

## **PEER REVIEWER COMMENTS**

### **External Peer Review Meeting on the *Toxicological Review of Urea* (CAS No. 57-13-6)**

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## I. INTRODUCTION

EPA's Integrated Risk Information System (IRIS) is a human health assessment program that evaluates quantitative and qualitative risk information on effects that may result from exposure to environmental contaminants. Through the IRIS Program, EPA provides the highest quality science-based human health assessments to support the Agency's regulatory activities. The IRIS database contains information for more than 540 chemical substances that can be used to support the first two steps (hazard identification and dose-response evaluation) of the risk assessment process. When supported by available data, the database provides oral reference doses (RfDs) and inhalation reference concentrations (RfCs) for chronic non-cancer health effects, and oral slope factors and inhalation unit risks for carcinogenic effects. Combined with specific exposure information, government and private entities use IRIS to help characterize public health risks of chemical substances in a site-specific situation and thereby support risk management decisions designed to protect public health.

The IRIS program within EPA's National Center for Environmental Assessment (NCEA) has developed a "Toxicological Review of Urea," an assessment which has not previously appeared on the IRIS database. The draft document slated for the external peer review contains a qualitative cancer assessment.

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## **II. CHARGE TO THE REVIEWERS**

The U.S. Environmental Protection Agency (EPA) is seeking an external peer review of the scientific basis supporting the human health assessment of urea that will appear on the Agency's online database, the Integrated Risk Information System (IRIS). IRIS is prepared and maintained by the EPA's National Center for Environmental Assessment (NCEA) within the Office of Research and Development (ORD). Currently an IRIS assessment of urea does not exist on the database.

The current draft health assessment includes an evaluation of the available data and a determination that the data are insufficient for the derivation of toxicity values. A cancer descriptor for urea is included. Below is a set of charge questions that address scientific issues in the assessment of urea. Please provide detailed explanations for responses to the charge questions. Please consider the accuracy, objectivity, and transparency of EPA's analysis and conclusions in your review.

### **General Charge Questions:**

1. Is the Toxicological Review logical, clear and concise? Has EPA clearly synthesized the scientific evidence for noncancer and cancer hazard?
2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.
3. Please discuss research that you think would be likely to increase confidence in the database for future assessments of urea.

### **Chemical-Specific Charge Questions:**

#### **(A) Oral Reference Dose (RfD) for Urea**

1. An RfD for urea was not derived. Is the rationale for not deriving an RfD scientifically justified and clearly described? Please identify and provide the rationale for any studies that should be selected as the principal study and any endpoint that should be considered as a critical effect.

#### **(B) Inhalation Reference Concentration (RfC) for Urea**

1. An RfC for urea was not derived. Is the rationale for not deriving an RfC scientifically justified and clearly described? Please identify and provide the rationale for any studies that should be selected as the principal study and any endpoint that should be considered as a critical effect?

**(C) Carcinogenicity of Urea**

1. Using EPA's 2005 Guidelines for Carcinogen Risk Assessment ([www.epa.gov/iris/backgr-d.htm](http://www.epa.gov/iris/backgr-d.htm)), the Agency concluded that there is "inadequate information to assess the carcinogenic potential" of urea. Please comment on the selection of the cancer descriptor. Is the cancer descriptor scientifically justified and clearly described?
2. EPA did not derive a quantitative estimate of the carcinogenic potential of urea. Do the data support an estimation of a cancer slope factor for urea? If a quantitative estimate is proposed, please identify the data set and a description of the method that should be used.

### III. GENERAL IMPRESSIONS

*Bruce C. Allen*

My general impression is that there is a lot of “information” presented for so little in the way of conclusions or determinations. I realize that there needs to be a full airing of the published literature, even when it is older or not-so-relevant to risk assessment purposes, but the lengthy listing of studies and their findings was perhaps not the approach that lends itself to the greatest clarity. As an alternative, I would suggest that the reader could be clued in to the “conclusions” early in the process, perhaps by providing two items of information prior to the list of studies. First, state the proposed mode(s) of action that are under consideration; then the reader can evaluate the findings from the individual studies with respect to that (those) mode(s) of action and evaluate the strength of evidence for or against them. Second, state the bottom line determination that none of the studies are suitable for risk assessment, along with some key deficiencies. Then the reader can have that determination and the reasons behind it in mind as s/he is considering the list of studies.

On a related issue, a table of the studies and their main findings would be very helpful. That was done for the genotoxicity studies (Table 4-6); the remaining studies would benefit from a similar tabular summarization.

With respect to the soundness of the conclusions, my only comments relate to my earlier observation about the mode of action. It seems that there is a logical basis for suspecting exogenous urea might have adverse effects, based on observations in individuals with kidney diseases. Yet, the document does not really put the pieces together from that perspective (or from any other possible perspective either) to indicate what might be happening, even when negative results were obtained. I was struck by the evaluation of carcinogenicity (Section 4.7, particularly the synthesis in Section 4.7.2) that had numerous references or statements to effects occurring only at *high doses* or changes noted to have been within physiological normal ranges even if slightly (perhaps statistically significantly) different from control values. As an example, consider the concluding sentence of the second paragraph of Section 4.7.2: “These results provide no relevant evidence that urea may play a role in tumorigenesis.” In fact, they provide very relevant evidence; they suggest that, while the biomarker levels may be changing, the changes at the doses tested have not reached a level that is clearly adverse (in the sense that they might indicate an increased risk of cancer), and that it would require higher exposures to (possibly) effect that level of change. These observations suggest to me a somewhat stronger conclusion than is given in this document, namely that if meaningful adverse effects are occurring or are going to occur after exposure to urea, they will be associated with higher exposure levels, levels above those reported in the experimental or epidemiological studies surveyed. If that kind of conclusion could be judged in light of the possible mode(s) of action suspected on the basis of endogenous urea’s association with kidney disease, then a much more relevant and supportable conclusion could possibly have been reached.

***Richard J. Bull***

Generally, the Toxicological Review of Urea is thorough. I have only minor concerns over the conclusions of the review. The review is well written. However, the presentation lacks clarity and there is little context for why an evaluation of urea is needed at all.

Most destructive to the document was the apparently unlimited presentation of studies and data that provide very little information relative to the toxicological review. This is exacerbated by an uncritical exploration of the literature creating an illusion that the data are of importance to judging the hazards and risks that might be associated with urea. The most obvious difficulty arises with the inclusion of studies directed primarily at the effects of excessive systemic urea on dialysis patients. There are also a large number of studies utilizing odd routes of administration relative to expected environmental exposures via diet and inhalation. Many of these effects are largely due to the overwhelming increase in osmotic pressure that is produced (exception may be the carbamylation of proteins – which might be developed into a biomarker of excessive exposure). *In vitro* studies where concentrations in excess of 10 mM are cited are also of little relevance (based on a stated normal urea concentration of 5 mM – page 49) unless plasma concentrations can be increased above this level with environmentally relevant external exposures to urea. The pharmacokinetic (PK) modeling suggests that it would be difficult to increase systemic concentrations of urea in humans (short of being in complete renal failure). Some of the studies do inform evaluation of environmental exposures to urea, but primarily to illustrate that rather huge amounts of urea can be administered without adverse effect. The predominance of literature with marginally relevant data that is presented in such great detail gets in the way of appreciating what among the data presented are critical.

The authors should find a way of indicating that the literature with marginal application was evaluated, explicitly citing the papers, including a short statement of the nature of the data (2-3 sentences) and a similarly short statement indicating why these papers really do not contribute to a safety assessment or risk assessment. There are some of the data that do have direct application to the consideration of environmental exposures to urea, but due to design flaws and poor reporting are less than useful. There are some data that fall in between these extremes that are important in understanding how urea might produce adverse effects, but their relevance to environmental exposures are not clear (e.g. most of the studies of UTs are directed to determining whether a uremic state could be produced and mostly contribute a conceptual framework as opposed to direct application to hazard and risk assessment). In such cases, the author should explicitly state why those data are relevant and provide a more complete description of these studies, but perhaps not to the level of detail present in the Toxicological Review. Then the authors should ensure that the critical studies are identified as such within the body of the review as well as in the concluding section (4.6) where the important studies were identified and evaluated. Incidentally, this is the most important chapter in the review and was generally well done.

***Alan H. Stern***

The document is generally well written. However, it suffers from a lack of focus relative to other IRIS technical support documents. In part, this is because urea is an endogenous chemical and only a contaminant with respect to the portion of the body burden that arises from exogenous

sources. This makes consideration of the normal physiological interactions of urea necessary in the document. Nonetheless, the overall focus and direction of the document should be to elucidate how increased exposure to urea above the normal endogenous levels results (or does not result) in adverse effects and, if possible, to describe the dose-response relationships for those effects in quantitative terms. In many sections of the document, it does not appear that the document is following a path to those goals. Rather, there is a sense that document is functioning as something of a data repository for all sorts of information about urea that doesn't move it toward the ultimate goals of the assessment. For example, it is not clear how the relatively long section on the urea transporters relates to a potential hazard assessment. Also, it is not clear what the relevance of the rather bizarre studies of colonic metabolism of urea is to the assessment.

While the role of this document is to elucidate the toxicology of urea and not specifically to focus on exposure, the extent of background exposure in the population is relevant because of the endogenous nature of much of the urea exposure. I would, therefore, have expected a section on the distribution of blood urea levels in the population. This would be particularly important given what is likely to be a wide distribution in the population due to inter-individual variability in protein intake as well as in metabolic function.

Given that the production of reactive oxygen species (ROS) associated with urea is raised in several places in the document as a possible mechanism of urea toxicity, there should be an explanation of the mechanism for ROS production. The mechanism for ROS production directly or indirectly from urea is not obvious to me and an evaluation of the plausibility for that mechanism to explain urea toxicity rests on an understanding of the mechanism.

***Bonnie R. Stern***

1. Although the first two sections of the document are clearly laid out and TK study summaries are well described, with conclusions, the remainder of the document was difficult to follow. There was very little information relevant to an IRIS document for deriving reference toxicity values and a cancer slope factor. It seemed to me that a thorough literature review and scoping document would have supported the conclusion that an IRIS Toxicological Review should not be done. A brief Internet search indicated that chemical uses of urea included synthesis of melamine-containing compounds, including various adhesives such as urea-formaldehyde or the [urea-melamine-formaldehyde](#) used in some types of plywood. These uses clarified to me why the toxicology of urea was of interest to EPA and the health of the general population (i.e., association with melamine and formaldehyde). These uses are not mentioned in the list on pages 2-3 and should be added. Also, at the teleconference meeting of the external peer reviewers, it was noted that the driving concern for writing a TR was concern by the Office of Air for human exposure to diesel fuel emissions containing urea. A statement to this effect would explain to the reader why the TR was written, given the paucity of data relevant to an IRIS evaluation.

2. Why is there no executive summary? An executive summary would provide a framework for the document and why it was written despite the paucity of information relevant to IRIS.

3. Urea is an endogenous water-soluble compound which is the primary end product of mammalian protein metabolism. Its formation in the liver and its metabolic functions are

required for survival and maintenance of health. (This is in contrast to endogenous formaldehyde which is the degradation product of abnormal cellular metabolism and other processes such as oxidant stress and energy metabolic imbalances, and induces adverse effects similar to those induced by exogenous formaldehyde, depending on concentrations and dose additivity (See L Lehman-McKeeman, 2010, “Paracelsus and Formaldehyde 2010: The Dose to the Target Organ Makes the Poison”). As such, the toxicology of urea must be viewed within a homeostatic framework, much like the toxicology of zinc (U.S. EPA, 2005: <http://www.epa.gov/ncea/iris/toxreviews/0426tr.pdf>).

Endogenous compounds which are requisite requirements for normal metabolism are handled very differently by the body than non-endogenous chemical substances with no essential physiological function. Homeostasis refers to the process whereby a complex and intricate set of physiological mechanisms tightly monitor and regulate production of urea in the liver, transport, distribution and excretion, according to body requirements.. Thus, the dose-response curve is U-shaped, because too much or too little urinary urea may be indicative of an underlying health condition requiring medical attention. Elevated urea levels may be associated with congestive heart disease, urinary obstruction, gastrointestinal disorders, as well as renal disease. Elevated urea levels may also be an indicator of dehydration, starvation, or shock. Urea levels below the normal physiological range may indicate over hydration, malnutrition, too little dietary protein in the diet or liver injury/disease. Adaptation may also occur in response to increased or decreased urea concentrations within physiological range of homeostasis. Therefore, significant changes in endogenous urea levels are due to impaired physiological functioning which maintain requisite levels in response to dietary and beverage intake (i.e. homeostatic control mechanisms are exceeded or overwhelmed), and experimental findings must be considered within a homeostatic context in order to be biologically and toxicologically relevant. Further, uremia, which is an illness caused by renal failure is not defined in the Toxicological Review, nor is azotemia, which is primarily used to denote clinically abnormal urea levels in the absence of clinically-evident disease. It is difficult to accurately characterize the adverse effects of exogenous urea unless baseline levels of endogenous urea concentrations are measured *apriori*. Therefore, a more thorough discussion of endogenous urea, its requisite physiological functions, and the effects of homeostasis and homeostatic control mechanisms on endogenous levels is needed at the beginning of the document, under a separate heading.

A section at the beginning of the Hazard section on this subject is warranted – under the title “Urea Homeostasis” or “Endogenous Urea,” or something similar. This section would focus on endogenous urea, what it normally does, how it is handled homeostatically by the body, and which diseases affect urea status and how.

4. No general statement about the limitations of conclusions drawn from findings in *in vitro* studies of cellular, molecular and genetic studies and their relevance to *in vivo* biological systems is made, but should be addressed. *In vitro* studies are hypothesis-generating, not hypothesis-testing, studies because the interactive effects of other endogenous compounds can significantly modify, repair or otherwise alter findings in *in vitro* isolated cell studies. This is especially germane to an endogenous substance like urea which is homeostatically regulated. Although the genomic era has resulted in an exponential increase in the number of molecular, genetic, enzymatic, and transgenic-model studies being reported in the literature, these studies are basic, hypothesis-generating research and, although of interest, are as of yet far from being relevant to

regulatory health risk assessment. Therefore, although interesting, inclusion of the sections on urea transporters, mechanistic data in the support of a mode of action, and gene expression studies in a Toxicological Review to support derivation of an RfD, RfC, and a cancer assessment is questionable. These data would be entirely appropriate for an ATSDR document.

5. To be relevant to a mode of action, a mode of action for a particular adverse effect, such as liver or kidney toxicity, must be hypothesized.

“Mode of action” is defined by U.S. EPA (2005a, p. 1-10) in terms of cancer endpoints; however, harmonization of approaches for the risk assessment for cancer and non-cancer outcomes make this term equally relevant to adverse health effects other than cancer. The mode of action is defined as “a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A “key event” is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element. Mode of action is contrasted with “mechanism of action,” which implies a more detailed understanding and description of events [*i.e.*, *key events*], often at the molecular level, than is meant by mode of action... There are many examples of possible modes of carcinogenic action, such as mutagenicity, mitogenesis, inhibition of cell death, cytotoxicity with reparative cell proliferation, and immune suppression.”

There is no hypothesized mode of action for urea-specific organ toxicity. Many of the data in many Toxicological Review sections pertain to changes in organs which are associated with excess exogenous urea. However, they are isolated events in an unknown mode-of-action chain of events. Second, many of the experiments significantly impair or eliminate normal kidney function to induce uremia and then examine the role of exogenous excess urea or other variables in already-existing disease. Thus, they are relevant to individuals with, or at known risk of, renal damage or disease. This is a highly susceptible subpopulation, with a reasonably large population incidence, but it is not characteristic of the general population. Third, even in this set of studies, a hypothesized mode of action is not given, just a mechanistic association. Therefore, the title of Section 4.5 is entirely misleading (except for the genotoxicity subsection) because the data cannot support a mode of action if no mode of action is proposed. It is not clear what useful information the molecular and transgenic data provide for health hazard assessment.

I recommend that these data be placed in a separate section entitled “Mechanistic Data” and that a distinction be made between data pertaining to mode of action (very little) and mechanistic data which are not relevant (as far as we know) to mode of action, but are of interest in hazard characterization of the compounds. Suggest also the liberal use of sub-headers: e.g., genomic data; transport protein data (please also define better); data from humans with pre-existing kidney/renal disease; data from animal models of kidney/renal disease.

6. Many of the human studies were conducted in patients with pre-existing disease. These are not relevant to the general population. Similarly, as noted above, uremia was induced in many of the animal studies and the effects of added exogenous urea were studied. The relevance to apparently healthy humans or humans without pre-existing renal disease is questionable. This should be noted.

Also, the document does not adequately discuss susceptible populations. Humans with pre-existing renal disease are a susceptible subpopulation. Studies done in these individuals and in animal models in which renal disease is induced are directed at studying how affected populations respond to exogenous urea load in order to better understand renal disease development. Are there any other susceptible populations? Individuals with liver disease? Children? Please expand this section.

7. The El Far et al. (2006) study describes enzymatic changes in an occupational cohort exposed to elevated air concentrations of urea; at the end of the study description, the findings are reported as being within normal physiological range. In the absence of additional data, this means that the findings are not toxicological significant or biologically relevant. Yet these findings are reported again and again (6 times total) in the report. Why?

8. The definition of urea transport proteins is incomplete. Transport proteins which have transmembrane domains may be involved in cellular efflux or influx in both renal and extra-renal tissues. Transport proteins within the cell (usually called chaperone proteins because they shuttle a substance to a receptor or organelle with great specificity) are involved in intracellular trafficking and processing. UT-A apparently has at least two isotypes and UT-B at least one, which may have different functions; isotypes are mentioned but not defined.

9. The cancer study description should be expanded for clarity and logic, and the published paper and the NCI report reviewed more comprehensively to determine if the inconsistencies reported in the Toxicological Review can be reconciled.

10. In the Synthesis and Dose-Response Assessments, it should be noted that (1) many of the available studies used the intraperitoneal, intravenous or subcutaneous route of compound administration and (2) these routes lack human relevance for derivation of reference toxicity values.

11. The functions of a number of the enzymes, genetic markers, and other molecular substances are not given, which is confusing to the reader. Also, the clarity of the Toxicological Review would be greatly increased if the acronyms (or scientific names) of all proteins and genes and enzymes mentioned in the report were added to the List of Acronyms. Some are there but most are not. Please update the list of acronyms.

## IV. RESPONSE TO CHARGE QUESTIONS

### General Charge Questions:

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

#### ***Bruce C. Allen***

As stated above, clarity could be enhanced by providing summary tables of the studies giving the main findings and by providing some thread (most likely based on mode of action considerations and the relationship between endogenous and exogenous urea) with which to tie the various results from the studies together. There appears to be very little in the way of synthesis; see my comments above. The document is missing a narrative arc that allows the reader to assemble the loosely assembled pieces of information into a coherent whole.

#### ***Richard J. Bull***

Based on the inclusion of a lot of information that is of marginal or no value to the Toxicological Review, this document cannot be labeled as being concise (discussed above).

There was a good discussion of PK work that has been done with urea, but (as far as I could tell) few practical benchmarks were provided. Most critical was that there was no direct reference to normal urea concentrations in the PK section or a clear statement of how much external doses of urea administered by a variety of routes actually raised that concentration. Some of this data might be extractable from the current PK write-up, but it would be difficult. Systemic concentrations are one of the criteria that I utilize to judge the relevance of *in vitro* data and I was unable to do so (without expending a lot more time than I have).

Fortunately, the obscurity created in the toxicological review does not interfere with bringing the sections that deal with hazard assessment and risk assessment to a logical conclusion.

Section 4.6 brought the important studies into perspective. For the most part, I agreed with the conclusions set forth in this section. I do think there needs to be some better perspective developed with respect to environmental exposures to urea. Do the authors' really believe that a uremic state has even a small probability of occurring in anyone except those in renal failure? If so, that should be explicitly stated, if not, that conclusion should also be stated. Much of the discussion in the mode of action section comes from studies of uremic states rather than issues related to possible environmental exposures to urea. Are those modes of action relevant to environmental exposure? If not, what effects of urea would be relevant to environmental exposure? The available PK data on urea in humans should provide the basis for estimating a dose of urea that would be necessary to produce a uremic state in a normal adult or even a young child.

***Alan H. Stern***

As per my comments under “General Impressions,” I think that the document lacks focus in terms of providing information that specifically advanced the reader toward an assessment of the hazard potential (and as possible, the dose-response relationship) for urea. Ultimately, the document addresses these goals, but one does not get the sense that the determination of the endpoints of the assessment proceeds directly or logically from the material presented. There is a large amount of material presented and (without having done an independent literature search) that information appears to be comprehensive, but it appears that much of the information presented is extraneous to the assessment of the IRIS goals (e.g., the relatively long section on urea transporters). Thus, the document does not appear to be appropriately concise.

In addition, the following text-specific comments are noted under Section V. SPECIFIC OBSERVATIONS.

***Bonnie R. Stern***

In my judgment, although well-written, the document was not logical and clear, and it certainly was not concise for a Toxicological Review. There was a significant amount of unnecessary redundancy. Further, a lot of information in the Mode of Action section was not about mode of action but about possible isolated mechanisms and genetic changes associated with urea exposure in cell lines or transgenic models. Data were not well integrated and synthesized in the Synthesis of Effects section. Please see above in general impressions.

**General Charge Questions:**

***2. Please identify any additional studies that would make a significant impact on the conclusions of the Toxicological Review.***

***Bruce C. Allen***

I know of no other studies.

***Richard J. Bull***

No other relevant studies were identified.

***Alan H. Stern***

Clearly, the critical missing piece of information in the urea database is a quantitative dose-response relationship for both ingested and inhaled urea. Kommadath et al. (2001) appear to have done a study that could have provided at least the minimal information necessary to establish an RfD, but failed to report their results in sufficient detail to make them useable for that purpose. Such a study with a straightforward design (e.g., dosing of rats and/or mice with a range of urea doses selected relative to the background serum urea concentration) is an obvious pre-requisite for developing an RfD. A straightforward study to establish the inhalation dose-response may be technically difficult. However, the limited human occupational data suggests that an RfC would likely be based on systemic, rather than respiratory endpoints. The dose-response basis for an RfC could, thus, probably be derived from the ingestion dose-response. Alternatively, EPA may want to contact Kommadath et al. directly and request the necessary supplemental information necessary to use their study in the derivation of an RfD.

***Bonnie R. Stern***

A search of PubMed using the following search terms, “urea toxicity,” “endogenous urea toxicity” and “exogenous urea toxicity” produced > 10,000, 174, and 95 papers, respectively. A scan of the titles of these papers, as well as some abstracts, demonstrated that either most studies used urea as a dependent variable, measuring changes in urea levels following administration of various toxic agents and other insults (e.g., bacteria), or described the protective effects of the urea-urease system in decreasing gastrointestinal irritation and other disorders induced by an exogenous toxic agent.

**General Charge Questions:**

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

***Bruce C. Allen***

Some research that addresses the mode of action and the relationship to endogenous urea could help. I would suggest a better long-term cancer bioassay, except that I do not see this as a high (or even medium) priority compound based on what has been presented in this document.

***Richard J. Bull***

The real question here is whether there are environmental exposures to urea that even approach levels that would be of concern given the data that are available. There are some potential issues with occupational exposures and perhaps some exploration of toxicity to animals due to the use of fertilizers containing high amounts of urea. However, there seems to be very little probability of environmental exposure of humans that would be within orders of magnitude of levels of possible concern. While there are data gaps in the toxicology of urea, filling in that database would seem of a very low level of priority.

***Alan H. Stern***

To a large extent, the research likely to increase confidence in the database would be that described in response to the previous charge question. However, in addition, a two-generation reproductive study would be the other major contribution to the increasing confidence in the assessment.

***Bonnie R. Stern***

The urea data base would benefit from a well-conducted subchronic adult rat study, following standard test guidelines which (1) establishes a baseline level of endogenous urea over a period of time (e.g., 1-2 weeks) prior to treatment; and (2) then monitors urea levels at repeated time points, in addition to other endpoints, over the course of the study. Dose range-finding studies would also be needed prior to conducting the subchronic study.

## Chemical-Specific Charge Questions:

### (A) Oral Reference Dose (RfD) for Urea

***1. An RfD for urea was not derived. Is the rationale for not deriving an RfD scientifically justified and clearly described? Please identify and provide the rationale for any studies that should be selected as the principal study and any endpoint that should be considered as a critical effect.***

***Bruce C. Allen***

I understand the data base is limited. However, the concluding sentences of Section 5.1.1 strike me as a bit disingenuous: “The use of this LOAEL for the derivation of an RfD is confounded by the significant uncertainty associated with the short duration of the study, extrapolation of this dose to humans, accounting for susceptible populations and the limited database of studies.” Do not the reasons listed in that sentence exactly correspond to the uncertainty factors that are routinely applied in an RfD derivation (subchronic-to-chronic, animal-to-human, inter-individual, and database UFs, respectively)? This hardly seems to be the rationale needed to support the lack of an RfD in the case of urea.

***Richard J. Bull***

The document indicates that an RfD could not be developed due to the sketchiness of the data. The data are sketchy, but the most important point is that there are substantial inconsistencies in doses that produce effects in different studies. Some of this can be explained by differences in experimental design [e.g. gavage administration for Kommadath et al. (2001) vs. administration of much higher doses in the dietary study]. The pharmacokinetic data suggest that such differences from acute dosing vs. gradual dosing over each day could be consistent with the reported results. The small number of animals per group in Kommadath et al. 2001 almost makes statistical analysis a useless exercise, even if the incidence data had been provided and it was not. On the other hand, the non-tumor pathology was very poorly reported in the Fleischman study, rendering this study virtually worthless as a substantive rebuttal of the results reported in the Kommadath study. The fact that the latter study was of only 28-day duration in the absence of dependable longer term studies should not be a reason for dismissing the data.

Whether an RfD could be derived under the circumstances is more of a matter of how important it is to have an RfD derived at this time. If there are pressing issues (i.e. occurrence in drinking water, air or food at high concentrations), a provisional value could be proposed based on the LOAEL in Kommadath study. Such a value would undoubtedly be conservative and should be revisable upward (i.e. should explicitly state that this figure should not be subject to non-degradation assumptions applied to some regulations that might be derived from this figure). In the absence of such a need and an agreement (or explicit recognition) that the value is likely to be revised upward if new data were to become available, no RfD should be issued. The reason should be that the data available are not resolvable because of inconsistencies in the database. I most strongly support the latter conclusion.

***Alan H. Stern***

I agree with the decision not to derive an RfD. There is only one study among those presented that potentially provides dose response information – the Kommadath et al. (2001) study. However, after reading that paper, I agree that EPA has correctly stated that the authors did not supply incidence information or dose information (i.e., body weights were not provided). This is a shame as it is likely that the necessary information was generated by the authors. It is additionally a shame because it seems likely, from the information that those authors do report, that a LOAEL for hepatotoxicity and/or nephrotoxicity exists within the dose range employed in that study.

As I suggest above, it may be worthwhile to contact those researchers to attempt to recover the missing data. Barring that, it does not appear that there currently exists a database that could permit the derivation of an RfD for urea. The rationale for this decision is clearly stated in the document. It is hard to believe that Kommadath et al. neglected to provide the necessary information and a reader may well question EPA's assessment of that study (as I did), but the deficits in that paper are, indeed, as reported in the document.

***Bonnie R. Stern***

The rationale was not well synthesized. The synthesis of major noncancer effects and study limitations were not well integrated. The summary of human studies in Section 4.6 excluded McKay and Kilpatrick (1964) which showed a dose-response relationship between urea concentrations and IUGR, and Bulpin and Breckenridge (1976) which showed a dose-response association between urea concentrations and mean blood pressure. No causal relationship in either direction could be inferred, but the correlations were strong and are considered to be part of hazard characterization. Second, in the animal studies, the report authors did not note that the limited number of whole-animal studies were also limited in assessment of endpoints; many were older studies and typically measured only survival and body weights. The cancer study by Fleischman et al. (1980) did not measure a wide range of endpoints, as was usual for carcinogenic bioassays of that era, but did do pathology and histopathology, and absence of noncancer pathology in this study is not clearly stated in either the study summary in the body of the text, or in the Synthesis section.

The study authors state (p. 63) that there are conflicting data between the reproductive studies by Teramoto et al. (1981) and Seipelt et al. (1969). In the Teramoto et al. (1981) study (p. 81), urea was used as a negative control and, unsurprisingly, did not affect any of the reproductive parameters examined. In Seipelt et al. (1969) (p. 32), it was reported that there were no statistical differences in kidney dry or wet weight in treated animals versus controls. The total number of pups delivered (N = 6 pregnant dams/group; only one dose was tested) was reported as **39 and 34** for treated and control groups, respectively. Yet the study authors report on p. 63 that “studies by Steipelt et al. (1969) suggest that maternal exposure may decrease the number of pups per litter.” This is erroneous. First, the treated group had a higher number of total pups. Second, even if this was an error and the higher number should have been in the control group, no statistical significance was reported, and based on inspection, the difference would not have been statistically different (6.5 vs. 5.67 pups/litter) either way. The cow data is not relevant to mammals with a single gut and this was not stated.

The two *in vivo* studies showing some urea-associated effects (Kommadath et al., 2001; Okada and Kobayashi, 1989) were too short for use in derivation of an oral RfD (28 and 14 days, respectively). This was not noted in the Synthesis section. Liver and kidney toxicity were noted in the first study, where urea was given in cow's milk to simulate urea-adulterated milk used in India (reason not given why urea was added to milk, whether it was accidental or intentional). However, there were dose-dependent effects at all dose levels compared with the control group given cow-milk vehicle. However, incidence data were not reported, so the study is not useful. Further, based on the short description in Okada and Kobayashi, 1989 on pancreatic effects in the mouse, it is possible that the observed effects were adaptive, rather than adverse. In this paper, too, incidence data do not appear to have been reported by the study authors as they were not described in the Toxicological Review.

In summary, the section on synthesis of major noncancer effects was not written in accordance with current risk assessment guidelines for writing this section. The majority of the studies were old and while they may have been well-conducted at the time, did not measure a sufficient number of endpoints, had small sample sizes; many did not do appropriate statistical testing, and were likely not well reported. Further, the study durations were too short for consideration for an RfD derivation. These types of studies are generally used in support of a well-conducted principal study and are too limited to be considered as principal studies themselves.

However, the report authors were correct in concluding that an oral RfD could not be derived.

## **(B) Inhalation Reference Concentration (RfC) for Urea**

***1. An RfC for urea was not derived. Is the rationale for not deriving an RfC scientifically justified and clearly described? Please identify and provide the rationale for any studies that should be selected as the principal study and any endpoint that should be considered as a critical effect?***

***Bruce C. Allen***

The case for no RfC is slightly stronger than that for no RfD, or at least the reasons cited appear to be more relevant. The lack of exposure levels for the human studies is a serious drawback.

***Richard J. Bull***

There should be no RfC for urea. The statement in 5.2 is right on.

***Alan H. Stern***

I agree with the decision not to derive an RfC for urea. However, the rationale (pg. 71-72) could provide a stronger justification. As stated, the rationale for not deriving an RfC from the human data is that the data are “limited and inconclusive.” This is correct as far as it goes, however, a more informative rationale would state that studies on human inhalation of urea lack a clear indication of significant effects and/or lack specificity in urea exposure (i.e., exposures are mixed) and/or exposure (dose) data in associated with health outcomes.

***Bonnie R. Stern***

The scientific rationale for not deriving an RfC was well developed. However, the interpretation of the two pulmonary function studies would have benefitted from additional information. The report authors state that that “lung function effects are minimal” and that both Bhat and Ramaswamy (1993) and Cade and Pain (1972) showed that urea inhalation induces mild impairment. In the first study, occupational exposure to urea decreased the PEFR/min when compared with controls but did not affect FVC or FEV1, which are screening markers for obstructive or restrictive pulmonary effects. PEFR/min is highly variable, and without co-occurring changes in FVC or FEV1, is not a reliable effect. The report authors note in the text (p. 23) and Dose-Response Assessment section, that the interpretation of this finding is also limited due to the small sample size, the lack of exposure assessment and the uncertainty that factors such as age were controlled in the study. The addition of this sentence to the Inhalation subsection of the Synthesis of Effects section would be useful. In the second study with symptom-free asthmatics, overall mild and variable statistically significant impairments of VC and FEV1 were observed, but there was no significant correlation between individual initial and post-exposure values of VC and FEV1, respectively. As differences between baseline and test responses are only biologically relevant on an individual basis, this indicates high variability and lack of toxicological significance. Further, the second study was an acute study, pulmonary function measurements were taken before and after 2-minute urea exposure. It is suggested that these studies be better described in the Synthesis section, as it is difficult to conclude that there was mild impairment of pulmonary function, given the findings.

### (C) Carcinogenicity of Urea

***I. Using EPA's 2005 "Guidelines for Carcinogen Risk Assessment" ([www.epa.gov/iris/backgr-d.htm](http://www.epa.gov/iris/backgr-d.htm)), the Agency concluded that there is "inadequate information to assess the carcinogenic potential" of urea. Please comment on the selection of the cancer descriptor. Is the cancer descriptor scientifically justified and clearly described?***

***Bruce C. Allen***

I would agree that the data are not sufficient to assess the carcinogenic potential of urea. On the other hand, I would not want to see that descriptor interpreted as indicating that there is a pressing need to collect better information; I am not convinced that the risk associated with urea exposure (at levels that have occurred in occupational settings, for example) are worth pursuing, relative to other, higher priority chemical exposures. On the other hand, EPA should make a stronger case for not choosing the descriptor of "Suggestive Evidence" given the (poorly documented but slightly problematic) results of the Fleischman et al. study. Again, relating these results to those expected based on endogenous urea or a suspected mode of action would strengthen the case.

***Richard J. Bull***

The document makes the correct decision on the potential carcinogenicity of urea. The presentation of the data in Fleischman et al. 1980 is very confusing. Although I agree with the conclusion of the carcinogenicity section, I was confused by the presentation of the data on malignant lymphoma. The authors seem correct that what is presented in the tables differs from findings quoted in the text. However, I was unable to identify the source of the data that was presented in the Toxicological Review. I suggest that the authors review that part of their paper and either clarify the source of the data or correct that data (this all occurs on p. 30 of the document).

One editorial comment is that I don't think it is the limited power that is really the reason that the Marsh et al. 2002 study could not be used. It is the possible co-exposure to other chemicals (and apparent inability to segregate out individuals with urea only exposures) that precludes the use of this study. That may be a power issue, but it does not have to be. It is more of a design issue.

***Alan H. Stern***

The Cancer Assessment section states that Fleischman et al. (1980) "...reported a nondose-related statistically significant ... increase in the incidence of malignant lymphomas." It is not clear to me why this response is characterized as "nondose-related." Based on the data presented from the Fleischman et al. text (as opposed to the table) for the malignant lymphomas, I calculate the incidence rate as 0.18, 0.11, 0.16, and 0.26 for the controls and successive dose groups. While it does not appear that the incidence rates for the two lowest doses are statistically significantly different from the controls, this does not imply that the response is nondose-related. Such a characterization would suggest that the response is inconsistent across doses. I do not see

evidence for this. Rather these data imply that the response is not significant until the highest dose.

The results for the interstitial adenomas similarly appear to have a high-dose elevation in the incidence rate. This is not at all the same as an inconsistent dose-response. While there are reporting inconsistencies in the Fleischman et al. (1980) data, these inconsistencies are relatively minor and do not appear to affect the qualitative assessment of a significant increase in tumor incidence at the high dose. While the Shear and Leiter (1941) study did not find treatment related tumors, the non-standard design of this study and the small number of surviving animals at study termination (11 months) do not allow for a comparison to the Fleischman et al. study. Given the findings of the Fleischman et al. (1980) study, I think that a reasonable case can be made within the context of the 2005 Cancer Guidelines for characterizing urea as having “Suggestive Evidence of Carcinogenic Potential.” This rests on the criteria of the 2005 Guidelines that include: “...a small, and possibly not statistically significant, increase in tumor incidence observed in a single animal or human study;” “evidence of a positive response in a study whose power, design, or conduct limits the ability to draw a confident conclusion (but does not make the study fatally flawed), but where the carcinogenic potential is strengthened by other lines of evidence (such as structure-activity relationships);” and “a statistically significant increase at one dose only, but no significant response at the other doses and no overall trend.” The possibility of carcinogenicity of urea is strengthened by the observation that urea has the potential to produce single strand (but apparently not double strand) breaks in DNA. While single strand breaks are not as directly related to tumor production as double strand breaks, they do suggest at least a high-dose mechanism (albeit with a possible threshold). Given the above, I don’t think that EPA has made a strong case as to why the characterization of carcinogenicity should necessarily be “Inadequate Information...” I think that given the evidence, EPA should also make the argument as to why the characterization does not justify “Suggestive Evidence...”

### ***Bonnie R. Stern***

The descriptor is correct. There is one NCI study (Fleischman et al., 1980) in mice and rats and the study duration was only 12 months, which is significantly lower than current standard bioassay guidelines (18 months for mice and 24 months for rats). In mice, the only statistically significant increase in tumors was reported to be malignant lymphoma in the mid-dose female group, and there was no dose-response. Further, the incidences in the text were different from those in the data table (p. 30). The incidence was reported as 9/50, 10/92, 7/43, and 10/38, in the control, low, mid-, and high-dose groups, respectively, in the text, and 9, 6, 9, and 8, in the control, low, mid-, and high-dose groups, respectively (no denominators given in the Toxicological Review) in the data table. It is entirely unclear why the denominator in the low-dose female group was 92; this must be an error. It is suggested that the report authors review the differences between the NCI report and the Fleischman et al. (1980) paper and reconcile the reported discrepancies. No differences in body weights or survival were noted in mice.

In the rats, body weights and survival were comparable except for the mid-dose males in which it was slightly reduced (significance not given). Among male rats, there was a statistically significant linear trend ( $p = 0.008$ ) for interstitial adenomas in the testes: 21/50, 27/48, 25/48, and 35/50 in the control, low-, mid-, and high-dose groups, respectively, but the only significant pairwise comparison was in the high dose group. Incidence inconsistencies similar to those

noted for the mouse study were also noted for the rat studies. There are two factors to consider with respect to adenomas: (1) they are benign tumors, although it is possible that they might progress to malignancy with longer exposure duration; and (2) the histopathological criteria for identifying an adenoma is difficult in terms of distinguishing it from hyperplasia or dysplasia, and can be highly variable among different pathologists reading the slides. Current NTP and industry studies use an independent pathologist or panel of pathologists to verify the study pathologist's report. Although the report authors considered a descriptor of *suggestive of evidence of carcinogenic potential*, this reviewer agrees that limitations in the study are consistent with the descriptor *inadequate information to assess carcinogenic potential*.

**(C) Carcinogenicity of Urea**

***2. EPA did not derive a quantitative estimate of the carcinogenic potential of urea. Do the data support an estimation of a cancer slope factor for urea? If a quantitative estimate is proposed, please identify the data set and a description of the method that should be used.***

***Bruce C. Allen***

I agree with the decision not to derive a quantitative estimate of the carcinogenic potential of urea.

***Richard J. Bull***

The data are not sufficient to make a quantitative estimate of cancer risk from urea. In all probability urea is non-carcinogenic.

***Alan H. Stern***

The data do not appear to justify a quantitative estimate of a cancer slope factor. However, as discussed above, the lack of sufficient quantitative data in a study that has suggestive evidence of carcinogenic potential is consistent with the characterization of “Suggestive Evidence...”

***Bonnie R. Stern***

No reliable, quantitative information is available to estimate a cancer slope factor for urea.

## V. SPECIFIC OBSERVATIONS

### *Bruce C. Allen*

One general observation that I will offer here relates to the use of the word “power” to describe the results, or limitations, of a study. In several instances in this document, it is stated that such-and-such a study lacked “power” for some reason. Care should be taken to avoid the connotation of power in the statistical sense. Statistical power is something that should be worried about *before* a study is conducted, it is the likelihood that the correct conclusion will be reached (through statistical hypothesis testing) when there is in fact something going on. Moreover, it is not a fixed, constant value; the power changes depending on (among other things) how the hypothesis is to be tested, the level of difference among the groups being compared, etc. After a study is completed, the results are what they are. “Limitations” associated with the completed study are better addressed or reflected in the confidence limits associated with estimates of interest.

So, for example, consider the statement at the end of the first paragraph of Section 5.3 (p. 72) that (in relation to the Marsh et al., 2002 study) “... the low incidence of bladder cancers [*sic*] deaths and the possibility of coexposure to other chemicals (nitric acid and acrylonitrile) limited the power of the study.” This makes no sense. The results are what they are; Marsh et al. reported an SMR with confidence limits and those should be used to understand how the number of cancer deaths (related to the size of the study population and the duration of follow-up) affected the precision of the results (and whether or not one should reject certain hypotheses). Co-exposure affects the accuracy of the results, to the extent that effects (on SMRs) that might be attributed to urea are actually the result (in whole or in part) of those co-exposures.

Other editorial comments:

p. 30, sixth line of first full paragraph: should be a close parenthesis after “control.”

p. 32, third line of last paragraph: s/b “elevated” rather than “elevate”?

p. 35, first paragraph in section 4.3.3: at first it says two UT genes, UT-A and UT-B. But then it has UT-A5. What is the relationship between UT-A and UT-A5?

p. 48, line 10: “myocolonus” should be “myoclonus.”

p. 68, second paragraph: Right after discussing the significant increase in interstitial adenomas in the testes of male rats at the high dose, reference is made to the fact that “female mice results were not statistically significant ...” This makes it sound like you were looking at female mice testes adenomas (or perhaps other testicular cancers), which clearly makes no sense.

p. 68, second paragraph, right after the citation immediately above: The statement is made that additional time of exposure/observation might have led to the formation of more tumors. While that may be true, it is just as true for the controls as it is for the treated groups (there are many tumors that occur primarily later in the life of test species even in the absence of exposure). So the apparent level of risk would not necessarily increase with additional duration of

exposure/observation; the remark in the document here should not give the impression that the results would indicate higher risk had the study been carried to 24 months, for example. Coming after the sentences describing the fact that the incidences of female mice cancers were higher in treated than in control animals, there is great potential for mistaken inferences.

***Richard J. Bull***

1. The basis for concern in terms of actual or likely exposures to urea was not spelled out in the document. We learned at the meeting that the primary program office concern related to the use of urea as a reductant (is the word redundant on page 4 a typo?) of NO<sub>x</sub> that are produced in diesel combustion. This nature of this concern needs to be spelled out in the document and exposure data developed or predicted. Other potential sources of exposure were identified in a general way in the short chapter on Chemical and Physical Information, but the document was completely devoid of any information related to actual or even estimated exposures to exogenous urea.

2. As mentioned in responses to the charge questions, little use was made of the pharmacokinetic data that has been developed for urea as a basis of judging whether the large body of data that are included in the document would be useful in a toxicological assessment. Urea is a natural product of protein catabolism and is essentially a means of ridding the body of excess ammonia. As a result, there is a large endogenous source of urea (urinary output is estimated to be 20-35 g per day on page 7). Understanding the risks to exogenous urea must be built on a firm understanding of how exogenous urea builds on endogenous urea concentrations in the system (or if it likely does in significant amounts given likely environmental exposure scenarios). Simply judging from the net excretion rate, it would take a very substantial exogenous exposure to significant increases in systemic urea concentrations in normal individuals. Clearly, individuals in renal failure (e.g. undergoing routine dialysis) do develop adverse effects, some of which might be attributed to urea. However, the question is how much exogenous urea would be necessary to raise the levels in a normal individual to such levels. Based on the limited data that have been described, this problem can only be approached using an pharmacokinetic model developed for normal individuals. The material provided for the Kaplan et al. (1999) study on pp. 17-20 appears sufficiently sophisticated to provide a basis for estimating key PK parameters. The main piece of data that appears to be missing in the description is a rate for systemic absorption. However, a relative simple modeling exercise could be done assuming a range of differing absorption rates based on the absorption and elimination of radiolabel derived from urea in the rat (Nomura et al., 2006). By all appearances, the amount needed to actually increase systemic concentrations in the two compartments will be very large. It is important to note that this approach would provide a reasonable framework for utilizing data on effects observed in uremic patients to provide perspective to the hazards that might be identified with excessive exogenous urea exposure.

Specific comments (largely editorial):

p. 8. There is some difficulty with the units in Table 3-1. It is indicated in the column that concentration is in mg. That figure needs a volume or weight associated with it to be called a concentration. There are other aspects of the Table that are confusing. There is a 91% recovery

listed for the control gum group, but there was no urea in that gum. I assume that there was no urea in the control gum? This table needs to be made clear.

p. 8 continued. The discussion of absorption in the first full paragraph is not clear. First it must be recognized that urea is going to be transferred in both directions through the oral mucosa. Therefore, it was not possible to judge absorption without using labeled urea (which was not done). The best that could be done is to estimate a net transfer in one direction or the other. The information presented is not sufficient to determine this. The authors' comments that are included do not make sense (i.e. some indication of net transfer could be made if there were measures of both blood and salivary concentrations of urea through time). Finally, I cannot figure out how to get to an absorption figure without also knowing how much of the urea remained in the gum (see Table 3.1). I am suspicious that this may more of an attempt to rationalize the inability of the authors of the Toxicological Review to come to a conclusion rather than an explanation of why the data are inadequate to the task that the authors of the review were attempting to put it to.

p. 8 continued. Based the description in the Review, it appears that the Nomura et al. (2006) study is structured completely around following the amount of radiolabel in plasma. Unless it is actual measurements of urea, itself, in these compartments (administered urea as well as systemic urea),  $^{14}\text{C}$  measurements do not actually characterize the concentrations of urea in plasma. It is only the movement of the labeled urea into and out of the compartment that is quantified. In the absence of measurements of endogenous urea, the changes in radiolabel may be confounded by isotope dilution. I may be incorrect on this, as I did not read the original manuscript. In any case, this description needs to be modified so that it is clear in terms of what is being measured (e.g. radioactivity or compound in each of the compartments). In the following pages, the text generally speaks of radiolabel distribution, which is why my concern is raised.

p. 11, line 7. Is this sentence trying to say that the  $^{14}\text{C}$ -label is moving through the choroid plexus at a rate slower than water? If so, that needs to be stated. I do not know how to interpret the statement "slower than in the skeletal muscle" in the absence of information about the movement of labeled water in both cases.

p. 11 and following. The experiments with differentially labeled urea are informative and should be useful if there is a need to refine the PK model (i.e. take it more to a physiological model as opposed to a compartmental model). An added comment is that on the sixth line from the bottom of page 13, there should be an explicit statement defining what is meant by recycling of urea carbon. This has to mean that little of the urea was converted to  $\text{CO}_2$ , because if it were it would be recycled through  $\text{CO}_2$ -fixation. I am confused because the remainder of the paragraph goes on to indicate that the urea is in fact being metabolized by gut bacteria. Perhaps this section needs to have a graphic that illustrates the time courses depicting how the two labels change in time in the two compartments. The basis of these two apparently contradictory statements is difficult to resolve within the material presented in the Review.

p. 16, paragraph that begins at the bottom of the page. This is a summary of all the ADME data. It does not belong under the subheading "Elimination." Separate it out as a section with a title. In this and other sections, the word concentration is used in the context of urea, when it appears

that the reference is expressed in terms of the amount of radiolabel, not of the compound itself. If that is the case, it needs to be corrected as the radiolabel may move through a compartment and make no contribution to the concentration of the studied compound.

p. 19, paragraph at the bottom of the page. The paragraph reads as though the authors do not recognize why inulin was used, as it states that only the data for urea are discussed. It should be made clear in the discussion that the inulin is probably there as a marker for extracellular water. It is also not clear whether endogenous urea is being measured or only the N<sup>15</sup>-labeled forms. In this case, it would be unusual not to measure both, but the discussion should nevertheless be explicit on this point. It is essential to know what is meant by plasma enrichment and disappearance. Based on the discussion of pools of urea, I suspect it has to do with 1) increases and decreases with respect to the total inulin space, and 2) examination of changing ratios of N<sup>15</sup> in the two included nitrogens on urea. That should be clear in the discussion and not left up to the reader to decipher.

p. 29, main paragraph. The description of the Al-Homrany study is not clear and the conclusions are odd based on the data that is described. Removal of urea increased LDH activity (presumably these are serum enzymes, but that is not stated) and addition of urea decreased GGT levels (again I presume in plasma), which would indicate that excessive urea is protective. It should also be noted that hemodialysis, itself may be altering these enzyme activities independent of the removal of urea. I suggest the authors look at other studies where effects of hemodialysis is examined independent of urea removal.

p. 39. Is it correct that urea is in equilibrium between brain and plasma in about 2.5 hours? This study involved sc and ip injections of urea to determine the time course of osmotic changes. However, it is not at all clear in the description how it was shown that the half-life of urea in the brain was 4.7 hours by using this means of administering the urea. What measurements was this estimate based upon?

### ***Alan H. Stern***

Pg. 9, Table 3-2 - The volume of distribution is a *volume*, and should have units of volume (e.g., L or mL), not mL/kg. Also, in column 8, it is not clear what the values of  $\beta$  in parentheses are. I would expect them to be the ranges of the mean value given above them, but that is not the case.

Pg.12, par. 2 and ff. - I am sure that the studies of colonic metabolism of urea by infusion of urea into the colon stem from a logical approach to answering a useful question. However, as presented, and without context, they seem bizarre and of unclear relevance.

Pg. 16, par. 1 (following the table) - Something should be said about the fact that only 54% of the dose was recovered in urine after 96 hr in the not-fasted animals compared to 95% in the fasted animals. Presumably, in the non-fasted animals, the unrecovered dose was metabolized and incorporated. However, it is unclear why this should be the case.

Pg. 17, par. 2 - It seems to me that the Marini et al. (2006) study would also have provided basic pharmacokinetic data (e.g.,  $T_{1/2}$ ). Were such data available? If so, they should have been reported in the ADME section.

Pg. 18, par. 1 - The logic here does not seem to follow. If  $F_{ab} = F_{ba}$ , then shouldn't the pool size in A and B equilibrate? Instead the text states that  $B \approx 5A$ .

Pg. 20, par. 1 - As per my earlier comment, the volume of distribution is a *volume* and should be expressed here in L rather than L/kg.

Pg. 22, par. 6 - With regard to the exposure to urea in mixtures increasing serum aspartate, how can the contribution of urea be determined?

Pg. 27, section 4.1.4 - It seems possible that for each of these effects, the association with urea could be secondary to overall kidney function (including maternal kidney function for birth weight associations).

Par. 3 - An odds-ratio (OR) is defined with respect to a dichotomous outcome. Therefore, the OR for lower blood pressure should be defined with respect to a specific blood pressure cut-off. This is important in understanding the magnitude of the response.

Pg. 30, par. 2 and 3 - It would be helpful if the incidence rate data from Fleischman et al. (1980) were presented as ratios (i.e., with values of 0-1) in addition to presenting them as fractions. This would help the reader understand the trend (or lack of trend) in tumor response.

Pg. 39, par. 1 - The reference to an osmolality of "312" requires associated units.

Pg. 40, par. 2 - "*Serum osmolality was significantly increased...*" To what extent does serum osmolality reflect the increased concentration of urea *per se* in the serum?

Pg. 47, Table 4-5 - This table should be presented relative to a dose. As presented it provides no information as to the dose resulting in these concentrations.

Pg. 60, par. 2 - "... *showed that with the relative fraction of DNA single strand breaks to relative toxicity...*" I don't understand this comparison.

**Bonnie R. Stern**

Many of my specific observations are described in I. GENERAL IMPRESSIONS.

Other specific observations:

P. vii: The acronym NOS is not on the list.

P.3: The first section on physical and chemical information is lacking a figure with chemical structure.

Uses of urea in melamine products, and formaldehyde and melamine-formaldehyde adhesives is not noted in Section 2.

P.7: Line 6 from bottom. A higher rate of saliva production may likely be a physiologically-adaptive response to urea in the chewing gum.

P. 31: In the Teramoto et al. (1981) study, urea was administered as a single high dose on GD 12 to rats and GD 10 to mice. This is rather late in the gestation period to induce significant reproductive/developmental effects, although effects do occur with other compounds using this single-dose regime.

P. 37: Bottom paragraph. Why would a death from excessive bleeding from an incomplete abortion not be considered treatment-related if it occurs in a monkey administered oxytocin in addition to a high urea dose?

P 39: Bottom paragraph. Why was the milk adulterated with urea in India? Was it accidental or intentional?

P. 41: First paragraph. Please clarify the dosing regimen. Were the animals habituated to a 2-hour/day feeding schedule for 2 weeks (fourth line in paragraph) and then treated for 5 weeks (seventh line in paragraph)? Were both the spaced-fed animals and the ad lib-fed animals first habituated to the 2-hour/day feeding schedule?

Note that the similar findings (in weight decrease and BUN) in these studies between spaced feeding group (low dose) and ad lib feeding group (much higher dose) strongly support homeostatic control of gastrointestinal absorption of urea.

P. 47: Discussion of the Chung et al. (1985) studies. So do these results support the hypothesis tested, that urea-induced myoclonus is similar to strychnine-induced myoclonus? Please draw a conclusion.

P: 45: Second paragraph, line 4. Please define Hsp. It is not obvious what it is; later in the paragraph heat shock response is noted, but nothing in that text relates it to Hsp. This is discussed later in the text, but would be appropriate here.

P. 51: First full paragraph, first line. Please change “In summary, urea may produce a variety of effects on the renal system.” to “In summary, elevated/excess exogenous urea may produce a variety of potentially adverse effects on the renal system.” Urea’s endogenous function is to produce a variety of effects on the renal system. Please distinguish between normal endogenous function and excess exogenous intake of urea.

P. 52: As noted previously, a better description of urea transport proteins is warranted.

P. 52: Last paragraph, first paragraph line. Given the large number of acronyms or short acronym-like scientific names for enzymes and proteins, it is preferable that chronic renal failure be written out in place of the acronym CRF. There are too many acronyms or names for enzymes and proteins which only consist of several letters to use this acronym for a clinical disease.

P. 53: First full paragraph, last two lines. Please note that a reduction in the expression of urea transport proteins in the kidney under conditions of kidney damage and renal failure, may be an adaptive, rather than an adverse, response.

P. 53: 12<sup>th</sup> line from bottom. What is a 7/8 unilateral nephrectomy?

P. 55: Second paragraph. Differential regulation of urea transport expression would be expected, as would changes in urea concentrations, depending on a variety of conditions, including dehydration (which increases urea concentrations) as does trauma, shock, and starvation. Thus, the responses of the transport proteins may be compensatory or adaptive.

P. 56: Last paragraph, first line: Please define a Ras protein.

Section 4.5.4 Genotoxicity: Please note that the majority of genotoxic effects occurred at the highest or higher dose(s) tested. Even if the authors do not report it, this suggests the possibility of cytotoxicity occurring at these doses, which invalidates the conclusion of genotoxicity. Please note this more clearly as a general statement, rather than restricting mention of cytotoxicity only to those studies in which the authors have reported it. In many studies, especially the older ones, cytotoxicity was not considered.

P. 63: Second paragraph. The study (not studies) by Seipelt et al. does not suggest that maternal exposure may decrease the number of pups/litter.

P. 63: Fifth line from bottom. Similarly, Okada and Kobayashi did only one study, not studies.

P. 63: Last two lines. Agree.

P. 65: Section 4.6.5. This is not a mode of action section. This is a mechanistic study section. First sentence in this paragraph should read “*Exogenous urea exposure* has been shown to *potentially/possibly* target a variety of organ systems...”

P. 69: Section 4.8. Susceptible Populations.

Populations susceptible to exogenous urea exposure are those with kidney injury or damage or renal disease. Patients with renal disease or pre-renal disease already have comprised urea processing so added urea would exacerbate this condition. Please note.

I don't think the findings in Cade and Pain (1972) support the supposition that asthmatics might be a susceptible subpopulation.

P. 74: Last two lines in second paragraph from bottom. Fleischman et al (1980) shows increased adenomas in the highest dose group. Based on study limitations and reporting inconsistencies, this effect is not sufficient to classify urea as *suggestive of carcinogenic potential*. Please be internally consistent.