Peer Review Report

External Peer Review of the 1995 Koch Industries Study Report

90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group

April 29, 2013

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CONTENTS

I. INTRODUCTION

The U.S. Environmental Protection Agency's (EPA) National Center for Environmental Assessment (NCEA) is currently developing a human health assessment of trimethylbenzenes (CAS No. 25551-13-7, 95-63-6, 526-73-8, and 108-67-8). In the 1990s, IIT Research Institute performed a 90-day oral gavage toxicity study of 1,3,5-trimethylbenzene (1,3,5-TMB) on the behalf of Koch Industries, Inc. The results of this subchronic, oral toxicity study were submitted to EPA in June 1995. The 1995 Koch Industries report, "90-Day Oral Gavage Toxicity Study of 1,3,5 Trimethylbenzene in Rats with a Recovery Group," is a potential principal or influential study for the IRIS assessment of trimethylbenzenes that is currently under development. However, this report has not been subjected to a formal peer review process. Such a peer review process is important in establishing the appropriateness, validity, and robustness of the study design, conduct, and interpretation of the reported findings. The purpose of the requested letter review is for EPA to receive written comments from individual experts.

Versar selected three senior scientists with expertise in the following disciplines to serve as peer reviewers: (1) neurotoxicology, (2) human health risk assessment, and (3) general laboratory animal toxicology studies

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II. CHARGE TO REVIEWERS

The IRIS Program has a strong preference for use of peer-reviewed, published studies as principal or influential studies. Such a peer review process is important to establishing the appropriateness, validity, and robustness of the study design, conduct, and interpretation of findings of the reported investigation. The purpose of the requested peer review is for EPA to receive comments from individual experts. It is important that selected outside experts evaluate the accuracy of the content and interpretation of the findings presented in this report.

Charge Questions:

1. Study Design - Based on your knowledge of toxicological protocols, please comment on the experimental design of the 90-day oral gavage toxicity study described in the Koch Industries report.

a. Please comment on any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

b. In consideration of the toxicological properties of trimethylbenzenes reported in the provided contextual references (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Korsak et al., 2000a, b; Wiaderna et al., 1998; Gralewicz et al., 1997a; Gralewicz et al., 1997b; Korsak et al., 1997; Korsak and Rydzyński, 1996; Korsak et al., 1995), please comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.

2. Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Koch Industries study.

3. Study Conclusions - Please comment on the discussion and conclusion sections of the Koch Industries report. Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made? Do you agree with the final conclusions of the Koch Industries report?

4. Study Reliability – Describe the reliability of the subject Koch Industries study for consideration in the qualitative characterization of noncancer risk and quantitative derivation of human health reference doses. Describe any major strengths or uncertainties with the study described in this report that might preclude them from being used as consideration for derivation of a noncancer reference dose.

III. INDIVIDUAL REVIEWER COMMENTS

Review by
M. Christopher Newland, Ph.D.

Peer Review Comments on the 1995 Koch Industries Study Report:
90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group

M. Christopher Newland, Ph.D.
Auburn University

April 22, 2013

I. GENERAL IMPRESSIONS

This 1995 report described a study designed to examine the effects of chronic (90-day) exposure to a single timethylbenzene compound, 1,3,5-trimethylbenzene (mesitylene). Multiple doses representing a broad range of exposures were employed. The study appears to have followed the design as specified by the sponsor (Koch Industries) as described in the first appendix. The report is clearly and succinctly written. The effects reported in the summary table accurately reflect the results as presented in the detailed tables. Some minor protocol deviations were reported, but these are not judged to present a challenge to the overall conclusion and their reporting is consistent with GLP requirements.

The test compound was analyzed at weekly intervals throughout the study and the actual concentrations of the corn-oil solution prepared for dosing was within 10% of the specified dose.

The purpose of the report is to provide data to support a NOEL for mesitylene. While the summary and conclusions are strictly accurate in describing where there were, and were not, effects of exposure, the overall design cannot support a NOEL for human exposure to mesitylene, in my opinion. There are two principle reasons for this conclusion. First, the route of administration, oral gavage, is not the principle route of human exposure and relating the oral route to the inhalation route is difficult under the best of circumstances. This difficulty is compounded by the absence of data relating biomarkers of exposure (e.g., blood concentrations) between the two routes. The second reason is that the effect-markers presented are insensitive to exposure. There is a substantial peer-reviewed literature linking trimethylbenzene (including mesitylene) exposure to functional deficits involving behavior, electrophysiology, and respiratory function. This literature provides strong evidence that effects are readily detected on these important functions during chronic, low-level exposure, many effects persist for many weeks after exposure has ended, and these effects appear at concentrations below those that affect body weight gain, important clinical signs, blood chemistry, or organ weights, markers used in the Koch Industries report. Thus, the "NOEL" identified in the report is a "no effect" level only because insensitive endpoints were investigated. If more sensitive measures had been taken then the NOEL would certainly be much lower.

II. RESPONSE TO CHARGE QUESTIONS

1. ***Study Design - Based on your knowledge of toxicological protocols, please comment on the experimental design of the 90-day oral gavage toxicity study described in the Koch Industries report.***

   This was a multi-dose study conducted under GLP standards with doses ranging from 50 to 600 mg/kg/day, plus a corn-oil control. Dosing was accomplished daily, five days/week, as a

single bolus delivered intragastrically. With the 5 day/week dosing regimen there was a two-day washout period every weekend. The range of doses did include an exposure level that produced detectable toxicity using the some of the methods employed in this study.

The route of administration was oral gavage in a constant volume. This route of administration is of questionable relevance since human exposure would be via inhalation. The absence of blood concentrations, pharmacokinetic data, or citations to studies containing such data makes it difficult to compare these doses to those likely to be experienced by humans.

There were 10 males and 10 females in each group. No power analysis was provided, so it is difficult to ascertain whether this was an adequate sample size to detect effects of many of the endpoints used. An additional high-dose (600 mg/kg/day) group was examined 28 days after dosing ended in an effort to detect long-lasting effects. The effects detected at the end of exposure had largely disappeared by this follow-up test.

A single species, the Sprague Dawley rat, was used as a test subject. The animals were fed a standard chow diet and purified drinking water. They were housed in wire-bottom cages, which are thought to produce some stress on the animal due to lesions on the bottom of the feet; these cages are no longer used (at least in most academic laboratories).

The storage conditions of the dosing solution were specified, except it was not stated whether it was covered (trimethylbenzenes are volatile). It can probably be assumed that it was covered and data indicate that an adequate concentration was always present.

a. Please comment on any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

The Sprague Dawley rat is a widely used experimental model. I cannot comment specifically on its use in testing laboratories in the 90s but I know of nothing to suggest that this would be an inappropriate model. A stronger assessment would have used multiple species. Terminal procedures used do not raise any special concerns.

Effects that were reported include increased phosphorus levels in the blood, decreased body weight, increased phosphorus levels in the blood, discolored inguinal fur, salivation, and increased liver and kidney weights. These effects were not detected in the 28-day post exposure group, so they are interpreted as being reversible.

Clinical signs, which involve subjective judgment, were apparently not conducted by an observer blind to treatment, so the possibility that bias appeared by knowing an animal's dose group cannot be ruled out conclusively.

Statistical analyses were conducted using ANOVA followed by Dunnett's tests to determine which dose was effective. It is not clear whether Dunnett's tests were conducted routinely or whether they were conducted only following a statistically significant main effect on the

ANOVA. This is an important issue since routinely conducting Dunnett's tests will undermine the protection against false positives ("Type 1 error") provided by the ANOVA. Statistically significant results are shown only as asterisks in the tables or narrative statements: no F tests or degrees of freedom are provided.

Apparently no statistical analysis was conducted on the qualitative clinical observations or pathology. The narrative notes that 18 males and 13 females from the high-dose group showed discolored inguinal fur. Since there were only 10 males and 10 females in this group, one must conclude that some animals showed discolored fur on more than one observation, and this is confirmed in the data tables. While these results are not confirmed with a statistical assessment, I do not dispute their conclusion that this effect was seen only in the high-dose groups. There was no mention of the problem of multiple comparisons, but this is unlikely to be a concern since the effects were confined to the high-dose group, which would be expected. There was no power analysis and no positive control for most endpoints so the ability of this overall test system to detect known effects is difficult to ascertain.

b. In consideration of the toxicological properties of trimethylbenzenes reported in the provided contextual references (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Korsak et al., 2000a, b; Wiaderna et al., 1998; Gralewicz et al., 1997a; Gralewicz et al., 1997b; Korsak et al., 1997; Korsak and Rydzyński, 1996; Korsak et al., 1995), please comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.

A series of studies conducted by scientists at the Nofer Institute of Occupational Medicine has been published in peer-reviewed journals in the toxicology literature. These provide a comprehensive neurotoxicological profile of three trimethylbenzenes, including mesitylene, when administered by inhalation. The other two trimethylbenzenes examined were pseudocumene (1,2,4-trimethylbenzene) and hemimellitene (1,2,3-trimethylbenzene).

The exposure levels used in these studies did not produce changes in food consumption, body weight, body weight gain, and lethality, and no changes were reported on "toxicologically significant" clinical signs. Specific effects on fur discoloration are unknown since this was not mentioned explicitly. The effects of acute, subchronic (30 day), chronic (90 day), and post chronic (one to two months after chronic exposure ended) were assessed. Therefore, effects of acute and chronic exposure, as well as irreversible effects of chronic exposure, were described. Several functional domains, including motor (rotarod), pain sensitivity (paw-lick latency on a hot plate), overall activity, cognitive (radial arm maze and passive/active avoidance), electrophysiological (EEG), and respiratory endpoints were examined.

A broad range of effects were noted on several functional domains with the trimethylbenzenes. Mesitylene was examined in some, but not all of these studies. The three trimethylbenzenes differed somewhat in potency but the effects profile was generally similar for these three compounds. Therefore, it is possible draw reasonable general inferences about mesitylene from the effects seen with the other two trimethylbenzenes.

Overall, these three trimethylbenzenes altered rotarod performance, impaired passive and active avoidance, produced deficits on the rotarod, and caused significant respiratory effects.

There were effects, though less consistently, on radial arm maze performance. Many of these effects were irreversible when tested one to two months after exposure ended. As noted (Korsak and Rydzynski, 1996), trimethylbenzene's toxicity resembles that of other organic solvents such as toluene, but it is more potent than toluene. This is of interest here because the extensive literature on toluene suggests that even more refined behavioral tests detect effects of inhaled toluene at concentrations that are similar to those reported here, or even lower. This is important because it suggests that the NOEL or LOEL for trimethylbenzenes might even be lower than in these peer-reviewed studies with more advanced testing.

In these studies, mesitylene in particular produced the following effects:

- Deficits on rotarod after acute exposure (EC50 = 963 ppm) (Korsak and Rydzynski, 1996).

- Deficit on hotplate after acute exposure (EC50 = 1212 ppm) (Korsak and Rydzynski, 1996).

- Decreased respiration rate after acute exposure (EC50 = 519 ppm)(Korsak et al., 1997).

- Passive avoidance after 30 day exposure to 25 ppm, and higher concentrations (Wiaderna et al., 2002).

- Diminished pain sensitivity after 30 day exposure to 100 ppm (Wiaderna et al., 2002).

- Changes in open field activity 25 days after 4 weeks of exposure to 100 ppm (Gralewicz and Wiaderna, 2001).

- Deficits in passive avoidance 39 to 40 days after 4 weeks of exposure to 100 ppm (Gralewicz and Wiadera, 2001)

- Decreased pain sensitivity 50 to 51 days after 4 weeks of exposure to 100 ppm. (Graleeicz and Wiaderna, 2001)

- Impaired acquisition of active avoidance 54 to 60 days after 4 weeks of exposure to 100 ppm. (Graleeicz and Wiaderna, 2001)

These studies are difficult to compare directly with the Koch report because of differences in the dosing regimen and, in particular, the route of exposure, but some points can be noted:

- The exposure levels used in the Nofer Institute studies did not change body weight or body weight gain; yet consistent and reproducible effects were detected in functional domains, including behavioral, electrophysiological, and respiratory endpoints.

- The route of administration in the Nofer Institute studies is more directly relevant to human exposure than that used in the Koch study.

- The route of administration used in the Nofer Institute studies likely produces a fairly stable blood concentration throughout exposure, whereas the oral gavage studies used in the Koch study would likely produce a brief, high blood concentration followed quickly by a return to baseline levels. This inference is not based on data provide in any of the studies reviewed but instead is based on a general understanding of the pharmacokinetics following inhalation or gavage administration of organic solvents.

2. ***Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Koch Industries study.***

The Koch Industries study appears to have been competently conducted and the conclusions are supported by the data presented. However, the relevance of the results to an evaluation of human exposure is highly questionable. This is because the route of administration is likely to produce a kinetic profile that is quite different from the chronic, low-level exposure experienced by humans. In addition, the toxicological endpoints selected for study are generally insensitive ones. No relevant behavioral, respiratory, or electrophysiological endpoints were examined. The pathology tests performed were generally of a gross nature so would be unlikely to detect effects of chronic low-level exposure.

3. ***Study Conclusions - Please comment on the discussion and conclusion sections of the Koch Industries report. Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made? Do you agree with the final conclusions of the Koch Industries report?***

The discussion was strictly limited to the results of the study and the discussion described the results accurately. It did not identify limitations such as the absence of a power analysis or the statistical analysis of the qualitative endpoints. There was no attempt to link these results to a broader (and extensive) literature on other organic solvents. In fairness, it must be noted that there was little literature on trimethylbenzenes at the time that this study was conducted, so it would have been difficult to relate the study under review to a broader literature on trimethylbenzenes in particular. However, there was an extensive literature on other organic solvents that could have been used for comparison.

I cannot disagree with the narrow conclusion that there is a NOEL of 200 mg/kg based narrowly on the studies reported. However, if endpoints more pertinent to human health and a more relevant dosing regimen were employed, then I am very confident that a lower NOEL would have been detected.

In addition, the statistical analysis employed and overall strategy relies on the detection of a NOAEL. As has been noted many times, this approach is of questionable value because an experiment can easily be designed to produce a NOAEL by making it underpowered (using too few subjects) or by using insensitive endpoints. The latter seems to be at issue here. Alternatively, had a benchmark dosing analysis been attempted, a different conclusion might also have been reached.

4. ***Study Reliability – Describe the reliability of the subject Koch Industries study for consideration in the qualitative characterization of noncancer risk and quantitative derivation of human health reference doses. Describe any major strengths or uncertainties with the study described in this report that might preclude them from being used as consideration for derivation of a noncancer reference dose.***

The study seems to be reliable as far as it goes. The implementation of the study appears to have been competently performed and the documentation is extensive. However, its validity (the ability to predict human toxicity) is questionable. I would have significant misgivings about using this study as a basis for the derivation of a noncancer reference dose.

- The dosing regimen and route of administration are of questionable relevance.
- No data on blood concentrations of mesitylene are presented.
- The endpoints used are insensitive.
- There is an extensive and reproducible peer-reviewed literature available that can be used to identify a LOEL or a NOEL (probably a LOEL). That literature would identify such a level in units (air concentration) that are directly relevant to human exposure and using more sensitive and relevant endpoints. While it can be noted that there are flaws in that literature, the strengths for identifying a LOEL or NOEL from the peer-reviewed literature far outweigh the weaknesses, especially in comparison with the Koch Industries report.

III. SPECIFIC OBSERVATIONS

All of my comments are noted above.

Review by
Carol S. Wood, Ph.D., DABT

Peer Review Comments on the 1995 Koch Industries Study Report:
90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group

Carol S. Wood, Ph.D., DABT
Oak Ridge National Laboratory

April 18, 2013

I. GENERAL IMPRESSIONS

The study report is well written and accurately reflects the findings in the study. This study was conducted appropriately based on standard guidelines for a 90-day subchronic oral toxicity study, with the exception of dose selection. Clinical signs of toxicity, including salivation and fur staining, were observed in high-dose males and females. These clinical findings were general in nature, mild, not observed in all high-dose animals, and not observed until after about three weeks of dosing. Other statistically significant findings are not considered by the reviewer to be adverse or biologically relevant. Taken together, the lack of findings indicating marked toxicity suggests that the animals could have tolerated a higher dose and that dosing was not adequate. A dose selection rationale was not given beyond the statement that the doses were chosen by the sponsor.

Potential neurotoxicity was not evaluated by the oral route in this study. Considering long-lasting, post-exposure effects observed in several of the contextual references, this endpoint is critical to evaluating the full toxicity of TMB by any route. Delayed onset of the clinical signs observed in the Koch Industries study supports potential long-lasting effects of TMB that were not evaluated. Similar to the current study, the standard subchronic inhalation studies (Korsak et al., 2000a,b) found very few effects when neurotoxicity endpoints were not evaluated. Based on the known neurotoxic potential of TMB, shown conclusively by the inhalation route, the clinical signs were too general to be predictive of neurotoxicity. The overt clinical signs were not observed during the recovery period, but this does not mean more subtle neurotoxic effects did not occur.

This reviewer does not think that the Koch Industries study is reliable for assessing noncancer risk because the endpoint of concern for TMB exposure, neurotoxicity, was not evaluated.

II. RESPONSE TO CHARGE QUESTIONS

1. ***Study Design - Based on your knowledge of toxicological protocols, please comment on the experimental design of the 90-day oral gavage toxicity study described in the Koch Industries report.***

As conducted, the study followed a standard accepted protocol for a 90-day oral toxicity study. The experimental design was adequate for a subchronic study with the exception of dose selection, which may not have been adequate.

a. Please comment on any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

No problems with the study conducted as a subchronic oral toxicity study.

b. In consideration of the toxicological properties of trimethylbenzenes reported in the provided contextual references (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Korsak et al., 2000a, b; Wiaderna et al., 1998; Gralewicz et al., 1997a; Gralewicz et al., 1997b; Korsak et al., 1997; Korsak and Rydzyński, 1996; Korsak et al., 1995), please comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.

Potential neurotoxicity was not evaluated by the oral route in this study. Considering the long-lasting effects observed in several of the contextual references, this endpoint is critical to evaluating the full toxicity of TMB by any route. Delayed onset of the clinical signs observed in the Koch Industries study supports potential long-lasting effects of TMB that were not evaluated. Similar to the current study, the standard subchronic inhalation studies (Korsak et al., 2000a,b) found very few effects when neurotoxicity endpoints were not evaluated. The reviewer acknowledges different isomers of TMB were used in these inhalation studies, but considers the toxicity of each to be similar.

2. ***Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Koch Industries study.***

The clinical signs of toxicity in high-dose males and females were the only adverse findings in this study. These clinical findings were general in nature, mild, and not observed in all high-dose animals. Based on the known neurotoxic potential of TMB, shown conclusively by the inhalation route, the clinical signs were too general to be predictive of neurotoxicity. The main findings of salivation and fur staining could have been due to other properties of the chemical such as irritation or bad taste. In addition, the clinical findings were not observed until after about three weeks of dosing (earlier in a few animals) and generally persisted until termination of dosing either by sacrifice or start of the recovery period. In several of the contextual references, neurotoxicity was shown to be long-lasting after cessation of exposure. The overt clinical signs were not observed during the recovery period, but this does not mean more subtle neurotoxic effects did not occur.

Other statistically significant findings are not considered by the reviewer to be adverse or biologically relevant. The increased relative kidney weight observed in the high-dose males is not a direct effect of TMB administration, but was entirely due to the slightly lower final body weight of these animals. Absolute liver weight was slightly increased in high-dose males and significantly increased in high-dose females, but the magnitude was small and not accompanied by histopathological correlates. Lack of hepatocellular hypertrophy suggests that the liver weight increases were marginally adaptive and not adverse. Increased phosphorus levels were consistent in both high-dose males and females, but the magnitude of change was not biologically significant.

Taken together, the lack of findings indicating toxicity (except clinical observations) suggests that the animals could have tolerated a higher dose and that dosing was not adequate. A dose selection rationale was not given beyond the statement that the doses were chosen by the sponsor. Conversely, dosing may have been adequate, but potential neurotoxicity endpoints were not evaluated and, therefore, could have been missed.

3. ***Study Conclusions - Please comment on the discussion and conclusion sections of the Koch Industries report. Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made? Do you agree with the final conclusions of the Koch Industries report?***

   Conclusions given in the report were consistent with the results as presented and no critical findings were omitted. This reviewer disagrees that increased relative kidney weight observed in the high-dose males should be part of the LOAEL because this effect was entirely due to the slightly lower final body weight of these animals. Increased phosphorus levels were consistent in both high-dose males and females, but the magnitude of change was not biologically significant and thus, of questionable relevance to the LOAEL. The clinical signs observed in high-dose males and females were the best evidence that dosing had occurred.

4. ***Study Reliability – Describe the reliability of the subject Koch Industries study for consideration in the qualitative characterization of noncancer risk and quantitative derivation of human health reference doses. Describe any major strengths or uncertainties with the study described in this report that might preclude them from being used as consideration for derivation of a noncancer reference dose.***

   This reviewer does not think that the Koch Industries study is reliable for assessing noncancer risk because the endpoint of concern for TMB exposure, neurotoxicity, was not evaluated. Thus, qualitative characterization of noncancer risk cannot be assessed from this study. A NOAEL was identified that could be quantitatively used to derive an RfD, but the endpoint of concern may not be protected against.

II. SPECIFIC OBSERVATION

None found.

Review by
Raymond G. York, Ph.D., DABT, ATS, ERT

Peer Review Comments on the 1995 Koch Industries Study Report:
90-Day Oral Gavage Toxicity Study of 1,3,5-Trimethylbenzene in Rats with a Recovery Group

Raymond G. York, Ph.D., DABT, ATS, ERT
R.G. York and Associates, LLC

April 21, 2013

I. GENERAL IMPRESSIONS

The 90-day oral gavage study of 1,3,5-trimethylbenzene (TMB) in rats with a recovery group was conducted by IIT Research Institute for Koch Industries in 1994. William Johnson, Ph.D., DABT, was the study director and the study was conducted according to the 1979 EPA Toxic Substances Control Act (TSCA) testing guideline 40 CFR 798.2650, and testing as required by CFR 799.5075 (Fed. Reg. Vol. 58 No. 216, pp. 59681-82).

In reviewing the study report, adequate numbers of animals were included in the experimental protocol and the study, for the most part, was adequately designed, conducted, and reported. The bulk chemical and dosing solution analyses, animal maintenance, study design (10 animals/sex/group for three dose groups plus one high dose group of 20 animals/sex, 10/sex for a recovery group; 100 total) and dosing 5 days/week are consistent with other subchronic 90-day studies conducted at that time. This study was conducted according to GLP regulations 40 CFR Part 792.

The conclusions reached for this subchronic study were supported by the rendered data. The 600 mg/kg/day high dose group clinical signs (including discolored and wet inguinal fur and salivation), cumulative decreased body weight gain in male rats, adverse clinical chemistry parameter of increased phosphorus blood levels in male and female rats, increased absolute liver weight for female rats, increased relative liver weights for male and female rats, and increased relative kidney weight in male rats were all considered treatment-related. All of the treatment-related effects were reversible, as none were present in the recovery rats after 28 days of cessation of treatment.

II. RESPONSE TO CHARGE QUESTIONS

1. ***Study Design - Based on your knowledge of toxicological protocols, please comment on the experimental design of the 90-day oral gavage toxicity study described in the Koch Industries report.***

   This study was adequately conducted under the toxicity guidelines and GLPs in effect during the mid-1990s. It employed appropriate experimental procedures, including animal model selection of appropriate age, sex, body weight, animal identification method, acclimation period, randomization, group size, husbandry, dose levels, dose level selection, route and period of administration, study endpoints measured, test and control substance preparation, characterization, storage, and sampling, as well as statistical evaluations of study results, quality assurance inspections and compliance. There were no significant issues with the experimental design of the study.

- Protocol: The experimental design for the study followed the then current EPA798.2650 Oral Toxicity Guidelines.

- Test Article Employed: 1,3,5-Trimethylbenzene (TMB) with the required purity of 99% and stored properly. The identity, composition, purity and stability of the test article (TMB) were confirmed by the testing facility. The identity, purity, composition, and stability of the vehicle (corn oil) were confirmed by the supplier (deviation noted). Reserve samples of each were retained by the Study Sponsor.

- Stock Dosing Solutions: Each concentration was prepared weekly and was not sequentially diluted from one stock solution (sequential dilution has the potential to multiple a preparation error). Aliquots from the top, middle and bottom of dosing solutions were determined to be homogeneous and within 10% of the mean concentration for each of the three dose levels. Concentration and stability analyses of dosing solutions were determined to be within specifications. No TMB was detected in any vehicle control samples.

- Route of Exposure: No comments except it could be important to know why the route of administration for the early 14 day study with the same test article (Project Number L08512, Study Number 1) was oral gavage. Were the diet or drinking water routes excluded due to palatability issues? Why was whole body inhalation, the route used in all the Nofer Institute studies and a known human route of exposure, not considered? The health effects of TMB toxicity were being required to be assessed because it was a drinking water contaminant. Drinking water and inhalation exposure (TMB is a volatile aromatic hydrogen) routes have more human relevancy and give much different absorption, metabolism and elimination profiles than bolus gavage administration.

- End Points Recorded: EPA798.2650 Oral Toxicity Guidelines require a measure of clotting potential such as clotting time or prothrombin time and should have been measured. Platelet counts only give current coagulation status. This was clarified in the 1998 EPA OPPTS 870.3100 and the 1998 OECD 408 Guidelines for a 90-day oral toxicity study in rodents by requiring both a measure of clotting time as well as platelet counts.

- Terminal Procedures: Procedures were followed according to the protocol. The report stated that the absence of significant ophthalmic lesions during the final week of dosing obviated the examination of the recovery rats 28 days later. This is not specifically defined in the guidelines and, if one of the aims of the guidelines is to "observe delayed occurrence of toxic effects during the post-treatment period," this omission should not have occurred.

- Statistical Analysis: Statistical analyses were appropriate. One shortcoming was the lack of a vehicle recovery control group for proper comparison. This has been addressed in the 1998 EPA OPPTS 870.3100 and the 1998 OECD 408 Guidelines for a 90-day oral toxicity study in rodents by requiring such a control group.

- Quality Assurance: Quality assurance procedures were correct.

- Report: All the elements required by the EPA 798.2650 Guidelines were included.

a. Please comment on any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?

Most of the physiological and toxicological endpoints collected were standard for protocols for this period of time. A current EPA OCSPP Harmonized Test Guideline 870.3100 study protocol should include:

- 7-day/week exposures for a continuous 91 days (if by gavage, 5 days/week still acceptable).

- Functional observation battery (FOB), if the two-week repeat study had clinical signs of depression of the CNS.

- Complete and contemporary statistical analyses.

- Keeping the volume of corn oil at 2 mL/kg or less may reduce the scattered and intermittent discolored and wet inguinal fur observed.

- Detailed clinical observations outside the home cage each week in the same standard arena and at the same approximate time.

- After week 11, assessment of motor activity, grip strength and reactivity to different sensory stimuli (visual, auditory, proprioceptive).

- Measure of clotting potential (prothrombin time or activated partial thromboplastin).

- Urinalysis determinations during the last week of study (appearance, volume, osmolarity or specific gravity, pH, protein, glucose and blood/blood cells) may be indicated since the liver was a target organ in the 14-day study (L08512) and kidney weights were increased in the 90-day study conducted.

- The study design is not as robust as it could have been since no vehicle control rats (extra 10/sex) were included in the core study to be retained for recovery observations and statistical comparisons to the high dose animals. The 1998 OPPTS 870.3100 Guidelines of a 90-day oral toxicity study in rodents require an additional satellite control group of 20 animals (10 animals of each sex) for comparison to the high dose satellite group.

- A dietary, drinking water, or inhalation route of exposure would have had more human relevancy.

- If gavage was determined to be the best route of exposure, 7 days/week administration would have produced a more robust study since Huo et al. (1989) demonstrated that 99% of TMB and its metabolites are eliminated in 24 hours, leaving 48 hours of non-exposure and extended clearance every 5 days.

b. In consideration of the toxicological properties of trimethylbenzenes reported in the provided contextual references (Wiaderna et al., 2002; Gralewicz and Wiaderna, 2001; Korsak et al., 2000a, b; Wiaderna et al., 1998; Gralewicz et al., 1997a; Gralewicz et al., 1997b; Korsak et al., 1997; Korsak and Rydzyński, 1996; Korsak et al., 1995), please comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.

The Contextual Reference articles provided convincing evidence that 4 or 13 week inhalation exposures at low-levels to TMB isomers results in: a) long-lasting neurobehavioral alterations in rats several weeks after exposure to low levels of the test article, b) neurotoxic effects occurring in a nonlinear concentration-effect relationship, and c) an altered emotional fear response.

However, there is difficulty in considering these studies as key in the development of the IRIS assessment of TMB:

- The exposure route for all the Contextual References was whole body inhalation at non-toxic levels, making it difficult to compare to any bolus oral gavage study.

- The Contextual Reference studies were all conducted at Nofer Institute of Occupational Medicine, Lodz, Poland. These were research studies and did not follow standardized USEPA or OECD guideline protocols.

- The studies were not conducted under any kind of GLPs and used non-validated methods and equipment.

- There were no negative or positive historical control data for adequate comparisons.

- The investigators measured an emotional fear response by using classical conditioning. Usually a relatively neutral stimulus is associated with an unconditioned stimulus. To measure an emotional fear response, they associated a painful stimulus with a fear-inducing experience. As a result, the formerly neutral stimulus elicits fear. For example, if seeing a dog (a neutral stimulus) is paired with the pain of being bitten by the dog (unconditioned stimulus), seeing a dog may become a conditioned stimulus that elicits fear (conditioned response). In the Nofer studies, a persistent foot-shock (non-neutral stimulus) was used to measure the paw-lick response to heat from a hot-plate test (unconditioned stimulus). This may not be a valid or equivalent paradigm usage for classical conditioning.

One more reference could be added for completeness: "Contrasting effects of 4-week inhalation exposure to pseudocumene or hemimellitene on sensitivity to amphetamine and propensity to amphetamine sensitization in the rat." Lutz, P., Gralewicy, S.,Wiaderna, D., Swiercz, R., Grzelinska, Z., Majcherek, W. Inter. J. of Occup. Med. and Environ. Hlth. 2010; 23(1):85-94. The study used two of the isomers of TMB, exposed rats by the same route, length of time, and the research was conducted at the same institute. The institution's previous work with TMB demonstrated long-lasting changes in behavior, so they were interested in determining if TMB exposure increased behavioral sensitivity to amphetamine.

2. *Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the Koch Industries study.*

Ophthalmology. Ophthalmology exams were not conducted on the recovery rats 28 days post-exposure, because the absence of significant ophthalmic lesions during the final week of dosing obviated the examination. This is not specifically defined in the guidelines. However, as one of the aims of the guidelines is to "observe delayed occurrence of toxic effects during the post-treatment period," this omission should not have occurred.

Body Weights. Fasted terminal body weights for the male rats in all the exposure groups were reduced, albeit none significantly (584g, 576g, 562g vs. 602g for the low, mid high vs. controls, respectively), and should have been mentioned. A trend analysis would most likely have been significant.

Clinical Chemistry. Multiple clinical chemistry endpoints for the male rats in the 600 mg/kg/day dose group were significantly altered at the day 30 interim blood draw and reported. However, the study director did not considered them to be related to treatment because: a) the same parameters were not significantly altered at the day 90 termination (which allows time for more complete enzyme adaptation to TMB exposure) and b) the values were within *or approximately within* (emphasis added) the range of the in-house historical control values. It needs to be noted that the in-house historical data do not give the dates of the information collected, or the sex (male, female or combined) of the rats. The significant increase in alkaline phosphate (ALK P) for the male rats in the 600 mg/kg/day dose group at termination was considered by the study director to be due to rats (61 and 63) that had exceptionally high values (257 and 213 IU/I). However, ALK P was increased almost 41% for the male rats (albeit not significantly) at the day 30 interim blood draw and it was the same two rats that had the highest levels. It is not clear why the significant increase in ALK P for the male rats in the 600 mg/kg/day dose group at termination was not included in determination of the NOEL by the study director.

Hematology. Mean monocyte counts (MONO) were significantly increased in the male rats at 200 and 600 mg/kg/day when compared to the vehicle control group at termination of exposure, but not considered by the study director as treatment-related. No reason was given. An increased number of monocytes in the blood (monocytosis) occur in response to chronic infections, in autoimmune disorders, in blood disorders, and in certain cancers. An explanation on why these findings were being excluded should have been included in the results section.

Organ Weights. Male relative kidney weights in the 600 mg/kg/day dose group were noted as being significantly increased; female relative kidney weights were also increased (7%) at this dose level, albeit not significantly, but should have been mentioned in the results section.

3. ***Study Conclusions - Please comment on the discussion and conclusion sections of the Koch Industries report. Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made? Do you agree with the final conclusions of the Koch Industries report?***

   The increased phosphorus levels in the 600 mg/kg/day dose group for the male and female rats should have been discussed in more detail. The kidneys excrete phosphate; therefore, the most common cause of increased phosphate levels (or hyperphosphatemia) is the kidney's inability to eliminate phosphate. This may have been related to the significantly reduced blood urea nitrogen (BUN) levels for female rats in the 600 mg/kg/day dose group.

   The conclusions reached for this subchronic study were supported by the rendered data. The 600 mg/kg/day high dose group showed clinical signs (including discolored and wet inguinal fur and salivation), cumulative decrease in body weight gain in male rats, adverse clinical chemistry parameter of increased phosphorus blood levels in male and female rats, increase in absolute liver weight for female rats, increased relative liver weights for male and female rats, and increased relative kidney weight in male rats. In the last line of the Summary, the adverse clinical signs in the 600 mg/kg/day dose group should have been included in the basis for establishing the observed toxicity level.

4. ***Study Reliability – Describe the reliability of the subject Koch Industries study for consideration in the qualitative characterization of noncancer risk and quantitative derivation of human health reference doses. Describe any major strengths or uncertainties with the study described in this report that might preclude them from being used as consideration for derivation of a noncancer reference dose.***

   The conclusions reached in this 90-day oral gavage study of 1,3,5-trimethylbenzene (TMB) in rats with a recovery group study were supported by the data. The adverse clinical signs, increase in blood phosphorus levels, and increase in liver and kidney weights in the 600 mg/kg/day dose group were clearly caused by the test substance and are the lowest-observed-effect-level (LOAEL). The 200 mg/kg/day dose group was established as the no-observed-adverse-level (NOAEL) in this study.

III. SPECIFIC OBSERVATIONS

Page	Paragraph	Comment or Question
86	Appendix 4	Control female rat # 14 was observed to have a swollen limb on day 92 (pathology confirmed a fractured tibia), which may account for the high platelet (p 126; Appendix 14) and monocyte counts (p. 135; Appendix 16) at termination and should have been deleted from analysis.