 0.16	4.77 ± 2.97
Nonsmokers	42	2.05 ± 1.46	0.021 ± 0.015	1.57 ± 1.37	0.29 ± 0.22	4.21 ± 3.67

AB.C. (%): aberrant cells carrying chromosomal aberrations, B/C: breaks per cell, F_G/100: genomic frequencies of stable chromosomal exchanges, *t*: translocations/1000 cells.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

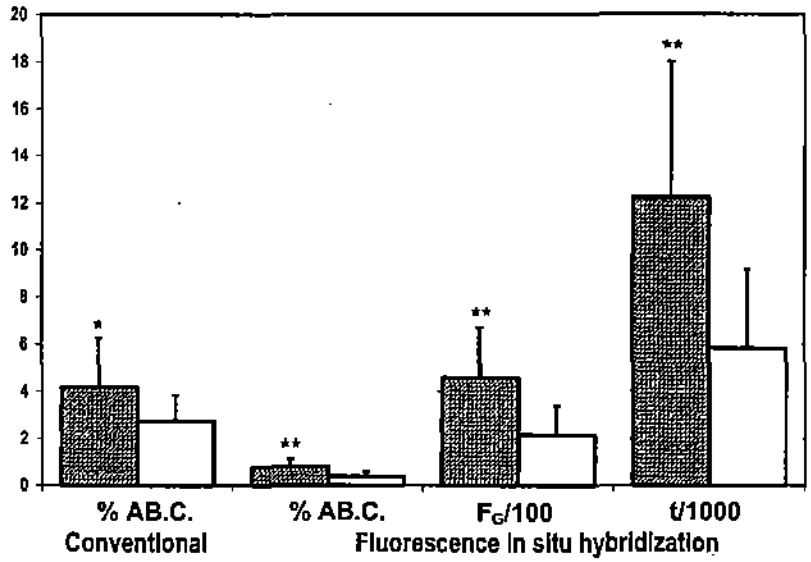

Fig. 3. Ethyl benzene: effect of implemented preventive measures. Conventional vs. FISH method. Hatch columns—sampling in January 2002, empty columns—sampling in November 2002. $^{*}P < 0.05$, $^{**}P < 0.001$, (%) AB.C: percentage of aberrant cells, F_G/100: genomic frequency of translocations by FISH, t/1000: translocations per 1000 cells.

effects and susceptibility for this type of occupational exposure was assessed (Binkova et al., 2002; Sram et al., 2002).

The conventional cytogenetic analysis did not reveal any differences between the group of police officers exposed to the ambient air pollution and the control group. Furthermore, there were no differences in smokers and nonsmokers between the groups and within the groups. The results are given in Table 4. In contrast, the FISH technique with whole chromosome painting detected a significant increase in all studied endpoints in the police officers ($P < 0.05$). This difference is the most apparent between exposed and unexposed nonsmokers F_G/100 (1.56 ± 1.34 versus 1.14 ± 1.02, $P < 0.06$).

We tried to identify the most important predictors of the conventional cytogenetic endpoints as well FISH endpoints using multivariate regression. The frequency of aberrant cells determined by conventional methods was associated with the polymorphisms of CYP1A1-Ile/Val, XPD exon 6 and XPD exon 23 ($R = 0.38$, $P < 0.001$). The frequency of

Table 4
Occupational exposure to carcinogenic PAHs

Group	N	Conventional analysis		FISH		t
		AB.C. (%)	B/C	F_G/100	AB.C. (%)	
Exposed	50	2.26 ± 1.52	0.028 ± 0.023	1.72 ± 1.57*	0.33 ± 0.25*	4.62 ± 4.21*
Smokers	17	2.18 ± 1.47	0.026 ± 0.021	2.02 ± 1.95	0.39 ± 0.32	5.41 ± 5.21
Nonsmokers	33	2.30 ± 1.57	0.028 ± 0.024	1.56 ± 1.34	0.29 ± 0.20*	4.21 ± 3.61
Controls	48	1.94 ± 1.29	0.022 ± 0.015	1.25 ± 1.11	0.24 ± 0.18	3.35 ± 2.99
Smokers	6	2.00 ± 2.10	0.022 ± 0.025	2.05 ± 1.51	0.40 ± 0.23	5.50 ± 4.04
Nonsmokers	42	1.93 ± 1.18	0.022 ± 0.014	1.14 ± 1.02	0.22 ± 0.16	3.05 ± 2.73

AB.C. (%): aberrant cells carrying chromosomal aberrations, B/C: breaks per cell, F_G/100: genomic frequencies of stable chromosomal exchanges, t: translocations/1000 cells.

* $P < 0.05$.

translocations determined by FISH was associated with age, smoking, folic acid levels, B(a)P-like DNA adducts (corresponding to the exposure of c-PAHs), polymorphisms of CYP1A1-Ile/Val, GSTP1, EPHX, and MTHFR ($R = 0.58$, $P < 0.001$).

4. Discussion

We attempt to compare the ability of conventional cytogenetic analysis and FISH technique with whole chromosome painting for chromosomes 1 and 4 to detect chromosomal damage in occupationally exposed populations. In this context, the advantages of the FISH techniques, such as better sensitivity, reliability and objectivity are usually discussed. However, practical experiences with the use of these techniques for biomonitoring of clastogenic exposures are limited. Therefore, we applied both methods on the same occupationally exposed groups and matched controls to see the differences in results. Most of all we were interested in the sensitivity of these methods under conditions common in public health practice.

Results showed significant clastogenic activity of ethyl benzene and benzene. The increase of chromosomal aberrations was interpreted by the occupational physician as the risk for exposed subjects. This became the reason to establish strict preventive measures, the use of personal protective equipment during each process, when workers are exposed to benzene and ethyl benzene. Substantial decrease of observed translocations after 10 months may be understood as a successful health intervention.

In the group occupationally exposed to ethyl benzene, the decrease of translocations seems to be more pronounced than the decrease of chromosomal breaks by conventional method. Usually, it is expected that stable translocations should circulate in lymphocytes longer than unstable chromosomal breaks. Hoffmann et al. (1999) evaluated the decline in cells bearing translocations to be ~13% per division. The information about exposure to carcinogens and persistence of translocations detected by FISH are lacking. Our data are first on this topic. They indicate, that substantial decrease in personal exposure to ethyl benzene and benzene in the course of more than 6 months can also decrease the level of stable translocations determined in peripheral lymphocytes. We can hypothesize that cells carrying several translocations do not circulate for years, but may be also eliminated as cells carrying chromosomal breaks. It seems to be pertinent to use FISH analysis repeatedly on the same subjects, trying to specify the relationship between the exposure and effect (expressed as stable translocations).

An occupational exposure to c-PAHs point out that exposure to ambient air pollution during working shifts may also induce DNA damage. As very important result is finding of the relationship between B[a]P-like DNA adduct and the frequency of translocations by FISH, corresponding to the exposure of c-PAHs. We may conclude that FISH analysis indicate that police officers in Prague represent a group of the increased genotoxic risk.

To summarize, all our results suggest, that the FISH technique is more sensitive than the conventional cytogenetic analysis. It was particularly apparent in case of police officers exposed to the levels of air pollution common in urban environment. In contrast to the FISH, the conventional method was not able to detect a chromosomal damage under these conditions.

In spite of the fact that most of features make the FISH superior to the conventional method, there is one circumstance, when an interpretation of results obtained by FISH and conventional method may differ. The FISH detects translocations, which are long lasting injuries likely transferred through many cell cycles. It means, that the FISH measures the type of chromosomal changes related to cancer and that these changes may circulate in PBL for a long period of time. However, we have observed the decrease in the level of chromosomal aberrations determined by the FISH within the period of 10 months. It seems, that it will be possible to use this method in the similar way as the conventional analysis not only to detect the exposure to clastogens but also to check an efficiency of improvements of working conditions.

The method of FISH seems to be a more sensitive method to detect clastogenic activity than conventional method. The translocations detected by FISH represent a sensitive biomarker of effect to evaluate the risk of occupational exposure to mutagens and carcinogens.

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